Investigating the management of potato and tomato late blight caused by *Phytophthora infestans* through host genetics and pathogen characters

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Phytophthora infestans (Mont) de Bary, is the causal agent of late blight of potato and tomato. In 2009 to 2013, four *P. infestans* clonal lineages US-22, US-23, US-24, and US-8 were isolated in several U.S. states including Wisconsin. In the present research two components of late blight management were investigated. 1) Biology and ecology of four recent clonal lineages of *P. infestans.* A phenotypic and genotypic characterization was performed on *P. infestans* collected in 2013 in Wisconsin. Results revealed that two clonal lineages, US-23 (A1 mating type, mefenoxam sensitive) and US-8 (mating type A2 and resistant to mefenoxam) were present. In an assessment of the virulence and oospore production with US-22 (A2), US-23 (A1), and US-24 (A1) isolates, results revealed that potato and tomato plants transformed with the *RB* gene and tomato Mountain Magic, carrying *Ph-2* and *Ph-3* genes, were resistant to inoculation with all of the individual isolates and no oospores were detected in these plants. In an investigation of survivability and infectivity of oospores, it was discovered that oospores were able to survive and remain infective to tomato leaflets after being exposed to natural field winter conditions in Wisconsin, and 5 temperatures ranging from 22° C to -20° C. 2). Breeding for resistance. Resistance to late blight from the wild potato species *Solanum verrucosum* has been introgressed into five diploid hybrid clones. Two of these clones have resistance to early blight from the wild potato species *S. commersonii*. All five clones have acceptable tuber yield, do not produce 2n pollen grains, and produce viable pollen. Using molecular approaches, transient expression of 18 RB orthologous genes from 7 *Solanum* species

were evaluated for the ability to recognize the IPI-O effectors from *P. infestans*. Our results revealed that none of the 18 RB orthologs were able to recognize the IPI-O effectors. The nucleotide sequence diversity observed between non-functional and functional *RB* genes can be used for future research to identify regions of importance for molecular characterization of the interaction between IPI-O effector and the RB gene.

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Chapter 1:

Introduction

Biology of *Phytophthora infestans*

Phytophthora infestans (Mont) de Bary, is the causal agent of late blight of potato (*Solanum tuberosum)* and tomato (*S. lycopersicum*) and is one of the main constraints of potato and tomato production worldwide (18,28). *Phytophthora infestans* is an oomycete pathogen, meaning that is belongs to a group of microorganisms sometimes refered to as the water molds. This pathogen traces its origin to either the Toluca Valley Mexico, or the Peruvian Andes, where it infects wild and cultivated *Solanum* species, and where *P. infestans* populations have a 1:1 ratio of the compatibility or mating types termed A1 and A2 (26,30,66). A virulent isolate of *P. infestans* is capable of initiating a late blight epidemic and destroying the crop in 7-10 days when highly susceptible cultivars are being grown under high relative humidity and optimun temperatures (18,20,30)

Phytophthora infestans can infect leaves, stems, potato tubers, and tomato fruits and is a hemibiotrophic pathogen with two phases of infection (19). The first is an early biotrophic infection during which the pathogen develops an appresoria-like structure for uptaking nutrients from living host plant cells (2). The second is a necrotrophic phase, in which nutrients required by the pathogen for colonization, reproduction, and sporulation are obtained from dead and dying cells in necrotic host plant tissues (33). The life cycle of *P. infestans* can be completed via asexual or sexual means (15). The asexual cycle is driven by dehiscent sporangia and zoospores, sources of short-living inoculum for polycyclic development, reproduction, and dispersion of the pathogen within a growing season. Usually, sporangia are dispersed by wind and after landing on host plant tissue can initiate an infection. If weather conditions include high relative humidity and a temperature around 20 to 25ºC sporangia can germinate and initiate a direct infection through formation of a germ tube that penetrates the host plant tissue. Under

cool temperatures around 10 to 14ºC, biflagellated zoopores (8-20 per sporangium) are released from the sporangia (15,38). The biflagellated zoospores are motile for a short period of time, usually no more than 60 minutes, before they encyst for direct germination and penetration of host tissue via a germ tube (63). During the 48 hours after the initiation of penetration, plants do not show any visible symptoms (51).

Phytophthora infestans is a heterothallic oomycete requiring two mating types, named A1 and A2, for sexual reproduction (21,56). A hormonal stimulation by the presence of both mating types triggers the production of oogonia and antheridia (37,44). Fertilization of oogonia by antheridia induces production of oospores, which are known to serve as a long-term, soil persistent source of inoculum for many years, and provide a source of genetic variation (22,49,60). Non-hybrid oospores have been reported when gametes are developed by a single mating type as products of hormonal stimulation by the presence of the opposing mating type in the same tissue but without physical contact with the opposite mating type (37). In addition to hormone stimulation from the opposite mating types, formation of gametes can be stimulated by other factors including: presence of opposing mating types of other *Phytophthora* species, age of the culture, and nutrients provided to cultures on media made from oats, lima beans, and V-8 juice (52,55).

Review of literature

The host range of *P. infestans* includes members of the Solanaceae family including *Solanum betaceum* (tree potato), *S. quitoense* (naranjilla, *S. dulcamara* (bittersweet nightshade), *S. physalifolium*, and *S. ochrantum* (hairy nightshade) in addition to the previously mentioned and most common hosts, *S. tuberosum* (potato) and *S. lycopersicum* (tomato) (1,9,10). The

pathogen can infect host plants at any phenological stage and is very aggressive and prolific. A single foliar lesion of late blight is estimated to produce around 300,000 sporangia per day that are easily dispersed by wind, splashed by rain to neighboring host plants, or into the soil where they infect potato tubers (19). One aspect of late blight that makes *P. infestans* a successful pathogen is that low levels *of P. infestans* are difficult to detect in the field and when foci of infection are visually detected, it is often too late to control the epidemic because the pathogen is already dispersed (17).

In 2009, late blight reached a pandemic status in the Eastern United States due the dispersion of a single clonal lineage in four weeks and no preventive actions (20). The life cycle of *P. infestans* can be completed in 5 to 6 days under ideal weather conditions and on a susceptible host plant. Epidemics of late blight in the last five years in the U.S. were caused by a few clonal lineages (11,35,54); but the source and type of primary inoculum is unknown. Mycelia, sporangia, and zoospores can survive only for a short period of time (minutes to hours) without a host plant and generally are eliminated by freezing temperatures in northern U.S. fields (18,59). In areas where only one mating type is present, the pathogen is capable of surviving from season to season as mycelium in infected potato tubers, plant debris (potato and tomato), and potato cull piles in the field (3,18). Diseased tubers are likely the primary mechanism of overwintering and account for the major sources of inoculum in the potato and tomato crops in the U.S. (39,45). Oospores can germinate under favorable environmental conditions and produce diploid sporangia that could be mating type A1 or A2 (22).

In many countries where both mating types are reported, oospores are the pathogen survival structure in the absence of host plants (14,21,31). Oospores in the field could become primary inoculum in subsequent years and a source of phenotypic and genotypic diversity.

Oospore infectivity has been reported after being exposed to winter field conditions in the Netherlands, the U.S., Canada, and Mexico (13,16,60). While the presence of both mating types in potato and tomato fields in the U.S. has been reported in the last 25 years without significant evidence of sexual recombination, this circumstance allows for the potential for oospore formation to play an important role in the epidemiology of late blight (12).

The populations of *P. infestans* in the U.S. have typically been introduced from other countries following asexual reproduction in the field. The asexual populations derived from a single genotype is named a clonal lineage (19). The genetic diversity and designation of genotype of the *P. infestans* population have been determined using different techniques. Genotyping is fundamental for implementation of integrated disease management and for host resistance breeding strategies (50). Since the early 1980s allozyme analyses with *glucose-6-phosphate isomerase (Gpi)* has been used for genotyping *P. infestans* populations in the U.S. due to the versatility, simplicity and speed of the method (29). Genotypes determined by the *Gpi* allozyme test correlate with mating type and mefenoxam sensitivity of the pathogen in the U.S. (54). However this linkage or correlation does not exist in countries where both mating types are present and *P .infestans* populations have high genetic diversity.

Genetic characterization using restriction fragment length polymorphism (RFLP) was developed in the early 1990s to evaluate the RG57 locus (27). In the last 10 years different techniques were developed based on DNA amplification, such as microsatellites or simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs). An inconvenience of using SNPs is that the tests are relatively high cost when compared to large-scale genotyping. In 2013, a set of 12 microsatellite markers was published for genotyping *P. infestans* populations (47). The 12 markers can be used in one-step multiplex PCR amplification followed by

electrophoresis in a capillary sequencer (47). The set of 12 microsatellite markers is a powerful tool that can distinguish genetic variation at the subclonal level.

Controlling late blight is a challenging task and is considered one of the most costly diseases in term the cost of control, and yield and postharvest losses (19,34). Prevention is the best tactic for management of the disease, which includes the use of late blight-free seedlings and seed tubers, the avoidance of formation of potato cull piles, removal/destruction of volunteer potato and tomato plants, elimination and/or disposal of late-blignt infected plants, allowance of air circulation between plants and rows to reduce canopy wetness, elimination of nightshade host weeds in and around fields, use of resistant cultivars, use of preventive fungicides, and adherence to prophylactic management triggers from appropiate disease-forecasting systems (18,61). The use of preventive and curative fungicides is the most popular method for control of late blight on susceptible cultivars, but the use of fungicides and their application can be very costly to farmers (25). Calendar-based fungicide applications are not always effective, especially under heavy infections and rainy days and such methods can at times cause applications of unnecesary fungicide if environmental conditions for disease have not been satisfied. The forecasting system BLITECAST uses temperature and relative humidity of the environment to predict when *P. infestans* is most likely active and ready to cause infection in potato and tomato (23). This forecasting system has been effectively modified for regional environmetal needs (58). Currently, in the U.S. several forecasting systems are utilized to trigger fungicide application including BLITECAST and SIMCAST (23).

The use of late blight-resistant varieties has economical, ecological, and human health benefits. Wild *Solanum* species have been sources of resistance to late blight, *Solanum*

demissum, S. bulbocastanum, S. berthaultii, and *S. pimpinellifolium* are among the species identified with potential usable resistance (36,43,57). The use of wild *Solanum* species is an excellent way to generate a broad genetic base for breeding populations. Potato and tomato breeders have developed some commercial cultivars with resistance to late blight (36). However, varieties with durable resistance and desirable characteristics in the market have not always been readily achievable or available for farmers and consumers. In the last decade, 2 late blight partial resistant genes were isolated: the *RB* gene from *S. bulbocastanum,* (57,62), and the *Ph-3* gene from *S. pimpinellifolium* (42,43). Both resistance genes encode nucleotide binding (NB) and Leucine-rich repeat (LRR) motifs and a coiled-coil (CC) N-terminal domain (43,57).

During early infection, several plant pathogens including *P. infestans*, secrete an arsenal of virulence proteins termed effectors into the host plant cells to alter the host physiology and suppress host defense. Effector proteins can be grouped in two classes, based on their target site in the host cells, 1) apoplastic effectors are released into the diffusional space outside of the plasma membrane or extracellular space, and 2) cytoplasmic effectors are secreted inside the cytoplasm of the host plant cell (40). The locus IPI-O (*in planta induced* genes O) encodes a family of cytoplasmic protein effectors present in almost all isolates of *P. infestans* and is involved in pathogenicity (64). *Phytophthora infestans* effectors in host cells induce effector-triggered susceptibility, leading to the development of late blight infection (4,6,40). Resistance protein receptors in resistant plants recognize the presence of pathogen effector proteins, thereby inducing the activation of effector triggered immunity or resistance to colonization, which can be expressed as a hypersensitive response (HR) in the area of infection and oxidative burst (6,41). However, *P. infestans* is a pathogen with a high potential evolution that can overcome the

genetic resistance of the plant host (50). Factors including natural mutation, genetic drift, and sexual recombination can contribute to the development of new populations with new genotypic and phenotypic characteristics (5,32,48,53). In many countries in Europe, sexual reproduction is occurring due to the existence of both mating types. As a result, *P. infestans* genoytpe EU13 A2 or "Blue 13" has developed and displaced other genotypes across Europe in less than three years (7). The EU13 A2 genotype has been reported to be more virulent to most potato varieties than prevalent genotypes of *P. infestans* in previous years (8,46,65).

From 2009 to present, *P. infestans* clonal lineages US-22, US-23, US-24, and US-8 have been isolated in the U.S. (24,54). Both mating types of the pathogen were isolated from field samples collected in Wisconsin in 2009, 2010, and 2013, posing a risk for sexual recombination and oospore production. Multi-faceted management measures are necesary in response to novel pathogen clonal lineages. In my dissertation research, two aspects of late blight were studied, including ecology and epidemiology of the pathogen, and potato breeding using conventional breeding and molecular approaches. The ability of *RB* and *Ph* genes in potato and tomato to minimize the risk of oopore production in the presence of mating pairs of *P. infestans*, and the ability of oospores to overwinter under soil natural field conditions and freezing temperatures under laboratory conditions were investigated. Conventional breeding was used to introgress the *RB* orthologous genes for resistance to late blight from the wild species *Solanum verrucosum* into diploid breeding clones; and evaluation of the transient expression of *RB* orthologous genes from different wild potato species, through its interaction with IPI-O effectors from *P. infestans*. Information generated in this study will contribute to current understanding of the epidemiology and ecology of recent clonal lineages of *P. infestans* collected in Wisconsin and throughout the

U.S., and diploid clones now be used for introgressing late blight resistance to potato breeding lines.

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Chapter 2

Genotypic and phenotypic characterization of *Phytophthora infestans* collected in Wisconsin during 2013

Abstract

Phytophthora infestans, the causal agent of late blight of potato and tomato, has been reported in Wisconsin in the last five growing seasons, after a 6-year-period of no reports of the disease. Thirty-nine isolates were collected from 16 Wisconsin counties in 2013. Genotypic characterization was performed with *Glucose-6-phosphate isomerase* (*Gpi*) allozyme locus analysis, mitochondrial haplotyping and genomic assessment with 12 microsatellite markers. Phenotypic characterization included mating type analysis, and sensitivity to the fungicide mefenoxam. *Gpi* allozyme testing revealed alleles 100/100 in 37 isolates (clonal lineage US-23) and alleles 100/111/122 in 2 isolates (clonal lineage US-8). Isolates belonging to the US-23 clonal lineages, from both potato and tomato tissues, were of the mating type A1 and were sensitive to mefenoxam. The two isolates of the US-8 clonal lineage were both from potato tissues and were of the mating type A2 and were resistance to mefenoxam. Microsatellite analysis did not identify polymorphism within the 37 isolates of the US-23 or the 2 isolates of the US-8 clonal lineage. However, when microsatellite analysis of 49 *P. infestans* isolates collected from 2009 to 2012 was performed, two variants of the clonal lineages US-22, US-23, and US-23 were detected. All *P. infestans* isolates collected from 2009 to 2013 were identified as members of the mitochondrial haplotype group Ia. Variation in *P. infestans* genotypic and phenotypic character from 2009 to 2013 indicated the need for continual disease management to limit the occurrence of opposing mating types in proximity.

Introduction

Late blight caused by the oomycete *Phytophthora infestans* (Mont) de Bary, limits production of potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum* L) worldwide (13). The disease is considered one of the world's most destructive plant diseases, affecting most commercially grown varieties of potato and tomato which are extremely susceptible to late blight. In Wisconsin in 2013, potatoes were grown on 25,300 ha, with production of a total of 1.4 million metric tons and value of $311 million farm gate value (42). While tomato production is relatively small in Wisconsin, around 1,600 ha, both crops have importance for local fresh market consumption, processing, and distribution to other states.

Phytophthora infestans can cause symptoms at any physiological state of the host plant, and can infect any plant tissue including leaves, stems, tomato fruits and potato tubers (11). As a heterothallic oomycete, *P. infestans* requires two mating types, termed A1 and A2 for sexual recombination (16). In many countries both mating types are present and sexual reproduction routinely occurs resulting in a population with high genetic diversity (9,12,17,40). Oospores are formed when mycelium from isolates of opposing mating types are in contact in or on leaves, stems, fruits, or tubers (17,26). Oospores hold great epidemiological importance in the disease cycle, because they serve as long-term, soil persistent sources of inoculum and create significant genetic variation (12,32,40).

P. infestans traces its origin to either the Toluca Valley in the highlands of central Mexico or the Peruvian Andes, where populations with high genetic diversity are present (44,18). The pathogen was introduced to the U.S. in the 1800s and, populations in this country have been reproducing primarily asexually, as the two mating types required for sexual reproduction have

not been readily detected in the same host plant or in close proximity (20). It has been presumed that the emergence of different but closely related lineages were generated by mutation, mitotic recombination or migration (20).

Historically, in the U.S., *P. infestans* populations have been classified into clonal lineages or genotypes according to an allozyme test of the *glucose-6-phosphate isomerase* (*Gpi*) locus, DNA fingerprint using the probe RG57, and mitochondrial haplotype (20,21,23). Phenotypic characterizations or grouping, have included races according to R-gene differentials, mating type, and sensitivity to mefenoxam (16,29,37). Due to lack of sexual recombination, therefore low genetic diversity observed in *P. infestans* populations in the U.S., linkage between the allozyme genotype, mefenoxam sensitivity, and mating type have been documented, allowing delivery of timely characterization information to growers for enhanced management (22,25,36). Recently molecular analyses using nucleotide sequences of the *P. infestans* genome have generated a one-step multiplex PCR method using twelve simple sequence repeat (SSR) markers for genotyping populations of *P. infestans* (28). This new set of molecular markers can be added to complement the markers that previously were developed for genotyping populations. Microsatellites are co-dominant markers and in diploid populations, can identify heterozygous individuals (43).

In the U.S., the clonal lineage US-1, which is mating type A1 and susceptible to mefenoxam, was present in predominance prior to 1990 (15). US-1 was gradually displaced by the clonal lineage US-8 in the subsequent years 1992-1996 (5,7,30). The introduction of US-8 brought new concerns for management of the disease, since this genotype is resistant to mefenoxam and is of the A2 mating type (14,30). The population of *P. infestans* in Wisconsin has been continually monitored in order to deliver appropriate recommendations to growers for

disease management (30,36,41). During the period from 2002 to 2008, there were no reports of late blight in Wisconsin (25,35). In 2009, the reemergence of *P. infestans* in Wisconsin and other areas in the U.S. was reported (6,36). New clonal lineages, representing both mating types were detected in potato and tomato fields during the growing seasons of 2009 to 2012 (36). However, both mating types were not detected in individual fields and remained geographically separate (35). Current management of late blight includes the use of certified disease-free seed; crop rotation; planting resistance or tolerant varieties; destruction of late blight infected plants, volunteer, and culls; and preventive use of selective fungicides often initiated by disease forecasting tools (13).

Mefenoxam is a fungicide that belongs to the phenylamide group, Fungicide Resistance Action Committee Group 4, and is one of the most effective and systemic fungicides to control late blight when the pathogen is sensitive to its activity (4). Mefenoxam was one of the most popular late blight fungicides, as it has protective, curative, and eradicative activity (34) which allows growers to extend spray intervals and prevent tuber infection. With the occurrence of resistant *P. infestans* genotypes, growers have reduced reliance upon this fungicide.

Understanding local pathogen diversity is the first step in a successful integrated late blight management program. The objectives of this study were 1) to assess the distribution, phenotypic, and genotypic characteristics of *P. infestans* collected from Wisconsin production fields and gardens in 2013; and 2) compare genotypes of *P. infestans* isolates collected from 2013 to isolates from 2009-2012.

Materials and Methods

Collection and isolation. *Phytophthora infestans* isolates were collected from late blight-infected tomato and potato foliage, fruits and tubers submitted to the Vegetable Pathology Lab at the University of Wisconsin-Madison, by growers, county extension agents, home gardeners, and professional crop consultants during the year 2009-2013. Phenotypic information on isolates from 2009-2012 was previously published (35,36). Forty-nine of these isolates were used in our genotypic analysis with 12 SSR markers. 2013 isolates were analyzed both phenotypically and genotypically. Portions of late blight lesions at the margin between healthy and diseased tissues were excised and placed on water agar in 9-cm diameter petri dishes and incubated at 18ºC in darkness for 48 hours to induce mycelial production and sporulation. To generate pure cultures from each sample of the pathogen, 5-10 sporangia from the sporulating lesion on the water agar plates were collected using a small (1-to 2 mm^2) piece of Rye A, on the tip of a dissecting needle. The piece of Rye A with sporangia was placed on new Rye A agar media (2) amended with rifampicin and ampicillin. All tissues on petri dishes were incubated in darkness at 18ºC for 14 days to induce further mycelial and sporangial formation in axenic culture of *P. infestans*. Three axenic cultures were selected from each field sample and single zoospore cultures were generated from each of the parental isolates, following a protocol proposed by Erwin and Ribeiro (11). All cultures were maintained on Rye A Agar media (2), amended with rifampicin and ampicillin for use in our experiments. Oospore production in single-isolate culture was never observed. For long term storage, isolates were maintained on infested hemp seeds in 1.5 ml tubes (33). To induce colonization of the hemp seeds by *P. infestans,* microcentrifuge tubes containing seeds and pathogens were incubated at 18º C for fourteen days and then transferred to 12º C and complete darkness in the Potato and Vegetable Pathology Laboratory at the University

of Wisconsin-Madison. Single representative isolates of each *P. infestans* clonal lineage were used as previous studies have indicated that recently identified lineages showed low variation in biological characteristics (8,25,44).

Allozyme assay. Characterization of the *Gpi* locus in each isolate was performed using the method of cellulose acetate agar overlay described by Goodwin et al (20). Sporulating late blight lesions were used for direct analysis, when possible. If there was no visible sporulation on the day of sample receipt, the infected plant tissue was placed on water agar in petri dishes and incubated at 18ºC in complete darkness overnight to promote sporulation. A small piece (16 mm^2) of the edge of the sporulating area was excised, placed in a 1.5 ml tube containing 1 ml of distilled water, and shaken with a vortexer to dislodge sporangia. Plant tissue was removed and the tube was centrifuged at 14,000 rpm for 1 minute to pellet the sporangia and all but 75-100 µl of the supernatant was removed. The remaining suspension and pellet were ground manually with a konte pestle. The extracts were then centrifuged for 1 min at 14,000 rpm, after being placed on ice, and used immediately to perform the allozyme assay as described by Goodwin et al (20). Standard clonal lineage controls for the *Gpi* banding patterns were included (US-8, -22, -23, -24). Migration of banding patterns of the *Gpi* proteins was determined by comparing the bands from the standard or known controls.

Mating type. Each test isolate was paired with an A1 and an A2 control isolate on Rye A media (7). Mycelia plugs (6 mm in diameter) from 3-week-old cultures of new isolates of *P. infestans* on Rye Agar media were placed 4 to 5 cm apart from mycelial plugs of a known A1 (US-1) or A2 (US-7) isolates on the media in separate petri dishes (90 mm in diameter). Plates containing the isolates were incubated at 18ºC in darkness for 14 days. For evaluation of oospore formation, a small portion of the culture at the hyphal interface of the two mycelial plugs was excised and

placed on a glass slide for microscopic observation at 100 x magnification. Isolates that formed oospores when paired with the A2 tester isolate were designated as A1. Isolates that formed oospores when paired with the A1 tester isolates were designated as A2. A negative control consisted of isolates of the same mating types paired on Rye A and positive control pairing isolates of opposing mating type on Rye A.

Mefenoxam sensitivity assay. *Phytophthora infestans* isolates were tested for sensitivity to the fungicide mefenoxam by comparing the mycelial growth on non-amended Rye A media to mycelial growth on Rye A amended with 100 ppm mefenoxam (Ridomil Gold SL, Syngenta Crop Protection, Greensboro, NC) after 14 days of incubation at 18º C in complete darkness. Eight-mm-diameter mycelial plugs from 14-day old *P. infestans* cultures on Rye A, were cut from the margin of the actively growing colonies. One mycelial plug was placed in the center of a 9-cm petri dish and incubated at 18ºC in darkness for 14 days. Mycelial growth of 4 mefenoxam-amended and 2 non-amended plates was measured in two perpendicular directions for each plate. The diameter of the mycelial plug was subtracted and the measurements were averaged. The relative mycelial growth (%) was determined for each isolate by dividing the average growth on non-amended media by growth on mefenoxam amended media. Isolates with values of 0 to <10% were considered sensitive, >10 to < 60% intermediately sensitive, and >60% insensitive (5,7,30).

Genomic DNA isolation. A DNA extraction protocol, adapted from Bok (1), was used. Mycelia of *P. infestans* were collected from 3-week old cultures on Rye A agar, by gently scraping the agar surface with a scalpel. Mycelial extractions were placed in 1.5 ml sterile microcentrifuge tubes and frozen at –80° C for at least 1 h before the overnight process of lyophilization. Lyopholized mycelia were broken into a fine powder using a toothpick and re-suspended in 700

µl of LETS buffer containing 20 mM EDTA (pH8), 0.5% SDS, 10mM Tris-HCl (pH8) and 0.1M LiCl. The suspension was mixed by inverting the tubes several times, and incubating on a laboratory benchtop for 5 minutes under ambient temperature conditions. Seven hundred-µl of phenol-chloroform-isoamyl alcohol (25:24:1) were added, mixed by shaking 10–15 times, and incubated on the bench top for 5 minutes. Samples were centrifuged for 10 minutes at 14,000 rpm. The supernatants were then transferred to new 1.5 ml tubes and 1 ml of 95% ethanol was added and mixed. To pellet the DNA, samples were spun for 5 minutes at 14000 rpm. The supernatants were discarded and pellets were washed with 1 ml of 70% ethanol, following centrifugation at 14,000 rpm to pellet DNA. The supernatants were discarded and pellets were dried for 15 min on a laboratory bench top under ambient temperature and light conditions. The pellets were re-suspended in 50 µl TE and 2 µl RNAse (10mg/ml stock) was added. To inactivate DNase and digest RNA, samples were incubated at 65° C for 30 min. Extracted genomic DNA was stored at -20° C.

Multiplex microsatellite marker analysis. Simple sequence repeats (SSRs) were analyzed from 39 *P. infestans* isolates collected in 2013 and 49 samples collected in 2009-2012. Isolates US-1,-7, -8, -20, -22, -23, and US-24 kindly provided by Dr. Bill Fry of Cornell University, were included as reference standards. Twelve polymorphic microsatellite loci were used for analysis, as described by Li (28). The QIAGEN Type-it Microsatellite PCR kit (QUIAGEN, Cat. No. 206243) was used. A multiplex PCR reaction using 12 primers (Table 1) in 25 µl was performed for each isolate. Master Mix was prepared according to manufacturer's instructions and primer concentrations per single reaction are shown in Table 2. Amplification reactions were conducted in a PTC200 thermal cycler (MJ Research, Waltham, Massachusetts, USA). The PCR conditions included an initial heat inactivation of 95° C for 5 min followed by 28 cycles of denaturation at

95° C for 30 s, annealing at 58° C for 90 s, and extension at 72° C for 30 s, and a final extension at 60° C for 30 min. One µl of PCR product from each reaction was mixed with 9.75 µl of deionized formamide (Hi-Di Formamide, No. 4311320 Applied Biosystems) and 0.25 µl of GeneScan-500LIZ standard (No. 4322682, Applied Biosystems). Electrophoresis in a capillary sequencer and visualization of PCR products were performed at the Biotechnology Center of UW-Madison. Electropherograms for each locus were scored using the GeneMarker Software (Softgenetics LLC, Pennsylvania, USA). Genetic distance was calculated using the software GenAlEx 6.5 (32) and phylogenetic analyses were conducted using MEGA 6 software (39).

Mitochondrial haplotype. Mitochondrial (mtDNA) haplotypes were determined for all samples collected during 2009 to 2013, according to the methods described by Griffith and Shaw (23). Amplification of the region P2, was performed using the primers P2F 5'TTCCCTTTGTCCTCTACCGAT-3' and P2R 5'TTACGGCGGTTTAGCACATACA -3', and for amplification of the region P4 was performed using the primer P4 F4 5'TGGTCATCCAGAGGTTTATGTT-3' and P4 R4 5'CCGATACCGATACCAGCACCAA-3'. Amplification reactions were carried out in 25 µl total volume containing 1 PCR buffer, 0.25 mM dNTPs, 2.5 mM Mag^{+}, 20 pmol of each primer and 5 U *taq* DNA polymerase and 50 ng of template genomic DNA. The PCR conditions were 3 min hot start at 95°C, followed by 35 cycles of 60 s at 94°C, 45 s at 60°C, and 120 s at 72°C. Reactions were completed with 5 min at 72°C. All PCR products for region P2 and P4 were digested with digestion enzymes *Msp*I and *Eco*RI, respectively. All digested PCR products were electrophoresed through a 2% agarose gel and visualized with UV light after ethidium bromide staining and scored as described by Griffith and Shaw (23).

Results

Allozyme clonal lineage. A total of 39 isolates of *P. infestans* (24 from tomato and 15 from potato) from 16 counties in Wisconsin were collected during 2013 (Table 2, Fig. 1). Two allozyme banding patterns were detected for the *Gpi* locus, 100/100 and 100/111/122, corresponding to US-23 and US-8 clonal lineages, respectively (Table 3). The 49 isolates, previously phenotypically characterized from 2009-2012 (36) represented US-22, -23, -24 clonal lineages, from 20 counties in Wisconsin (35).

Mating type. The 37 samples identified as US-23 were identified as mating type A1. The samples identified as US-8 formed oospores with the A1 tester isolate, and were identified as mating type A2. Both mating types were present within Portage County Wisconsin in 2013, but not in the same field.

Mefenoxam sensitivity. Overall, 37 isolates of *P. infestans* collected from tomato and potato that were classified as clonal lineage US-23 grew less than 10% relative to the control (0 ppm) and were considered sensitive to mefenoxam. In this group there was no variation on the response of *P. infestans* to mefenoxam in relation to the plant host. The 2 isolates belonging to US-8 grew more than 60% relative to the control, and were considered resistant to mefenoxam (Table 2).

Mitochondrial haplotypes. All *P. infestans* isolates collected in Wisconsin in 2013 yielded two fragments for the region P2, with size of 720 and 350 bp after digestion with *Msp*I. Three fragments were obtained in the P4 region with size of 394, 361 and 209 bp. These results indicated that clonal lineages US-8 and US-23 collected in Wisconsin in 2013, had the Ia mitochondrial haplotype (Table 2).

Microsatellite analysis SSR. SSR analysis was performed to determine the genetic diversity of the *P. infestans* isolates from Wisconsin. Allele sizes were compared with previously published data (6,25,28), data obtained from the reference isolates, and data from isolates from 2009-2012 included in this study (Fig. 1). Overall, SSR genotyping using a set of 12 primers in *P. infestans* collected in Wisconsin 2013 successfully distinguished two clonal lineages US-23 and US-8 (Table 3). In the clonal lineage US-23, 37 isolates had identical allele sizes amplified at the 12 loci, and had similar allele sizes with a US-23 isolate used as control. Two isolates were placed in the clonal lineage US-8 according the allele sizes, and no polymorphism was detected using the 12 SSR primers. The samples belonging to the US-23 clonal lineage were collected throughout the growing season 2013. The samples of the US-8 clonal lineage were both collected from Portage County, with the first sample on 30 July and the second sample on 21 August.

Three clonal lineages US-22, US-23, and US-24, were identified in the genotypic analysis of 49 isolates of *P. infestans* collected from 2009 to 2012. These results are in concordance with results obtained by Seidl-Jonhson using the *Gpi* allozyme (36). In 19 samples classified as the US-23 clonal lineage, the same allele sizes and no polymorphism was detected, except in a single isolate collected from potato leaf tissue in Adams County in 2012. The isolate had different allele sizes for the loci PinfSSR4 and PinfSSR11. Two variants were identified within the 21 isolates of clonal lineages US-22 collected in 2009, 2010, and 2011. Nineteen isolates had the typical allele sizes as the US-22 control, 2 identical isolates differed from the group of 19 at loci D13, PinfSSR11, and PiG11. These 2 isolates were collected in 2010, one sample isolated from tomato leaves, and the other from potato leaves collected in Brown and Columbia Counties respectively. Nine samples belonging to the US-24 clonal lineage were identified. Seven of the

9 had the typical alleles size as the US-24 control, and the remaining 2 had different allele sizes at loci PinfSSR11, Pig11, and Pi4B (Table 3).

Discussion

The clonal lineage US-23, of the mating type A1, was the predominant genotype in Wisconsin in 2013 comprising 95% of the samples, and infecting both potato and tomato. This genotype was also identified as the causal *P. infestans* clonal lineages of late blight epidemics in several states during the growing seasons of 2012 and 2013 (41). US-8 *P. infestans* of the mating type A2, was also identified in the 2013 late blight epidemics of Wisconsin. The two clonal lineages, US-23 and US-8, were, identified based on both genotypic and phenotypic characteristics. The finding of the 2 clonal lineages suggested that late blight epidemics in 2013 in Wisconsin were initiated by 2 different sources of inoculum.

The first report of the US-23 clonal lineage in Wisconsin was in 2009. Since that time, the lineage has been detected in each of the subsequent 4 years, and was the most predominant lineage in 2012 and 2013 in Wisconsin and nationally (6,36). Two important pathogenic fitness parameters make US-23 isolates successful including higher sporulation rates on potato and tomato (35) and higher lesion growth rates on potato than isolates of the lineages US-22 or US-24 (35), despite US-23 having sensitivity to phenylamide fungicides such as mefenoxam..

The second, and only other clonal lineage detected in 2013 was US-8 that is of the A2 mating type. It has been present in potato fields in the U.S. since 1989, and was the most predominant clonal lineage from 1994 to 2002 (8,18). In 2002 and subsequent years, US-8 was

nearly displaced by other clonal lineages such as US-7, US-11, and US-20 (3,5,18). However, there are reports of sporadic late blight outbreaks caused by US-8 in multiple states from 2002 to 2013 (25). Isolates of US-8 have some important pathogenic fitness parameters such as shorter latent period, larger lesions, resistance to mefenoxam, and higher sporulation rate on potato leaves, than the US-1 genotype (27). These characteristics contributed to the displacement of US-1 by US-8 in the 1990s (7,27). The 2 US-8 isolates from 2013 had almost same alleles size with the US-8 genotype used as control from the laboratory of Dr. William E. Fry at Cornell University Lab. The only allelic difference was at locus PinfSSR4, with sizes 290/294 and 285/294 respectively. The difference observed in both US-8 with respect to the US-8 reference could be a product of mutation or mitotic recombination, since *P. infestans* has a high evolution rate.

The initial source of late blight inoculum in Wisconsin during 2009 - 2013 is not known with certainty. The genetic profile generated by microsatellite markers showed no polymorphism among 56 isolates of US-23 collected in 2009 to 2013, except in one isolate. This result provide evidence that the pathogen is reproducing exclusively asexually and this clonal lineage was likely harbored in infected potato tubers, in volunteer potato or tomato plants, in infected cull tubers, or in *Solanum* weeds in or around production fields. The presence of one variant of US-23 could be the product of mutation or mitotic recombination and was likely eliminated with the control of late blight in the area where it was detected in 2010.

The SSR analysis of the 21 isolates of the US-22 clonal lineage collected in 2009 and 2010 revealed that 2 members, identified as US-22a, differed at the locus PiG11, SSR11 and D13 from the main group. Interestingly, the US-22a in the locus D13 had alleles with size 135/135,

while the US-22 with 19 members does not have these alleles. A mutation at the locus D13 was also reported by Danies et al (7), but the alleles size were 147/147.

The SSR analysis of the 9 isolates of the US-24 clonal lineages collected in 2009-2010 revealed that just 2 members, identified as US-24a, had different allele sizes at locus Pig11, PinfSSR11, and Pi4B. Isolates of US-24 were reported to have variability in their sensitivity to mefenoxam, ranging from 14% to 73% or intermediate resistance to full resistance (35), even though genetic diversity was detected by the microsatellite analysis and variation in the sensitivity to mefenoxam, this clonal lineage had lower sporulation rates and lesion size in potato leaves than US-23, and could be why this lineage was less fit and was not again detected in Wisconsin from 2010 to the current time.

Increased genetic diversity in *P. infestans* in many European countries has been detected, due to the sexual nature of the pathogen populations (9,10,38). In Wisconsin, both mating types were detected during the growing seasons of 2009, 2010, and 2013, bringing, about the concern of sexual recombination and oospore formation. Fortunately these mating types were separated in space and time and were effectively managed. However, the concern of sexual reproduction remains, and the fact that both clonal lineages US-23 mating A1 and US-8 mating type A2 were detected in neighboring fields in Portage County is cause for continued and elevated concern. Constant monitoring of the population of *P. infestans* is important to optimize late blight management, to predict disease risk, and to eliminate chances of sexual reproduction.

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Table 1. SSR loci, primer sequences, dye, final concentration of primers, and product size range of each locus in *Phytophthora infestans* isolates.

SSR locus	Primer Sequence	Dye	Final conc. (µM)	Product size range
PiG11	F TGCTATTTATCAAGCGTGGG	NED	0.05	130 - 180
PiG11	R GTTTCAATCTGCAGCCGTAAGA		0.05	
PinfSSR3	F ACTTGCAGAACTACCGCCC	NED	0.05	255 - 275
PinfSSR3	R GTTTGACCACTTTCCTCGGTTC		0.05	
PinfSSR11	F TTAAGCCACGACATGAGCTG	NED	0.05	325 - 360
PinfSSR11	R GTTTAGACAATTGTTTTGTGGTCGC		0.05	
D13	F TGCCCCCTGCTCACTC	FAM	0.16	100 - 185
D13	R GCTCGAATTCATTTTACAGACTTG		0.05	
PinfSSR8	F AATCTGATCGCAACTGAGGG	FAM	0.3	250 - 275
PinfSSR8	R GTTTACAAGATACACACGTCGCTCC		0.3	
PinfSSR4	F TCTTGTTCGAGTATGCGACG	FAM	0.05	280 - 305
PinfSSR4	R GTTTCACTTCGGGAGAAAGGCTTC		0.05	
Pi04	F AGCGGCTTACCGATGG	VIC	0.05	160 - 175
Pi04	R GTTTCAGCGGCTGTTTCGAC		0.05	
Pi70	F ATGAAAATACGTCAATGCTCG	VIC	0.05	185 - 205
Pi70	R CGTTGGATATTTCTATTTCTTCG		0.05	
PinfSSR6	F GTTTTGGTGGGGCTGAAGTTTT		0.05	230 - 250
PinfSSR6	R TCGCCACAAGATTTATTCCG	VIC	0.05	
Pi63	F ATGACGAAGATGAAAGTGAGG	VIC	0.05	265 - 280
Pi63	R CGTATTTTCCTGTTTATCTAACACC		0.05	
PinfSSR2	F CGACTTCTACATCAACCGGC	PET	0.05	165 - 180
PinfSSR2	R GTTTGCTTGGACTGCGTCTTTAGC		0.05	
Pi4B	F AAAATAAAGCCTTTGGTTCA	PET	0.30	200 - 295
Pi4B	R GCAAGCGAGGTTTGTAGATT		0.30	

Table 2. Summary of the phenotypic and genotypic characteristics of *Phytophthora infestans* isolates collected in Wisconsin in 2013. For mefenoxam test: S= sensitive, R= resistance

County	No. isolates	Host	Plant tissue	Size of the *Gpi* alleles	Clonal lineages	Mating type	Mefenoxam *
Adams	4	Potato	Leaves	100/100	US-23	A1	S
	1	Tomato	Leaves	100/100	US-23	A1	S
Juneau	2	Potato	Leaves	100/100	US-23	A1	S
Sauk	2	Tomato	Leaves and fruits	100/100	US-23	A1	S
Dunn	1	Potato	Leaves	100/100	US-23	A1	S
Portage	3	Potato	Leaves	100/100	US-23	A1	S
	2	Potato	Leaves and stems	100/111/122	US-8	A2	R
	5	Tomato	Leaves	100/100	US-23	A1	S
Brown	2	Tomato	Leaves	100/100	US-23	A1	S
Langlade	4	Potato	Leaves	100/100	US-23	A1	S
	1	Tomato	Leaves	100/100	US-23	A1	S
Racine	2	Tomato	Leaves	100/100	US-23	A1	S
Waushara	2	Tomato	Leaves	100/100	US-23	A1	S
Milwaukee	3	Tomato	Leaves	100/100	US-23	A1	S
Forest	1	Tomato	Leaves	100/100	US-23	A1	S
Oconto	1	Tomato	Leaves	100/100	US-23	A1	S
Walworth	1	Tomato	Leaves and fruits	100/100	US-23	A1	S
Waukesha	1	Tomato	Leaves and fruits	100/100	US-23	A1	S
Polk	1	Tomato	Leaves and fruits	100/100	US-23	A1	S

* R = resistance to mefenoxam

S = sensitive to mefenoxam

Table 3. PCR product sizes observed for 12 loci in isolates of 4 clonal lineages of *Phytophthora infestans* amplified using 12 SSR fluorescent primers.

SSR locus	Clonal lineage						
	US-8 (2)*	US-22 (19)*	US-22a (2)*	US-23 (55)*	US-23a (1)*	US-24 (7)*	US-24a (2)*
PiG11	155	132	157	155	155	155	155
PiG11	155	157	157	155	155	155	157
PinfSSR3	268	268	268	268	268	268	268
PinfSSR3	270	270	270	270/272	270/272	270	270
PinfSSR11	341	341	341	331	341	331	341
PinfSSR11	356	341	356	241	356	341	341
D13	106	Null	135	142	142	106	106
D13	110	Null	135	142	142	110	110
PinfSSR8	262	262	262	262	262	262	262
PinfSSR8	268	268	268	268	268	268	268
PinfSSR4	290	285	285	288	288	285	285
PinfSSR4	294	294/296	294/298	294/296	290/296	288/296	288/292
Pi04	171	171	171	175	175	171	171
Pi04	175	175	175	175	175	175	175
Pi70	191	191	191	191	191	191	191
Pi70	191	194	194	191	191	194	194
PinfSSR6	246	246	246	246	246	246	246
PinfSSR6	246	246	246	246	246	246	246
Pi63	280	280	280	280	280	280	280
Pi63	280	280	280	280	280	280	280
PinfSSR2	173	173	173	173	173	173	173
PinfSSR2	173	175	173	175	173	173	173
Pi4B	215	215	215	215	215	219	214
Pi4B	228	215	215	220	220	228	228

*number of isolates in this group.

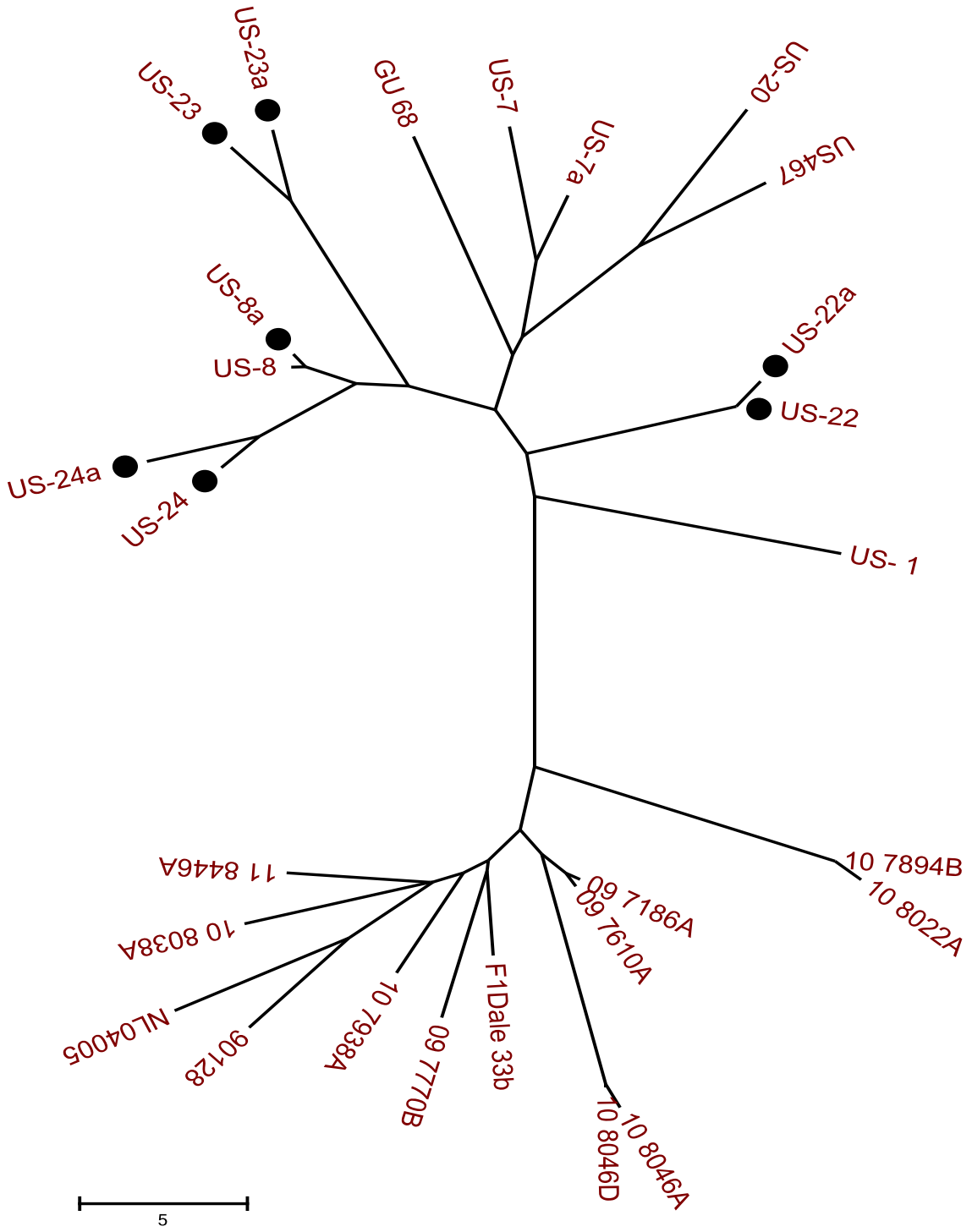

Figure 1. Dendrogram based on analysis of 12 SSR loci from diverse members of *P. infestans*. Isolates with black dot were collected in Wisconsin 2013. U.S. isolates for reference were provided by Dr. Bill Fry, Cornell University. The other SSR locus sizes were obtained from Li (28)

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Chapter 3

RB and Ph resistance genes in potato and tomato minimize risk for oospore production in the presence of mating pairs of *Phytophthora infestans*

Abstract

Late blight caused by the heterothallic oomycete *Phytophthora infestans,* is a main constraint of potato and tomato production worldwide. Currently, pathogen populations within the U.S. are primarily asexual with limited recombination or soil persistent oospores occurring. Both mating types, however, were isolated from Wisconsin during recent production seasons, posing a potential risk for oospore formation. Late blight resistance genes can be useful in offering a reduction in disease against the currently predominant *P. infestans* clonal lineages. To assess the potential for disease and oospore production with US-22 (A2), US-23 (A1), and US-24 (A1) isolates, hosts with different resistance genetics were inoculated with sporangial suspensions. Potato and tomato plants transformed with the *RB* gene and tomato 'Mountain Magic,' carrying *Ph-2* and *Ph-3* genes, were resistant to infection by representative isolates of each clonal lineage. Oospores were observed 15 days after co-inoculation with both mating pairs in leaf and stem epidermal tissue of all susceptible and moderately resistant hosts. No oospores were detected in tomato carrying *Ph2* and *Ph3* genes or potato and tomato with *RB.* Our findings suggest that the use of cultivars with *Ph2* and *Ph3*, and/or *RB* genes could be an important part of an integrated late blight management program by limiting the sexual phase of the life cycle. We demonstrated the potential for oospore production between recent clonal lineages of different mating types on susceptible cultivars. The deployment of *Ph* and *RB* resistance could reduce, if not eliminate, the risk of oospore production and sexual recombination thereby reducing further population variation and development of soilborne inoculum source of *P. infestans.*

Introduction

The oomycete *Phytophthora infestans* (Mont) de Bary, is a widely distributed phytopathogen and is the causal agent of late blight of potato (*Solanum tuberosum*) and tomato (*S. lycopersicum)* (18). Late blight was a key factor in the Irish Potato Famine, which occurred around 160 years ago, and continues to be a problem when susceptible crops are grown under cool and humid environmental conditions (17). In commercial potato production, late blight can cause serious economic losses as a result of yield reduction, post-harvest storage losses, and increased cost of fungicide applications (32,33). *Phytophthora infestans* is a heterothallic oomycete with two compatibility groups termed mating type A1 and type A2. When the two opposing mating types interact in or on infected leaves, stems, fruits, or tubers, this can result in the production of thick-walled, soil persistent oospores (13,19). Oospores can also be the product of self-pairing influenced by hormones when both mating types are present (34,37). Mating type A1 was the only type present in the US from the earliest report of the pathogen in 1843 up until 1987 (24). In 1987, one A2 mating type was detected in Pennsylvania (9) and in the 1990s clonal lineages US-7 and US-8 quickly replaced the A1 mating type as the most prevalent in the U.S. Despite the introduction of the A2 mating type in the U.S. and Canada and its coexistence with populations of A1 lineages, evidence of sexual recombination has been rare.

In addition to tomato and potato, the host range of *P. infestans* includes other members of the Solanaceae family including *Solanum betaceum* (tree potato), *S. quitoense* (naranjilla), *S. dulcamara* (bittersweet nightshade), *S. physalifolium* (hairy nightshade), and *S. ochrantum* (1,5,7,15). On susceptible crops, virulent clonal lineages of *P. infestans* can initiate an epidemic of late blight and, under continued favorable conditions, destroy the crop in 7 to 10 days (14,22). The use of resistant cultivars is an important component of an integrated late blight management program for both potato and tomato, and is the most economical and safest for human and environmental health.

For at least five decades, potato and tomato breeders have been developing cultivars with late blight resistance genes from wild *Solanum* species. In the 1950s the wild species *Solanum demissum* was the source of 11 race-specific genes for resistance to late blight in potato (53). However, after their release in commercial potato cultivars these resistance genes were overcome by virulent genotypes of *P. infestans* (45,47). In 2003, the late blight resistance gene *RB* was isolated from *S. bulbocastanum* but it is not currently used in commercially available varieties (50,54). A limited number of potato cultivars with late blight resistance are commercially available and include Jacqueline Lee (12), Missaukee (11), Defender (43), Palisade Russet (44), and Yukon Gem (55). However, these cultivars have not been widely adopted in commercial production. The wild tomato species *Solanum pimpinellifolium* (formerly *Lycopersicum pimpinellifolium)* is the source of three resistance genes termed *Ph-1*, *Ph-2*, and *Ph-3* (16,42). The dominant resistant gene *Ph-1* was identified in 1946 and five years later was overcome by the pathogen (4). Gene *Ph-3* which is incompletely dominant, confers partial resistance in tomato when it is exposed to different isolates of *P. infestans* (2).

At least five tomato cultivars, including Mountain Magic, Plum Regal, Mountain Merit, Defiant and most recently Iron Lady, with *Ph* resistance genes have been commercially deployed in production over the past few years with success in limiting late blight caused by isolates of new *P. infestans* genotypes (20,36). Late blight-resistant cultivars of tomato and potato are typically evaluated by screening the plants against single isolates of *P. infestans* genotypes. In some cases, multiple genotypes have been co-inoculated onto plants for a more comprehensive screen. To date, none of the studies have evaluated resistance to the oospore phase of the pathogen cycle when tissues were co-inoculated with both A1 and A2 isolates.

It is of continued importance to test putative late blight resistance against multiple isolates of new *P. infestans* genotypes, as the population of the pathogen change due to multiple factors including migration, natural mutation, genetic drift, and sexual recombination (3,28). Such factors contribute to the development of shifts in the population structure with greater genetic diversity resulting in new clonal

lineages with changes in pathogenicity that can potentially overcome host resistance as well as resistance to commonly used single site mode of action fungicides (24,40,46). Assessing the virulence of isolates of new genotypes of *P. infestans* on commercial potato and tomato cultivars with and without recognized resistance genes is important in developing non-chemical strategies to manage late blight.

In 2009, 2010, and 2013, both *P. infestans* mating types were isolated from late blight-symptomatic tomatoes and potato plant tissues from Wisconsin. However, mating pairs were not collected from individual fields at the same point in time (22). It is important to better understand the compatibility and the likelihood of oospore production, henceforth referred to as "oospore potential" of new clonal lineages, to optimize late blight management and more accurately assess disease risk. Oospores, whether sexually or asexually produced, impact disease epidemiology and the efficacy of management strategies.

The objective of this study was to assess the late blight response of select potato, tomato, and nightshade weed species, with and without known resistance genes. US-22, US-23, and US-24 *P. infestans* isolates were used to evaluate oospore formation on leaf and stem tissue of the selected solanaceous plant species co-inoculated with opposing mating type pairs.

Materials and Methods

Phytophthora infestans culture maintenance. One representative isolate from each of three clonal lineages collected from different commercial field epidemics in Wisconsin in 2010 was used in this study (Table 1) (22). Single zoospore cultures were generated from each of the axenic parental isolates and maintained on Rye A Agar media (3) amended with rifampicin and ampicillin. Oospore production in single-isolate culture was never observed. Isolates used for the survival experiment was transferred every four months in culture after isolation to maintain the original characteristics of the isolates (27,30,52). For long term storage, isolates were maintained in hemp seeds (38) in the Potato and Vegetable Pathology Laboratory at the University of Wisconsin-Madison. Single representative isolates of each *P. infestans* clonal lineage

were used since previous studies indicated that recently identified lineages showed minimal variation in biological characteristics (8,41)

Selection of solanaceous crop and weed species used in disease response and oospore studies. Potato and tomato cultivars were selected based on their reported late blight response and potential involvement since they are commonly planted cultivars. Tomato cultivars utilized in our work that were considered susceptible to late blight: 'Brandywine Red' (Johnny's Selected Seeds, Waterville, ME), is an heirloom variety; 'Green Zebra' (Johnny's Selected Seeds, Waterville, ME), is a uniquely colored and flavored small fruited tomato grown by some specialty direct market growers; 'Mountain Spring' (Totally Tomatoes, Randolph, WI), is a standard commercial variety with determinate growth; and 'VF36' (C.M.Rick Tomato Genetics Resource Center, UC Davis) has determinate growth and resistance to verticillium and fusarium wilts. Tomato cultivars considered resistant to late blight: 'Mountain Magic' (Johnny's Selected Seeds, Waterville ME) is a newly released commercial cultivar carrying two late blight resistance genes *Ph-2* and *Ph-3* (20); and 'Tomato *RB'* (Dennis Halterman, USDA, Madison WI) is an experimental line of VF36 transformed with the *RB* gene that is commercially available.

Potato cultivars used in the experiment: 'Russet Burbank' (WI Certified Seed Potato Grower, Antigo, WI) is a moderately late-blight resistant and commonly grown cultivar in the U.S.; 'Defender', 'Satina' (Jansky Potato Breeding Program, Madison, WI) both are moderately late-blight-resistant cultivar often grown for the organic market in the Midwestern U.S.; 'Jaqueline Lee' (David Douches, Michigan State University) is a US-8 late blight-resistant cultivar (12); 'Katahdin' (Halterman Lab, USDA ARS Madison, WI) is an old standard variety, with late blight susceptibility; and 'SP951' (Halterman Lab, USDA, ARS Madison, WI) is 'Katahdin', with a single copy of the *RB* resistance gene. The weeds *Solanum dulcamara* (bittersweet nightshade) and *Solanum physalifolium* (hairy nightshade) were also included in the experiment. Both species are common in and around commercial potato fields

in Wisconsin and were considered good candidate hosts for *P. infestans* (14,48). Both nightshade weeds were sourced from collections made from natural field infestation in Hancock, WI in 2010.

Late blight resistance assays. One plant of each cultivar of potato, tomato, and solanaceous weed as a replication in the experiment. Each plant was grown in a pot containing 500 g of peat moss in a late blight-free greenhouse at 26°C, for 5 weeks. On the day of inoculation, plants were moved to a mist chamber with conditions of 100% relative humidity, a temperature of 18°C during day and night, and natural ambient lighting.

To generate sporangial inoculum, single-zoospore-generated isolates were cultivated on Rye A Agar in 90-mm diameter Petri plates at 18°C for 21 days in complete darkness. Sporangia were collected from cultures by adding 5 ml of sterile water to each plate and gently scraping the agar surface with a glass spreading rod. Sporangial concentrations were calculated using a hemocytometer and adjusted to a concentration of 50,000 sporangia per ml. The sporangial suspensions were incubated for 2 h at 12°C under darkness to promote the release of zoospores. Three milliliters of zoospore inoculum suspension was applied to both abaxial and adaxial surfaces of the leaflets of each plant using a spray bottle. Disease reactions to *P. infestans* inoculation were evaluated at 11 days after inoculation (dai) with a categorical visual rating for each plant that was based on percentage of symptomatic tissue: 1: > 90%; 2: 81-90%; 3: 71-80%; 4: 61-70%; 5: 41-60%; 6: 26-40%; 7:11-25%; and 8 < 10% (6). The experiment was conducted twice, data were pooled across both experiments and statistical analysis was performed using an ANOVA and Kruskal-Wallis test.

Assessing oospore formation on detached leaflets: Plants were grown in a late blight-free greenhouse at 28°C, 80% relative humidity with a 16 hour photoperiod. Fully expanded leaflets were collected from the middle canopy of 6-week-old plants of each cultivar of potato, tomato, and solanaceous weed.

Phytophthora infestans sporangia were collected from Rye A agar media as previously described, and the suspension was adjusted to 5,000 sporangia per ml. Three detached leaflets were placed in a petri dish containing a moist paper towel. For each treatment, 6 leaflets were used (in 2 dishes). Inoculation was performed by applying 10 µl of sporangial suspension of opposing mating types 1 cm apart onto the leaf surfaces (adaxial). Two mating pair co-inoculations were evaluated: US-22 (A2) x US-23 (A1) and US-22 (A2) x US-24 (A1). Control inoculations of water and individual isolates of each clonal lineage were also performed. After inoculation, leaves in plates were incubated under complete darkness at 3 temperatures, 12°C, 16°C, and 20°C for 21 days. Five days after co-inoculation, distilled water was added to each dish to float the leaflets.

Leaflets were then removed from the dishes and tissue was clarified to remove chlorophyll by boiling in ethanol for 5 minutes. A glass slide was prepared for each clarified leaflet and oospores were observed and quantified by microscopy at 100x magnification, using a Zeiss Axio Scope A1 microscope (Gottingen, Germany). The area with the highest concentration of oospores was identified and oospores within an area of one square millimeter were counted for each leaflet. The experiment was conducted twice, data were pooled across both experiments. The data were analyzed using Two and Three Way Analysis of Variance with the SigmaPlot software (Systat Software, Inc. San Jose, CA, USA).

Assessing oospore formation on whole plants: This experiment was conducted to assess the ability of oospore formation in leaves and stem epidermal tissue of the solanaceous plants. Four plants of each cultivar of potato, tomato, and solanaceous weed were used in each treatment of the experiment. Plants were grown in a late blight-free greenhouse for 6 weeks at 22°C under 14 h lighting. On the day of inoculation, plants were moved to a mist chamber with 100% relative humidity and temperature of 18°C. *Phytophthora infestans* sporangia were collected from Rye A agar media as previously described, and the suspension was adjusted to 50,000 sporangia per ml. Each plant was inoculated by spraying 3 ml of a

sporangial suspension containing an equal mixture of opposing mating types onto leaves and stems with a spray bottle. Two mating type combinations were evaluated: US-22 (A2) x US-23 (A1) and US-22 (A2) x US-24 (A1). A single inoculation using US-22 was included as a control in the experiment, and plants sprayed with distilled water were used as a negative control. Plants were analyzed for oospore production at 8 and 15 dai by harvesting 50 leaflets from each plant and epidermal tissue of necrotic areas on stems. Plant tissues from each treatment were clarified in boiling ethanol for 5 minutes as previously described. A glass slide was prepared for each clarified plant tissue and oospores were quantified as previously described. The experiment was conducted twice, a statistical analysis was performed using a Two Way Analysis of Variance with the SigmaPlot software (Systat Software, Inc. San Jose, CA, USA).

Results

Late blight reaction of solanaceous whole plants to P. infestans. Inoculation of plants with US-22, US-23, and US-24 isolates of *P. infestans* resulted in severe late blight infection on potato, tomato, and solanaceous weed plants without *Ph-2*/*Ph-3* or *RB* genes (Figure 1). Cultivars VF36, Mountain Spring, Brandywine Red, Bonny Best, Green Zebra and Katahdin were susceptible to all pathogen isolates. Visible symptoms of late blight on these cultivars began at 3 dai, and by 7 dai, susceptible plants had 100% symptomatic tissue corresponding to a score of 1 for each of the three clonal lineage isolates. In contrast, tomato cultivar Mountain Magic, and the *RB*-transgenic tomato and potato plants exhibited small water soaked lesions consistent with a hypersensitive response (HR) on the lower leaves. The mean resistance score of tomato *RB* was 7.7, SP951 was 7.1, and Mountain Magic was 7.7. An ANOVA showed that there was no statistically significant difference (P=0.05) among resistance scores for each *P. infestans* isolate representing each of the three clonal lineages. Potato cultivars Russet Burbank and Jacqueline Lee each had a mean resistance score of 1.6 across *P. infestans* isolates US-22, -23 and -24. The mean resistance scores of potato cultivars Satina and Defender were both 2.8 across clonal lineages.

For cultivars Defender and Satina, inoculation with the US-23 isolate resulted in more disease than did inoculation with US-22 or US-24, numerically. Each representative isolate of the 3 novel clonal lineages of *P. infestans* was able to infect hairy nightshade and bitter nightshade. The mean score of resistance for bittersweet nightshade was 5.8; for hairy nightshade it was 5.2 across *P. infestans* lineages isolates (Figure 1). None of the water-inoculated control plants exhibited disease symptoms.

Oospore formation in detached leaves in vitro. Inoculation of detached leaflets, with pairs of opposite mating types of *P. infestans* resulted in the formation of oospores in plant tissue of most of the solanaceous species tested at each of three temperatures (Table 2 and figure 4). No oospores were observed at 3 weeks after inoculation of tomato with the *RB* gene or potato SP951 (with *RB*) with either mating type combination of *P. infestans* at 12°C. There was no statistical difference ($P<0.05$) between the number of oospores formed in tissues of tomato cultivar Mountain Magic, SP951, and tomato *RB* at 12°C and 20°C with any combination of mating types, or with the combination of US-22 x US-24 at 16°C. Both solanaceous weeds allowed oospore formation when inoculated with US-22 x US-23 and US-22 x US-24 (Table 2).

There were statistical differences ($P<0.05$) in the mean number of oospores produced in plant leaflets across different temperatures with the 2 combinations of clonal lineages of *P. infestans*. More oospores were formed in plant tissue after co-inoculation with US-22 x US-24, with a mean of 87.95 oospores/mm^2 compared to the combination of US-22 x US-23 with a mean of 77.74 oospores/mm^2. Statistically significant differences ($P<0.05$) were also identified among the mean number of oospores formed in leaflets across solanaceous species at different temperatures with 64.5, 105.2, and 80.0 oospores/mm^2 at temperatures of 12°C, 16°C, and 20°C, respectively. No oospores were observed in tomato or potato leaves inoculated with a single mating type isolate or with the water control.

Oospore formation in whole plants. Inoculation of plants with pairs of opposite mating types of *P. infestans* resulted in the formation of no oospores at 8 dai for all solanaceous species and temperatures tested. At 15 dai, however, oospores were present in leaves and stems of solanaceous weeds, and potato and tomato cultivars without *Ph-2*/*Ph-3* or *RB* genes. Oospores were counted in leaves attached to the plant and in leaves that had fallen off of plants that were in contact with the soil. In tomato 'Mountain Magic' and the *RB*-transgenic tomato and potato plants, no oospores were formed in the plant tissue (Figure 2). There was a statistically significant difference ($P<0.01$) in the mean value of oospores/mm^2 between plants without resistance genes *Ph-2*/*Ph-3* and *RB*, compared to plants with resistance genes, with mean values of 14.1 oospores/mm^2 and 0 oospores/mm^2, respectively. Susceptible tomato and potato were better hosts for oospores than the two Solanaceous weeds, with a mean of 14.1 oospores/mm^2 of leaf area of potato-tomato and 6.9 for Solanaceous weeds.

Among potato cultivars, Katahdin was the best host for oospore formation with 40% of leaflets containing oospores with a combination of US-22 x US-24, and 36% with a combination of US-22 x US-23. Among tomato cultivars, Green Zebra had the highest percentage of leaflets containing oospores, with 35% hosting oospores when inoculated with US-22 x US-23. Tomato cultivar Bonny Best had 33% of the leaflets with oospores with a combination of US-22 x US-24 (Figure 3). No oospores were observed in tomato and potato leaves inoculated with a single mating type isolate or with the water control.

Discussion

Potato and tomato plants expressing the *RB* gene and tomato cultivar Mountain Magic, expressing the *Ph-2* and *Ph-3* genes, were resistant to representative isolates of three clonal lineages of *P. infestans* (US-22, US-23, and US-24) collected from Wisconsin. The resistance present in these hosts blocked the formation of oospores in plant tissue of potato and tomato inoculated with the two mating types pairs.

We found that US-22, US-23, and US-24 *P. infestans* isolates were virulent and able to produce oospores when co-inoculated with the opposite mating type on potato cultivars Satina, Defender and Jacqueline Lee. These three cultivars have been previously reported as resistant to late blight infection (12,43). The ability of *P. infestans* to infect and carry out sexual recombination on these cultivars indicates the necessity for further protection of crops in areas where complementary lineages of *P. infestans* are present. To date, opposite mating types have not been identified in the same field, but they were identified in neighboring fields in Portage County, Wisconsin in 2013. Constant monitoring and managing of late blight through the use of varietal resistance and fungicides is essential in order to efficiently and effectively control late blight and maintain geographical separation of mating types.

The *RB* gene has been classified as a resistance gene conferring broad-spectrum resistance to isolates of multiple clonal lineages of *P. infestans* including US-1, US-1.7, US-8, US-10, and US-14 (50). RB resistance can be overcome by isolates of clonal lineages containing certain combinations of virulence factors, but isolates with this ability are rare and have not been identified in the U.S. (29). In this study, the phenotype observed in plants carrying the *RB* gene indicated that the isolates of lineages US-22, US-23, and US-24 of *P. infestans* that we investigated were not able to overcome resistance. Each representative isolate of the US-22, US-23, and US-24 clonal lineage of *P. infestans* was able to infect the two solanaceous weeds, which supports a 2010 report of late blight infection on *S. dulcamara* caused by US-22 (10). Based on these results, there is a potential risk that both solanaceous weed species can be reservoirs of *P. infestans* even in the absence of potato and tomato hosts in the field, leading to a source of inoculum that could spread to commercial plantings during the growing season.

The reproduction of *P. infestans* currently in the U.S. is considered to occur primarily asexually, which has led to relatively uniform populations of each clonal lineage (17). Until 1987, only *P. infestans* mating type A1 was present in the U.S., at which time one A2 mating type was detected in Pennsylvania (9). In subsequent years, a greater number of *P. infestans* isolates belonging to the A2 mating type were detected in field samples from several states (25), coinciding with increased disease severity in the field.

In Wisconsin, the presence of both mating types, capable of producing oospores in leaves and epidermal tissues of the stems of susceptible cultivars under laboratory and greenhouse conditions, indicates the potential for increased variation in the population, longer term soil persistence, and greater epidemiological impact of late blight. The occurrence of sexual recombination in the pathogen population poses a serious risk for development of fungicide resistance and increased virulence.

Solanaceous weeds, such as hairy nightshade and bitter nightshade, have been reported to be hosts of *P. infestans* under natural conditions. However, a relatively low number of oospores and low percentage of leaflets containing oospores were observed on these hosts in this work. Moderate resistance in bitter nightshade could be explained by the presence of the resistance gene *Rpi-dlc*, which has been isolated from some accessions of *S. dulcamara* in Europe, and has been shown to provide partial resistance to late blight (23). No resistance genes have been identified in *S. physalifolium* and therefore it is difficult to speculate whether reduced oospore formation is due to the presence of a specific resistance gene or whether hairy nightshade is a poor host species. The virulence of other isolates of US-22, US-23, and US-24 could differ slightly from the results presented here since several factors can affect virulence, such as genetic diversity within each clonal lineage, plant age, method of inoculation, pathogen culture conditions, protocol, environment conditions, and genetic variation in *Solanum* weed species (51).

Investigation of a single representative isolate of each *P. infestans* clonal lineage was appropriate for this study, as virulent variation in non-sexually recombining populations of *P. infestans* has been shown to be low. Goodwin et al. (26), concluded that only 37% of the variation in virulence that they found in a *P. infestans* population was from within lineages, but the majority was from between lineages. Mizubuti and Fry (41) also concluded that there was very little variation in several biological characteristics for isolates belonging to the same lineage. 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 (26,39). The US-22, US-23, and US-24 lineages were only recently identified (31,35,49) and Danies et al. (8) showed low variation in several biological characteristics

among isolates of US-22, US-23 and US-24. As such, testing of single representative isolates from each clonal lineage is adequate for profiling the current host range status of the population. Despite this, it is important to note that *P. infestans* is a pathogen that is constantly evolving. History has repeatedly shown that major resistance genes deployed in a commercial cultivar will eventually be overcome due to changes within the *P. infestans* populations and subsequent selection on the cultivar with resistance. As such, a strategy of gene pyramiding in a breeding program should be considered in order to maintain functional and durable *RB, Ph-2* and *Ph-3* genes.

Our results suggest that the use of *RB*-transgenic Solanaceous plants and use of cultivars containing *Ph-2* and *Ph-3* genes could be an important part of an integrated management program for late blight, with less reliance on fungicide applications. Additionally, the prevention of oospore formation in the tissues of these resistant Solanaceous plants provides an important late blight management strategy reducing soil persistence and further variability within-pathogen populations.

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Table 1. Characteristics of *Phytophthora infestans* isolates used in this study. All isolates were collected from symptomatic plant tissue in 2010.

Clonal lineage	**WI county of collection**	**Mating type**	**Host**
US-22	Vernon	A2	Tomato
US-23	Waukesha	A1	Tomato
US-24	Waushara	A1	Potato

Table 2. Mean number of oospores (per square mm of leaf tissue) detected in leaf tissues of solanaceous plants co-inoculated with *P. infestans* mating pairs at 3 weeks after inoculation.

Host[1]	***P. infestans* pair**	**Mean # of oospores ± standard deviation**					
		12°C		**16°C**		**20°C**	
Mountain Magic	US-22 x US-23	2	±3	11	±7	0	0
	US-22 x US-24	1	±2	4	±5	2	±3
Tomato RB	US-22 x US-23	0	0	1	±1	0	0
	US-22 x US-24	0	0	2	±3	1	±2
SP 951	US-22 x US-23	0	0	2	±2	0	0
	US-22 x US-24	0	0	1	±3	1	±3
Bittersweet	US-22 x US-23	10	±9	10	±9	6	±6
nightshade	US-22 x US-24	12	±10	5	±4	9	±7
Hairy nightshade	US-22 x US-23	16	±8	18	±13	11	±6
	US-22 x US-24	6	±5	82	±35	15	±9
Defender	US-22 x US-23	4	±3	4	±2	6	±4
	US-22 x US-24	3	±3	8	±4	5	±4

Satina	US-22 x US-23	14	±8	9	±6	12	±7
	US-22 x US-24	4	±3	4	±2	15	±9
Russet Burbank	US-22 x US-23	156	±25	275	±63	93	±25
	US-22 x US-24	170	±27	86	±12	140	±11
Jacqueline Lee	US-22 x US-23	123	±15	262	±79	101	±24
	US-22 x US-24	133	±24	167	±66	120	±18
Katahdin	US-22 x US-23	148	±15	195	±29	99	±27
	US-22 x US-24	149	±27	235	±28	138	±10
VF36	US-22 x US-23	195	±17	195	±16	129	±30
	US-22 x US-24	175	±20	258	±14	153	±17
Mountain Spring	US-22 x US-23	147	±33	191	±40	110	±24
	US-22 x US-24	171	±37	199	±46	139	±24
Brandywine Red	US-22 x US-23	108	±27	132	±34	74	±26
	US-22 x US-24	116	±32	244	±34	117	±15
Bonny Best	US-22 x US-23	121	±39	118	±38	79	±21
	US-22 x US-24	129	±29	158	±27	139	±16
Green Zebra	US-22 x US-23	144	±41	121	±24	68	±35
	US-22 x US-24	142	±43	258	±42	154	±26

[1] Hosts were ordered based on their placement within Figure 1 (most resistant to most susceptible)

Figure Legends

Figure 1. Resistance score of different solanaceous hosts eleven days after inoculation with the US-22, US-23 and US-24 clonal lineages of *P. infestans*. The resistance score was determined based on the percentage of symptomatic tissue: 1: > 90%; 2: 81-90%; 3: 71-80%; 4: 61-70%; 5: 41-60%; 6: 26-40%; 7:11-25%; and 8 < 10% (6).

Figure 2. Mean number of oospores (per mm^2 of leaf tissue) generated from crosses of *P. infestans*, clonal lineages US-22 x US-23 and US-22 x US-24, on species of tomato, potato, and solanaceous weeds. Oospores were counted from both attached leaf tissues and leaf t tissue hat had fallen to the soil at 15 days post inoculation.

Figure 3. Percentage of leaflets with oospores generated from co-inoculation of *P. infestans*, clonal lineages US-22 x US-23 and US-22 x US-24, on solanaceous species.

Fig. 4. Appearance of a typical oospore observed in leaf tissue of susceptible solanaceous host with US-22 x US-23 and US-22 x US-24 co-inoculations**.**

Figure 1

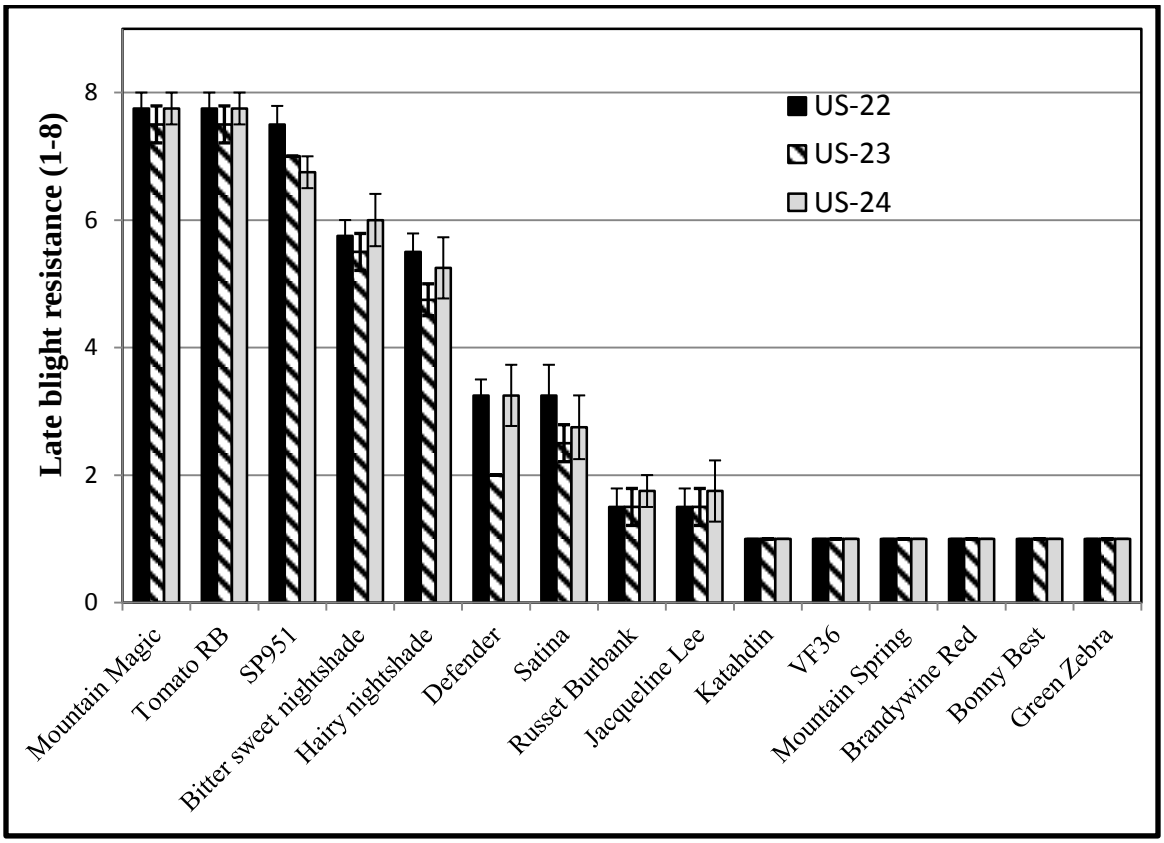

Late blight resistance (1-8)
8
6
4
2
0
US-22
US-23
US-24
Mountain Magic
Tomato RB
SP951
Bitter sweet nightshade
Hairy nightshade
Defender
Satina
Russet Burbank
Jacqueline Lee
Katahdin
VF36
Mountain Spring
Brandywine Red
Bonny Best
Green Zebra

Figure 2

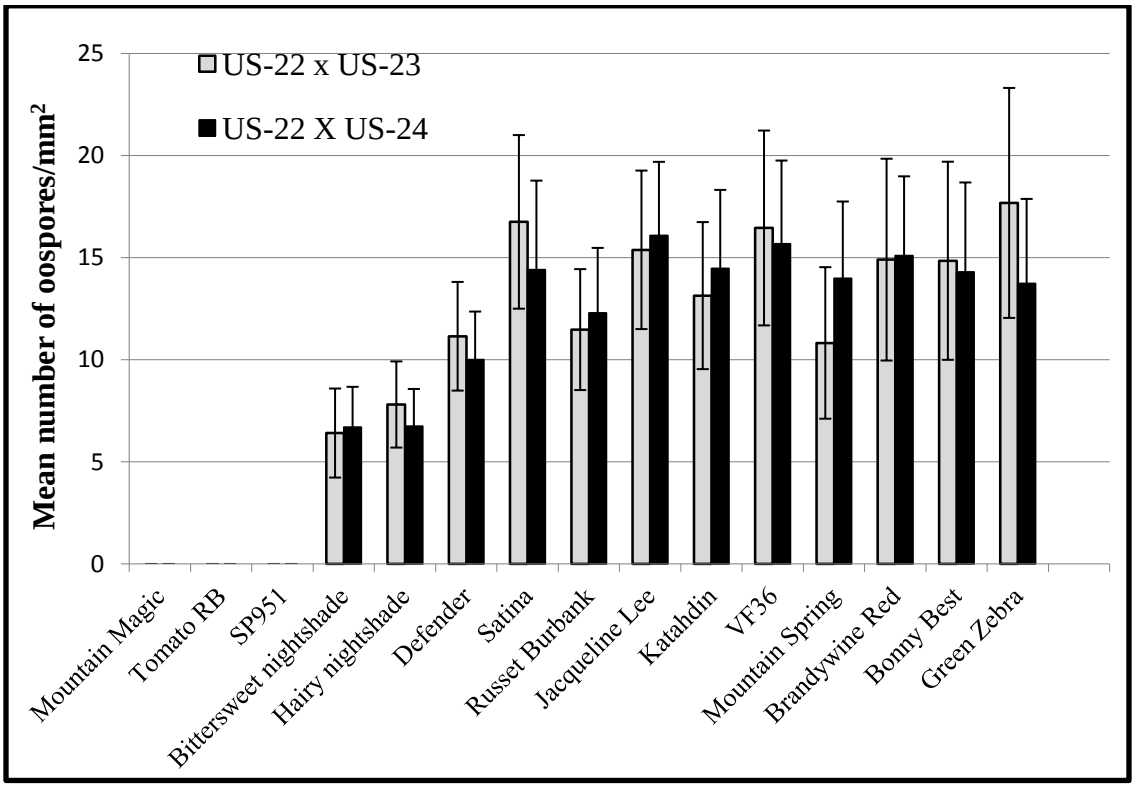

US-22 x US-23
US-22 X US-24
Mean number of oospores/mm²
25
20
15
10
5
0
Mountain Magic
Tomato RB
SP951
Bittersweet nightshade
Hairy nightshade
Defender
Satina
Russet Burbank
Jacqueline Lee
Katahdin
VF36
Mountain Spring
Brandywine Red
Bonny Best
Green Zebra

Figure 3

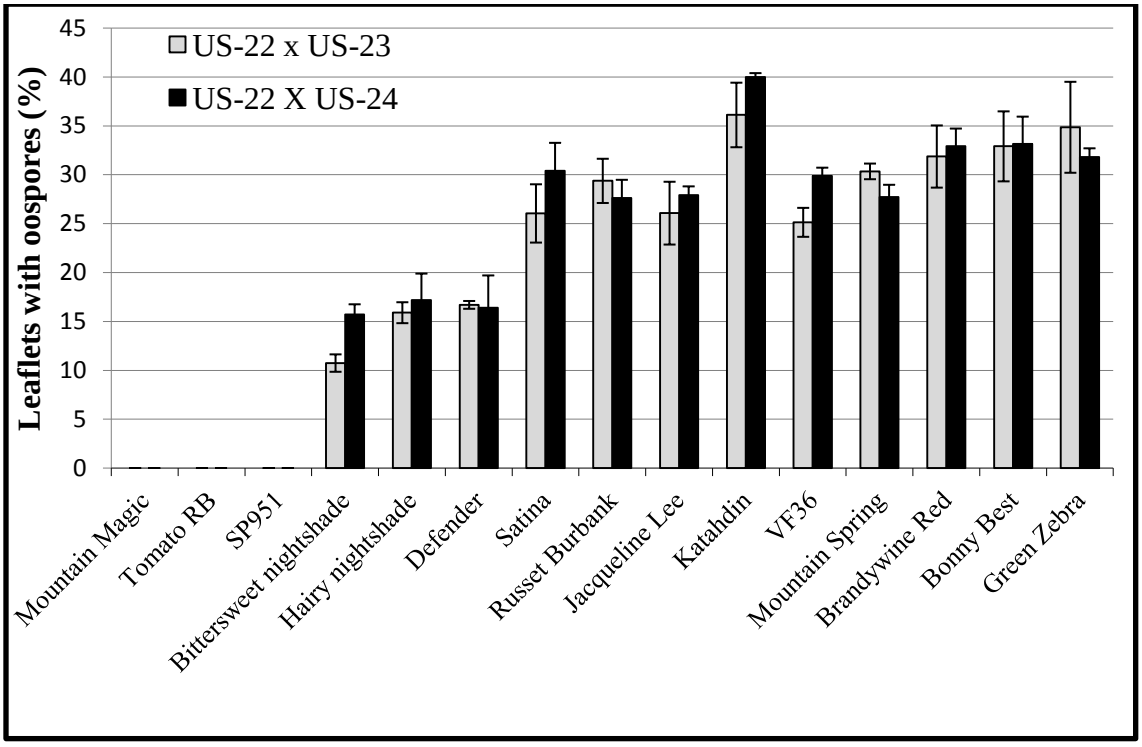
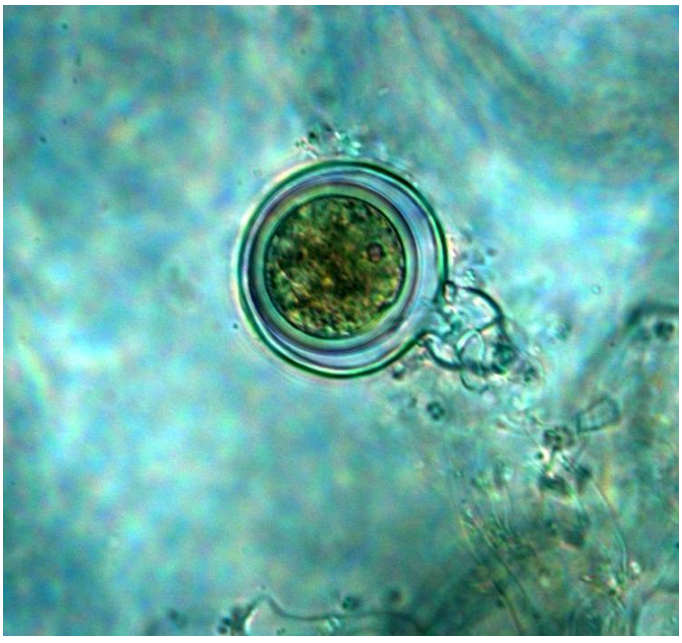


Figure 4



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Chapter 4

Survivability and infectivity of oospores generated by mating pairs of *Phytophthora infestans* collected in Wisconsin

Abstract

Phytophthora infestans, is a heterothallic oomycete causing late blight of potato and tomato. The pathogen can produce oospores when both mating types A1 and A2 have physical interaction. In the history of late blight in the U.S., *P. infestans* populations have been largely asexual and detection and tracking of clonal lineages has aided in rapid pathotype characterization and prescriptive management. However, in other countries, sexual recombination occurs making for increased strain diversity, soil persistence, and enhanced challenge in disease control. In our study, oospores were produced under controlled laboratory conditions in tomato leaflets by crossing two mating pairs of *P. infestans* collected from Wisconsin, during 2009-2013. US-22 (A2) x US-23 (A1) and US-22 x US-24 (A1). Extracted oospores were then incubated for 5 months under 6 different temperature regimes including one in soil under natural field conditions in central Wisconsin, from Nov to Mar of 2011-12 and 2012-13. The remaining 5 temperatures were controlled in the laboratory and ranged from 22° C to -20° C. Post incubation, the viability of oospores was determined using plasmolysis and vital staining methods. Our results showed that oospores were viable after being exposed to natural field winter conditions with temperatures ranging from 5° C to -10° C at depths from 2 inches to 7 inches. Both viability assessment methods indicated a reduction in percent viability with increased cold conditions. Oospores incubated at 22°C had the highest percent of viability of roughly 23% compared to just 5% viability after incubation at -20°C. In our assessments of infectivity, we determined that oospores exposed to different cold temperatures could be a source of inoculum and initiate late blight on tomato leaflets. Oospores incubated at 22°C caused late blight lesions in 9% of tomato leaflets. Averages of 4% of leaflets were infected in the bioassay with soil exposed to winter in field conditions. Oospores could not initiate late blight infection

after exposure, in soil to -20°C. Nor were oospores able to initiate disease on potato plantlets emerging from whole seed potatoes planted into oospore-infested soil. We demonstrated the potential for oospores produced by new clonal lineages of *P. infestans* to survive and remain infective after incubation under freezing temperatures in controlled laboratory environments, as well as under field soil conditions during two Wisconsin winters 2011-12 and 2012-13.

Introduction

Late blight caused by *Phytophthora infestans* (Mont) de Bary, continues to be one of the most devastating diseases of potato (*Solanum tuberosum*) and tomato (*S. lycopersicum)* crops worldwide (6,10). The oomycete pathogen can infect leaf, stem, and tuber tissues of susceptible potato; and leaf, stem and fruit tissues of susceptible tomato (8). *Phytophthora infestans* is heterothallic with two compatibility groups termed mating type A1 and A2. Oospore formation is induced when hyphae from isolates of opposite mating types interact in or on infected plant tissues (12,26). While asexual sporangia and zoospores are the primary source of ephemeral inoculum for polycyclic development, reproduction, and dispersion of the pathogen within a growing season here in the U.S., sexually derived oospores can create a genetically diverse, long-term, soil persistent source of inoculum for many years as is occurring in several countries (1,6,7,8,9,37). The production of antheridia and oogonia within a single isolate of *P. infestans* can be stimulated by hormones when an opposing mating type is also present in the plant tissue, leading to the formation of oospores as products of self-pairing (24,26).

Oospores provide two important functions in the disease cycle of *P. infestans.* First, they are pathogen survival structures during times when no host plants are present in production

fields. Secondly, oospores may be products of sexual recombination and may introduce greater genetic and phenotypic diversity into the pathogen population. Diverse phenotypes may include differences in virulence, host range, fungicide resistance, and other critical ecological and epidemiological characteristics which influence fitness (7,8,39).

While the center of origin and diversity of *P. infestans* can be evidenced in the Toluca Valley and in the Peruvian Andes, where both mating types are present in a 1:1 ratio of the two mating types termed A1 and A2 (14,18); in the Toluca Valley oospores are readily produced under natural field conditions and can remain infective on potato for up 24 months (12,18,32,33). In a study conducted in the Netherlands, Turkensteen et al (37), reported viability of oospores in natural sandy soil conditions for up to 48 months. Recombinant oospores from crosses of clonal lineages US-17 (A1) and US-8 (A2) were able to survive and maintain their infectivity on tomato and potato leaves after exposure to natural winter conditions in New York, U.S. (29). In a study performed in Central Mexico by Fernandez-Pavia, soilborne oospores of *P. infestans* survived the winter period and were infective to potato plants in the field (9). Oospores of *P. infestans* remained viable and infectious after exposure to natural winter weather in the Netherlands during 1992-93, and subsequently incubated under 5 controlled temperatures between 35°C and -80°C for 48 hours (6).

From the earliest reports of late blight up until 1987, mating type A2, was absent in U.S. pathogen populations (5,15) and there was no opportunity for sexual reproduction and oospore formation. Recent introduction and migration of new clonal lineages have brought about the presence of the A2 mating type in potato and tomato production fields in the U.S. (5,22,35). While isolates of the A2 mating type have been reported in the U.S. since 1988 (5,30), sexual reproduction has not been detected with significance in the U.S. (16). During the last 7 years,

populations of *P. infestans* in the U.S. consisted of a mixture of clonal lineages US-8 (A2), -11 (A1), -22 (A2), -23 (A1), and -24 (A1) (4,22,34), creating the risk for oospore formation in several potato and vegetable production regions. In Wisconsin, we have identified opposing mating types of *P. infestans* within individual counties, but not in the same field. The proximity of opposing mating types poses great risk for oosporic soilborne inoculum and for further genetic diversification that could impact the epidemiology and management of potato and tomato late blight. This scenario makes up a serious threat to potato and tomato production in traditional agricultural systems. With the recent persistence of the US-23 clonal lineage, current research is needed to better understand the risk of oospore production, viability, and infectivity in crosses with relatively novel lineages.

The objectives of this study were to investigate the viability of progeny oospores formed by crosses of current predominant *P. infestans* clonal lineages exposed to winter field conditions and controlled freezing temperature under laboratory conditions, and to evaluate the infectivity of progeny oospores on potato and tomato tissues.

Materials and Methods

Phytophthora infestans *isolates selection, culture maintenance and inoculum preparation.* One representative isolate from each of 3 *P. infestans* clonal lineages collected from different commercial field epidemics in Wisconsin in 2010 were used in this study (Table 1) (34). Single zoospore cultures were generated from each of the axenic parental isolates and maintained on Rye A Agar media (2) amended with rifampicin and ampicillin for use in our experiments. Oospore production in single-isolate culture was never observed. Isolates used in this study were

transferred minimally in culture after isolation to maintain the original characteristics of the isolates. For long term storage, isolates were maintained in hemp seeds (17) in the Potato and Vegetable Pathology Laboratory at the University of Wisconsin-Madison. Single representative isolates of each *P. infestans* clonal lineage were used since previous studies indicated that recently identified lineages showed low variation in biological characteristics (4,22,34).

Sporangial inoculum, for use in oospore production experiments, was generated from single-zoospore-derived isolates cultivated on Rye A Agar in 90-mm-diameter petri plates at 18°C for 21 days in complete darkness. Sporangia were collected from cultures by adding 5 ml of sterile water to each plate and gently scraping the agar surface with a glass bacterial spreading rod. Sporangial concentrations were calculated using a hemocytometer with compound microscopy and adjusted to a concentration of 50,000 sporangia per ml. The sporangial suspensions were incubated for 2 h at 12°C under darkness to promote the release of zoospores.

Oospore production on tomato leaflets. Oospores were generated in leaf tissues of late blight-susceptible 'Brandywine Red' tomato (Johnny's Selected Seeds, Waterville, ME) (35). Tomato plants were grown in a late blight-free greenhouse at 24°C, under 80% relative humidity, with a 16 hour photoperiod. Eighty fully expanded leaflets were collected from the middle canopy of 6-week-old plants. For each treatment, 40 detached leaflets were placed in an enclosed plastic container with moistened sterilized paper towels for enhancing humidity. Inoculations were performed by spraying 3 ml of sporangial suspension, previously described, containing a 1:1 ratio of A1 and A2 mating type inoculum using a 100 ml plastic spray bottle. Two mating type

combinations were evaluated: US-22 (A2) x US-23 (A1) and US-22 (A2) x US-24 (A1). After inoculation, distilled water was added to each dish to float the leaflets and inoculated tissues were incubated under complete darkness at 18°C for 35 days. Oospores which formed in the leaflet tissues were extracted by collecting all of the leaflet tissue and blending them in 100 ml of sterile deionized water at high speed for 30 seconds in a commercial food blender, Waring (Model 51BL16, Waring Laboratory Science, Torrington, CT). Oospores were separated by sequential filtering through 100 and 45-µm sieves, in addition to a 20-µm nylon filter and then washed with sterile deionized water and re-suspended in 10 ml sterile deionized water. To ensure that any residual mycelium was destroyed, oospores were subjected to two rounds of freezing, thawing, and drying by keeping them at -5°C for 24 h, thawing 1 h, and following by desiccation for 5 days at 22°C (9,37). Oospores were then re-suspended in 20 ml sterile deionized water and the concentration of oospores was calculated using a hemocytometer with microscopy and adjusted to 10,000 oospores per ml. To confirm the viability of oospores generated in tomato leaflets in the laboratory, oospore suspensions were inoculated onto water agar plates and incubated at 22°C and constant lighting to promote germination. Germinated oospores were transferred to Rye A Agar media amended with rifampicin and ampicillin. Mycelia and sporangia from the germinated oospores were observed using a microscope and confirmed as *P. infestans* based on morphological features (13).

Assessing overwinter survival of oospores in the field and under freezing temperatures in the laboratory: The viability of oospores under natural field soil conditions was evaluated from November 1 to April 1 to represent winter conditions of 2011-2012 and 2012-2013 in a contained field experiment at the University of Wisconsin Hancock Agricultural Station

(HARS), Hancock, Wisconsin. An experiment of a completely randomized design with 3 treatments and 3 replicates was used. Treatment 1 was comprised of oospores of the cross US-22 (A2) x US-23 (A1). Treatment 2 was comprised of oospores of the cross US-22 (A2) x US-23 (A1) and, treatment 3 was our negative control containing no oospores. Each replicate consisted of one plastic 5-gallon bucket containing sandy loam field soil holding 2 experimental pots, each containing 500 g of sandy loam field soil collected from an area cropped to field corn for 5 consecutive years at the HARS. To facilitate drainage, each bucket had six 1-cm holes cut into the bottom. Approximately 20,000 oospores were mixed with 500 g of sandy soil and placed in each plastic pot. To avoid dispersion of the oospores by wind, a layer of 2 cm of soil without oospores was distributed on top of each pot. Field sandy soil was used to create a 10 cm layer in the bottom of each bucket and to fill the space between the two pots inside of each bucket. The 5-gal buckets were buried with the top edge at ground level. The temperature at 2 inches depth of soil was measured during the experiments, with a Watch Dog B Series temperatures buttons (Spectrum Technologies Inc., Aurora, IL).

For the evaluation of oospore survivability at freezing temperatures in the laboratory, oospores were incubated at 5 temperatures (22°C, 0°C, -5°C -10°C, -20ºC), for 5 month intervals, similar to the time period for the field overwintering experiments. Viability experiments were established using a completely randomized design with 3 treatments and 3 replicates. Treatment 1 was comprised of oospores of the cross US-22 (A2) x US-23 (A1). Treatment 2 was comprised of oospores of the cross US-22 (A2) x US-23 (A1). And, treatment 3 was our negative control containing no oospores. Each replication consisted of 2 Zip Lock plastic bags, with each bag containing 100 g of sandy soil and 20,000 oospores homogeneously dispersed throughout the soil. All plastic bags were closed and incubated at specified

temperatures. For incubation at 22°C plastic bags were placed in a Percival incubator (Model 136NL, Percival Scientific, Inc. Perry, IA). For incubation at 0, -5 and -10°C, plastic bags with inoculated soils were placed in a Programmed Refrigerator Incubator (Hotpack Corp. Phila, PA, USA). To achieve -20ºC incubation, bags with inoculated soil were placed in a Frigidaire freezer (Electrolux, Inc., Cleveland, OH). Temperatures in the incubators were continuously measured using a Watch Dog B Series temperatures buttons (Spectrum Technologies Inc., Aurora, IL). During the time of the experiments, temperatures fluctuated no more than 1ºC from the desired temperatures.

Oospores extraction from soil: To assess the viability of oospores exposed to different temperatures, oospores were first extracted from soil by adding 70 ml of distilled water to 10 g of infested soil in 100-ml beaker followed by stirring with a glass bar. The resulting soil suspension was then passed through two sieves with pore sizes of 100 and 40-µm, and a filter paper with pores sizes of 20-µm. The residue collected on the 20-µm filter paper was re-suspended in 20 ml distilled water and immediately added to 30 ml of a 70% sucrose solution for separation from soil particles by centrifugation at 1000 G for 3 min (11).

Assessing oospore viability: Two methods were used to evaluate the viability of oospores after incubation at different temperatures. The first method was a vital or viability stain using tetrazolium bromide (MTT = 3-(4,5-dimethylthiazol-2)-2,5-diphenyl-2H-tetrazolium bromide) (36). The second method was a plasmolysis approach using 2 M NaCl (21). To establish non-viable control treatments, 400 freshly harvested oospores from tomato leaves were boiled in

water for 15 minutes and half of them were used as nonviable oospore controls for each method of viability testing. For evaluation of oospore viability 400 oospores from each replication were extracted from soil, as previously described, and 200 oospores were isolated and used for each viability method. For the vital stain method, 200 oospores from each replication were incubated in 0.1% MTT in 0.01 M phosphate buffer (pH6.2) and incubated at 35ºC for 48 hours. After the incubation period, oospores were microscopically examined using a Zeiss Axio Scope A1 microscope (Gottingen, Germany). During the viability test, tetrazolium compounds are reduced by the hydrogen enzymatic transports system of the cell to form an insoluble pink or red compound called formazan and cells become stained (31). Pink-red stained oospores were counted as viable, and black or non-stained were counted as nonviable (11). For the evaluation of oospore viability using the plasmolysis method, 200 oospores from each replication were incubated in 1 ml of 2 M NaCl for 60 minutes. The plasmolysis process occurs when a living plant cell is immersed in a highly concentrated solution of salt or sugar, and the water from the viable cell (oospore) diffuses out of the cell, moving from an area with low concentration to an area with high concentration of solute. Plasmolyzed cells lost their turgor pressure and became flaccid and shrunken. Non-viable oospores lost the differential permeability of the cellular membrane (23). After the incubation period in 2M NaCl, oospores were examined using a Zeiss Axio Scope A1 microscope (Gottingen, Germany), and plasmolyzed oospores were considered as viable. Viability experiments were replicated two times.

Assessing oospore infectivity on potato minitubers. To investigate whether oospores exposed to natural and controlled laboratory freezing conditions were infective, potato plantlets were inoculated in a set of mist chamber experiments. Soil containing oospores from the previously

described survival experiments in the laboratory were homogenized with 400 g of autoclaved sandy loam soil and placed in plastic pots. Pots containing oospore-infested soil were placed in a mist chamber with conditions of 100% relative humidity, a temperature of 18°C, and natural ambient lighting on 1 April 2012 and 1 April 2013. Using a method described by Fernandez-Pavia, et al (9), four minitubers (1 cm diameter) of potato cultivar Russet Burbank, produced by the University of Wisconsin Seed Potato Certification Program, were planted in each pot. Potato plantlets were examined for late blight lesions on the stems and leaves up to 60 days after planting. On the last day of the experiment, daughter tubers from the pots were harvested and visually assessed for late blight symptoms.

Assessing infectivity of oospores in tomato leaflets: In order to evaluate the viability of oospores from the previously described freezing conditions experiments, spore-baiting bioassays were performed (6). Pots containing oospore-infested soil were placed in plastic containers and distilled water was added to each pot to flood up to 3 cm above the soil level. Five tomato leaflets collected from the middle canopy of cultivar Brandywine Red were placed upside down on the water surface in each pot, and subsequently replaced every 5 days until day 25. Exposed leaflets were placed in petri dishes containing a moist paper towel. Two leaflets in each petri plate were incubated under complete darkness at 18ºC and examined for late blight lesions every two days for 16 days. Leaflets were rated for disease incidence as late blight infected, or not infected. The experiment was repeated 2 times.

Results

Germination and viability of fresh harvested oospores from tomato leaves. Fresh harvested oospores generated from pairs of *P. infestans* clonal lineages US-22 x US-23 and US-22 x US-24 were viable and were able to germinate (Table 2). The mean percent of viability of non-boiled oospores from mating pair US-22 x US-23, was 84% using the vital stain test and 76% using the plasmolysis test. For oospores from the mating pair US-22 x US-24 percent viability was 81% and 78% using the vital staining test and the plasmolysis test, respectively. Evaluation of viability of boiled oospores in the non-viable control treatment using the vital staining method gave false positive results with mean percent viability of 5.5% and 7.5% for mating pairs US-22 x US-23 and US-22 x US-24, respectively. False positives were not obtained with the plasmolysis method and so data from this particular test were utilized for further discussion.

The mean percent oospore germination on water agar was 39% for oospore progeny from US-22 x US-23, and 34% for progeny from US-22 x US-24. There were no statistical differences ($P<0.05$) between the percent of oospore germination generated by either combination of mating types. There were no statistical differences ($P<0.05$) between the percent viability oospore progenies from the *P. infestans* mating pairs of US-22 x US-23 and US-22 x US-24. There were statistical differences ($P<0.05$) between the percent viability of oospores tested with the plasmolysis method and the percent viability of oospores tested for germination on water agar. The percent viability was greater than the percent germination on agar.

Overwinter survival of oospores in the field and under freezing temperatures in the laboratory.

Oospores exposed to natural field weather conditions during winter or incubated at 5 different temperatures in the laboratory during the periods of 2011-12 and 2012-13, were viable when evaluated by both vital staining and plasmolysis methods, (Fig. 2). There were no statistical differences ($P<0.05$) between the mean values of oospore viability from two years of the study and no statistical differences ($P<0.05$) were determined between survivability of oospores generated by either *P. infestans* mating pairs (Fig.2). The mean numbers of viable oospores obtained with the vital staining method using tetrazolium bromide were consistently higher with oospores of both mating type crosses than the mean values recorded using plasmolysis with a mean value of 23.0 and 15.9, respectively. Oospores incubated at a temperature of 22ºC had the highest mean viability obtained with the plasmolysis method, compared with the mean values of oospores incubated at different freezing temperatures, indicating that oospore viability was affected significantly ($P<0.05$) by the freezing temperatures. There was no significant difference ($P<0.05$) between the mean viability of oospores incubated at 0 ºC, -5 ºC and under winter weather conditions in the field with either viability assessment method. Overall, oospore viability decreased with decreasing temperatures from a mean of 28% viable at 22 ºC to just 6.9% at -20 ºC.

The minimum daily soil temperatures recorded during the 5 month interval of the field study (November 1 to April 1) 2011-2012 and 2012-2013, at 2 inches deep were between 5º and -5ºC with the exception of an extremely cold period in February 2013, where the below-ground temperature reached -11ºC (Fig. 5).

Assessing oospore infectivity on potato minitubers. Potato plants from minitubers grown in oospore-infested soil did not show any symptoms of late blight 60 days of planting with either oospore progeny from *P. infestans* US-22 x US-23 and US-22 x US-24. Daughter tubers generated from the plants were exhumed and exhibited no symptoms consistent with tuber late blight at 60 days after planting.

Assessing oospore infectivity on tomato leaflets. Late blight lesions were not observed on tomato leaflet tissues in the floating inoculation system during the first 5 days of the experiments. However, small lesions were observed on some leaflets collected on days 10 and 15 after flooding the soil with progeny of *P. infestans* pairs US-22 x US-23 and US-23 x US-24 and incubating at 22, 0, -5, and -10ºC (Fig.6). At the time of leaflet collection, late blight lesions were very small and mycelia were not observed. However, mycelia were seen when leaflets were incubated at 18ºC in the petri dishes containing a wet paper towel for enhanced humidity. Small pieces of tomato leaflets with sporangia were transferred to Rye A agar media amended with rifampicin and ampicillin, and morphological features of mycelia and sporangia consistent with *P. infestans* were observed by microscopy. Leaflets collected from the pots containing oospores at days 20 and 25 did not have any lesions and did not develop lesions at 18ºC under darkness. Tomato leaflets floated on flooded soil containing oospores incubated at -20°C did not show late blight symptoms.

Discussion

Results from this study demonstrated that oospores generated by *P. infestans* mating pairs collected from Wisconsin, survived, and were infective for at least 5 months after incubation

under six different temperature environments including one under natural soil conditions in an experimental field in Hancock, Wisconsin. Oospores generated by mating of relatively new clonal lineages of *P. infestans* (US-22 x US-23 and US-22 x US-24) have the potential to significantly impact our current understanding and management of late blight in WI, and the U.S. Epidemiologically the presence of oospores could represent a serious threat for potato and tomato production if planted in fields that had previous reports of *P. infestans* of both mating types, even though infectivity from oospores on leaflets ranged only between 2 to 4%. The low percent of infected leaflets could be explained as asynchronous germination that is a typical phenomenon that has been reported likely as a strategy of the pathogen to ensure a continuous supply of inoculum in the soil (21,25). In our study, oospore viability, as determined by either the plasmolysis or staining assays, demonstrated a reduction in viability with increasing cold temperatures, which corroborated with the work of Hanson and Shattock (19).

In work by Turkensteen et al (37), oospores survived for 48 months after exposure to prevailing local weather conditions in the Netherlands. Infectivity of these oospores in baiting bioassays had a mean of 10% of infected potato leaflets. It has been documented that oospore progeny from crosses of US-1 x US-8 and US-11 x US-8 had overall viability ranging from 11 to 29% and 12% germination after 7 months exposure to prevalent winter conditions in northeastern North America in 1995-96 and 1996-97 (30). Our oospores survival data from either mating pair (19%) is in concordance with results obtained by Medina and Turkensteen (30,37). The difference in the mean percent survivability observed between our data and the published data could be due to genetic difference of *P. infestans* genotypes used. It is important to mention that the winter conditions are relatively mild in agricultural fields in the Netherlands

compared with the winter conditions in Hancock, Wisconsin. In the two years of our experiment, soil temperatures typically persisted between 0°C and -5°C. Early winter snow cover on agricultural fields provides an insulating layer that keeps soil temperatures relatively stable and may protect the viability of oospores.

The ability of oospores to survive and initiate infection indicates the potential risk of oospores serving as primarily inoculum. Sexual reproduction could further have impact in the epidemiology of late blight on potato and tomato by generating more genetically diverse *P. infestans* populations, resulting in serious consequences for management of late blight. Overwintering oospores present in production soil could germinate when weather conditions are favorable and initiate infections in potato or tomato, or even in *Solanum* weeds and volunteer potato plants present in the field. In Michigan, a state with somewhat similar weather conditions as Wisconsin, oospores of *Phytophthora capsici* have been documented as having survived for 5 or more years in the soil, and are the main source of primary inoculum each growing season (27). Epidemics of Phytophthora crown, fruit, and root rot of cucurbits and solanaceous crops in Michigan, and other vegetable production states are caused by oospores of high genetic diversity as they are progeny of sexual reproduction resulting from a 1:1 ratio of both mating types in field soils (20).

In our work oospores were not capable of initiating late blight on potato plantlets emerging from seed potato minitubers planted into heavily infested soil. It is difficult to speculate what factor triggers the germination process and infectivity of oospores, but it is likely that water content in the soil plays an important role in this process, since infections were observed when infested soils were flooded, but not when soils were maintained moist in a mist chamber with 100% of relative humidity. Lapwood (28) suggested that oospores were likely the

source of primary inoculum for late blight infections in Toluca Valley, Mexico since initial infections appeared only on leaves located a few centimeters below the soil surface after heavy rain days. It is hypothesized that soilborne oospores can germinate and produce motile zoospores that are able to swim and find leaves in contact with soils to initiate late blight infections during periods of high rainfall (6). Assessing germination is perhaps the best method of evaluating oospore survivability (3). The detection of oospore germination is possible when oospores are generated on sterile media under laboratory conditions, but difficult when oospores are recovered from field soil and cultured on nutritive media where saprophytic microorganisms grow faster than *P. infestans*. Several unknown factors likely also affect dormancy and germination of oospores.

Further investigation is necessary to determine what specific factors, or combination of factors, may be involved in the stimulation of germination of oospores progeny from the US-22, US-23, and US-24 clonal lineages of *P. infestans.* Soilborne oospores of *P. infestans* as a source of inoculum for late blight infection under natural conditions have been reported outside of the U.S. (1,6,9,33). The overwintering survival of oospores generated by *P. infestans* in Wisconsin could have great epidemiological implications resulting from enhanced diversity and phenotypic differences within the pathogen population. Reports of late blight infection on potato and tomato have been more frequent in recent years throughout many areas of agricultural production in the U.S., but the nature and exact sources of primary inoculum is unknown (38). Consequently, maximum effort should be taken to control any foci of late blight infection in any field or garden, and keep both mating types geographically separated.

Table 1. Characteristics of *Phytophthora infestans* isolates used in this study. All isolates were collected from symptomatic plant tissues in 2010 (34).

Clonal lineages	**Allozyme banding pattern**	**WI county of collection**	**Mating type**	**Host**
US-22	100/122	Vernon	A2	Tomato
US-23	100/100	Waukesha	A1	Tomato
US-24	100/100/111	Waushara	A1	Potato

Table 2. Percent germination and viability of fresh harvested oospores generated by *P. infestans* pairs collected from commercial fields in Wisconsin. Viability was evaluated with tetrazolium bromide and plasmolysis methods.

P. infestans pairs	% germination [a]	MTT test [c], % viability[a]		plasmolysis test, % viability[a]	
	Non-boiled [b] oospores	boiled[d] oospores	non-boiled[b]	boiled[d] oospores	non-boiled[b] oospores
US-22 x US-23	39[e] ±3	5.5 ±4	84 ±7	0	76 ±5
US-22 x US-24	34 ±4	7.5 ±5.5	81 ±7	0	78 ±6

[a] Percent of germination and viability. Tests were performed using fresh harvested oospores.

[b] Non-boiled oospores = are intact oospores.

[c] MTT test = Tetrazolium bromide method

[d] Boiled oospores: oospores were boiled for 15 minutes, and were used as a non-viable control.

[e] Data are expressed as percentages and their standard deviations. Percentages are the average of two observations of 100 oospores each.

Figure legends:

Fig. 1. Characteristic appearance of viable and non-viable oospores resulting from viability tests using the plasmolysis method (2M NaCl) and vital staining method (tetrazolium bromide).

Figure 2. Mean percent viability of oospores from two years. Oospores were incubated under natural winter conditions and at 22°C, 0°C, -5°C, -10°C and -20°C, during 5 months from November 2011-March 2012 and November 2012-March 2013. Oospores were generated by *P. infestans* pairs US-22 x US-23 and US-22 x US-24. Percentages of viability were determined using vital staining and plasmolysis methods.

Figure 3. Soil temperatures during winter 2011-2012 and 2012-1013 at the Hancock Agricultural Research Station, Hancock, WI.

Figure 4. Percentage of leaflets with late blight lesions in the bioassay experiment to evaluate infectivity of oospores after incubation for 5 months in the winter in the field and at 22°C, 0°C -5°C, -10°C and -20°C.

Figure. 1

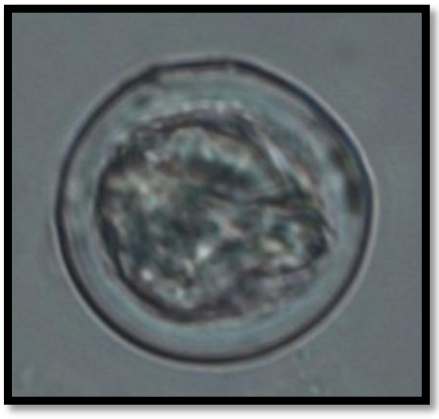
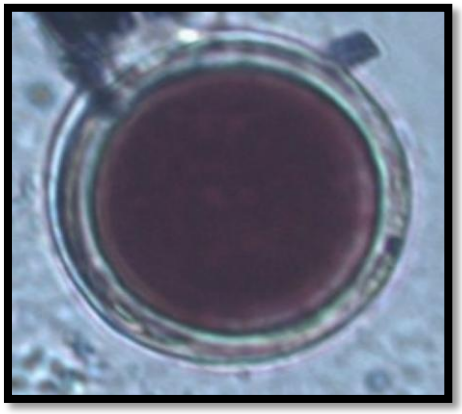
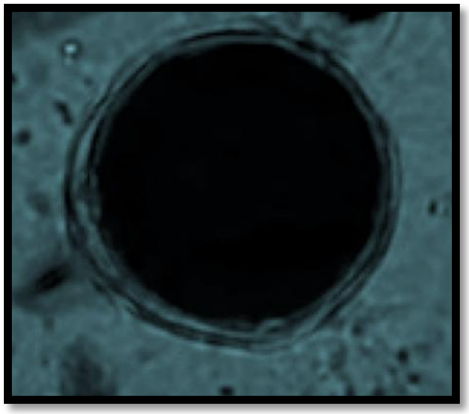
Method	Viable oospores	Non-viable oospores
Plasmolysis (2M NaCl)		
Vital staining (Tetrazolium Bromide = MTT)		

Figure 2.

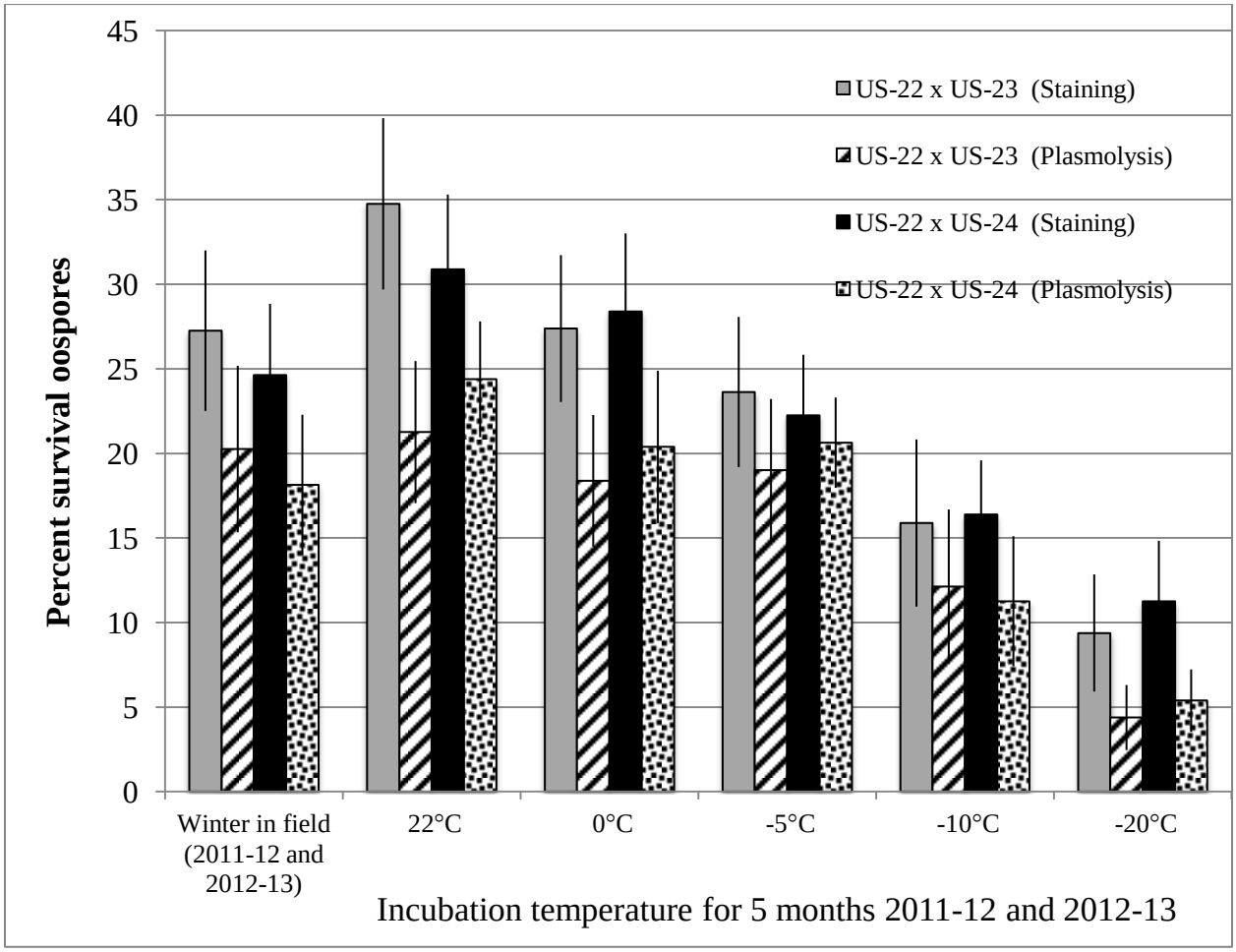

45
40
35
30
25
20
15
10
5
0
Percent survival oospores
US-22 x US-23 (Staining)
US-22 x US-23 (Plasmolysis)
US-22 x US-24 (Staining)
US-22 x US-24 (Plasmolysis)
Winter in field (2011-12 and 2012-13)
22°C
0°C
-5°C
-10°C
-20°C
Incubation temperature for 5 months 2011-12 and 2012-13

Figure 3.

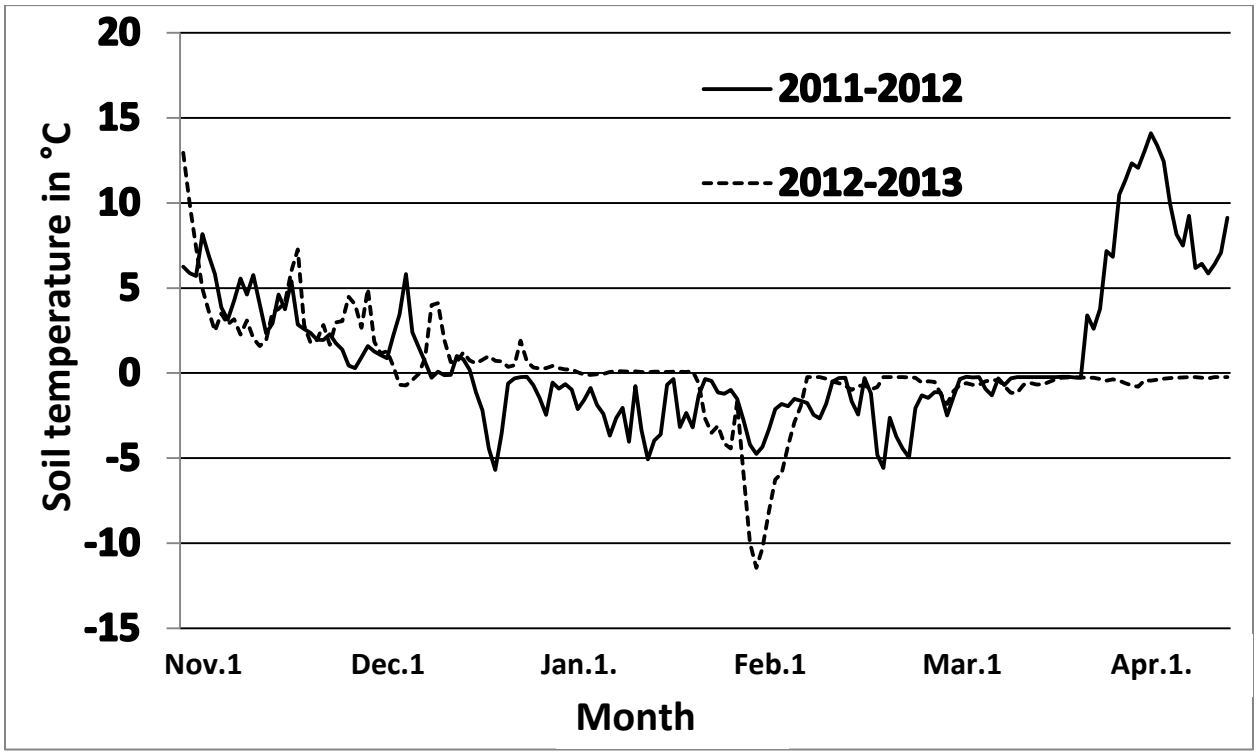
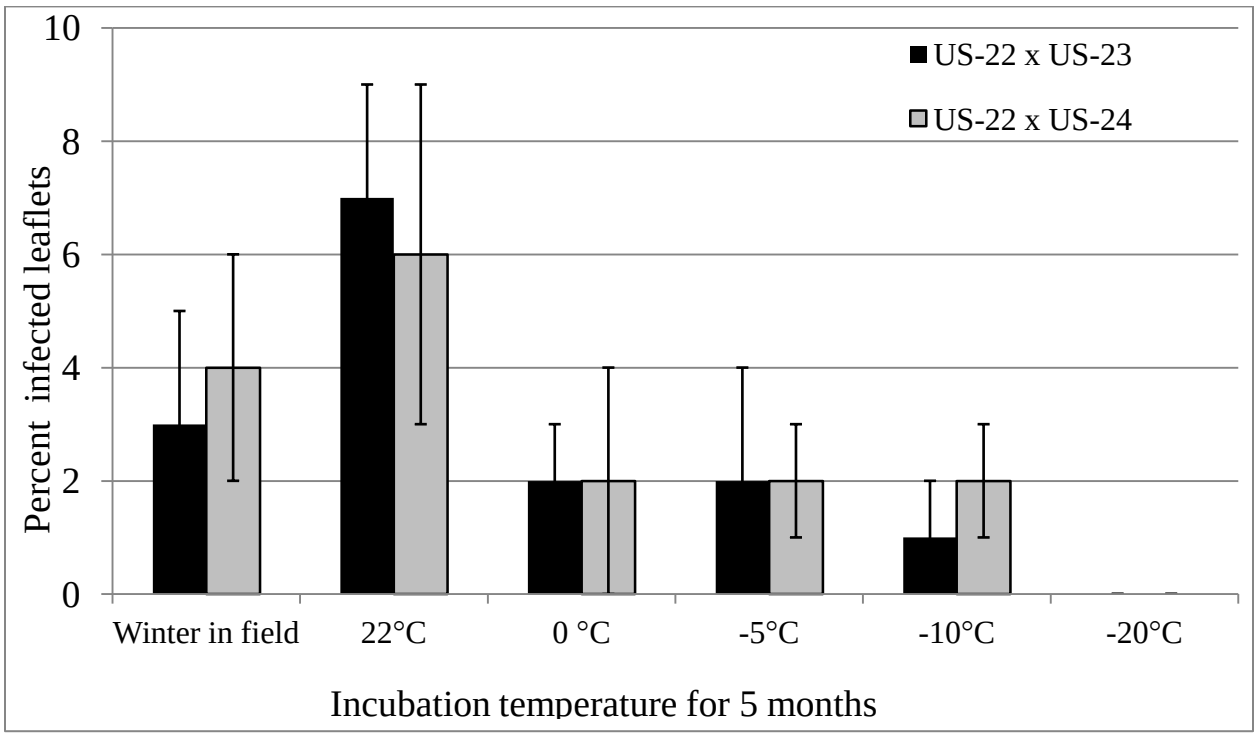

20
15
10
5
0
-5
-10
-15
Soil temperature in °C
2011-2012
2012-2013
Nov.1
Dec.1
Jan.1.
Feb.1
Mar.1
Apr.1.
Month

Figure 4.


10
8
6
4
2
0
Percent infected leaflets
US-22 x US-23
US-22 x US-24
Winter in field
22°C
0 °C
-5°C
-10°C
-20°C
Incubation temperature for 5 months

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Chapter 5

Evaluation of transient expression of *RB*-orthologous genes from wild potato species

Abstract

Potato and tomato late blight, a disease caused by the oomycete *Phytophthora infestans*, is one of the main constraints of potato and tomato production worldwide. Several wild potato species have been sources of resistance to different diseases of Solanaceous plants. In the present research, the interaction between the protein from the host plant *RB* resistance gene and the protein from *P. infestans* IPI-O effectors was used as model for gene for gene screening of resistance to late blight in 18 accessions from 7 wild *Solanum* species. Analysis of amino acid sequences of the 18 accessions showed that fourteen accessions encode potential resistance proteins of 979 amino acids and four accessions encode potential resistance proteins of 876 amino acids. Evaluation of transient expression of the 18 *RB* orthologous genes determined that none were able to detect the IPI-O1 or IPI-O4 effector from *P. infestans*. The 18 *RB* orthologous genes had a conserved CC domain with few mutations and three kinase motifs with similar amino acid sequences as the functional *RB blb*. In the present study we found that 4 *RB* orthologous genes have a deletion of 103 amino acids corresponding to 4 complete LRR units; and the group of 14 *RB* orthologous genes containing 21 LRR units, with an insertion of 20 amino acids in the LRR unit No.13. The information generated in the present study and the sequence diversity between non-functional and the functional *RB* genes can be used for future research to identify regions of importance.

Introduction

Late blight of potato and tomato is caused by the oomycete plant pathogen *Phytophthora infestans* (Mont) de Bary (6). Late blight was a key factor for the Irish potato famine in the 1840s and 160 years later in modern agriculture systems, continues to be one the world's most potentially devastating disease (24).

Control of late blight is considered one of the most costly disease in terms of the cost of control as well as yield and postharvest losses (6,9). The implementation of an integrated management of late blight is the best strategy to control the disease, which includes: use of late blight-free seedling and seed tubers, avoidance of cull piles in the field, removal of volunteer potato and tomato plants, elimination and disposition of late-blighted plants, use of resistant cultivars, and use of preventive fungicides. Control using fungicides is the most common method to control late blight (27). However, the use of fungicides and its application represent a high cost for the farmers, which sometimes farmers in developing countries cannot afford. The use of cultivars with durable resistance is an important strategy for control of late blight. Potato cultivars with durable resistance and with characteristics suitable for the market are still missing, even after more than 50 years of efforts of breeding for resistance to this disease. Pathogen populations are constantly evolving, which leads to new strains that are able to overcome major resistance genes in commercial varieties (28). Some wild *Solanum* species are sources of resistance genes to *P. infestans.* Eleven race-specific genes for resistance to potato late blight from *Solanum demissum* were identified and introgressed into potato varieties in the 1960's (23), but these genes were overcome by new virulent genotypes of *P. infestans* a few years after their release (22,23). The wild tomato species *Solanum pimpinellifolium* (formerly *Lycopersicum pimpinellifolium)* was the source of 3 resistance genes named *Ph-1, Ph-2* and *Ph-3* (5,15,21).

The plant innate immune system in plant pathogen interaction is a multifaceted process, and it is explained by the zigzag model proposed by Jones and Dangl (12). The first phase of plant defense is constituted by a broad-range proteins defense, able to detect conserved molecules secreted or in the surface of the pathogens (11). This molecule are called microbe or pathogen associated molecular patterns, MAMPs or PAMPs, which can be recognized by plant receptor, resulting in PAMP-triggered immunity (PTI). The pathogen must overcome the front-line defense to initiate infection. In the second phase, the pathogen secreted different virulence proteins termed effectors that suppress or contribute to manipulate plant innate immunity, resulting in effector triggered susceptibility (ETS) and suppression of PTI (7). In third the phase, resistance proteins in the plant host with resistance gene can recognize either indirectly or directly a specific effector from the pathogen, triggering of an arsenal of plant defense responses that result in effector-triggered immunity (ETI), and conferring disease resistance expressed as hypersensitive cell death (HR) at the site of infection. ETI represents the second line of defense. In the fourth phase effector evolve via natural selection or getting additional effector to evade ETI; in this phase resistance genes evolve to establish detection of new effectors (12).

For infection, *P. infestans* and other microbial pathogens secrete numerous virulence effectors, allowing the pathogen to grow and reproduce (13,30). IPI-O encodes a family of effectors from *P. infestans* that are involved in pathogenicity (32). *P. infestans* effectors in host cells induce effector-triggered susceptibility, leading to development of late blight infection (2,13). Resistance proteins recognize the presence of effector proteins, therefore inducing the activation of effector triggered immunity or resistance, which can be expressed as a hypersensitivity response (HR) in the area of infection and oxidative burst (14,25). The wild potato species, *Solanum bulbocastanum*, is the source of the *RB* gene (*RB blb*) which encodes a

protein that is able to recognize some IPI-O effectors from *P. infestans* and trigger plant defense (3,26,31). However, *RB* from *S. bulbocastanum* does not recognize IPI-O4 effectors, so *P. infestans* carrying the IPI-O4 effector can cause late blight infection (8). The interaction of RB protein with the IPI-O effector protein is an example of the model of gene for gene interaction. This model of resistance proteins of the host plant recognizing the pathogen effector to active the plant defense, can be used to screen resistance in *Solanum* species to late blight. In order to have an effective and sustainable management of potato late blight it is necessary to find new sources of resistance genes in wild potato species that are able to recognize the pathogenicity effectors from *P. infestans.*

The *RB* orthologous gene named RB^{ver} was isolated from the wild potato species *Solanum verrucosum*. Stable introduction into a susceptible cultivar via transformation and the functional confirmation demonstrated that this gene is likely of ancient origin and probably present in other wild potato *Solanum* species (16). Having more resistance genes available for a strategy of gene pyramiding in commercial potato cultivars could be possible, with benefit to reduce the opportunity of breaking resistance, reduction of the severity of disease, and reducing the frequency of fungicide application.

The objective of the present study was to evaluate the ability of *RB* orthologous genes from 7 wild potato species to recognize IPIO effectors from *P. infestans.*

Materials and Methods

Genomic DNA extraction, amplification, and sequencing of *RB* orthologous genes were performed by Sarah Stephenson in the Halterman Lab.

Plant growth and preparation of genomic DNA

Seeds of a total of 18 accessions of *Solanum* wild species were obtained from the National Research Program (NRSP) potato Gene Bank in Sturgeon Bay, Wisconsin, which include: 3 plant accessions of *Solanum stoloniferum*, 3 plant accessions of *S. fendleri*, 1 plant accession of *S. hjertingii*, 2 plant accessions of *S. trifidum*, 2 plant accessions of *S. pinnatisectum*, 4 plant accessions of *S. demissum*, 3 plant accessions of *S. polyadenium.* Seeds were planted and three weeks later seedling were transplanted to an individual pots and were grown in a greenhouse under 23º C during day and 16º C at night and 14 hours. Genomic DNA was extracted from young leaves of the 18 *Solanum* species, using a standard method of DNA extraction. Concentration of genomic DNA was determined.

Amplification of *RB* orthologous genes from *Solanum* species

Based on the sequence of the *RB* gene from *S. bulbocastanum* (26), a pair of oligonucleotide primers F1 5'- CTT CCC ATT TCA TTC CAA CTA GCC -3' and R14 5'-CCT TCT CAC ACC GCT TGA TCA G – 3' were designed for amplification of the open reading frame of the genes, but lacking the last 36 nucleotides due the DNA variation in this region. To compensate for missing nucleotides, we used the last 36 nucleotides (upstream of stop codon) of *RB* gene from *S. bulbocastanum (RB blb)*. PCR amplifications were performed using 50 ng of total genomic DNA in 25 µl containing 22 µl of Platinum PCR SuperMix High Fidelity (Invitrogen, CA), 1 µl of 5 picomole of each primer. PCR conditions were as follows: 1 min at 94º C, followed by 40 cycles of 40 sec at 94º C, 30 sec at 55º C, 8 min at 68º C and 15 min at 68ºC. PCR products were electrophoresed in agarose gel at 1%, and visualized with UV light after ethidium bromide staining. PCR products were extracted from the gel using the MoBio

UltraClean 15 Gel Isolation kit. Extracted DNA fragments from gel were cloned in pENTR vector (Life Technologies), as follows: 4 µl (250 ng/ µl) of the DNA fragment to insert, 6 µl (250 ng/ml) of pENTR vector, 6 µl distilled water, 2 µl of 10X ligation buffer, and 2 µl T4 Ligase. Ligation reactions were incubated overnight at 16º C. Transformations were performed next day, for each *RB* orthologous gene, 5 µl of the pENTR ligation reaction were mixed with 55 µl of competent *E. coli*, and using heat shock at 42º C. Transformed *E. coli* were transferred to 600 µl of SOC media in a 1.5 ml tube, and cultured by shaking for 2 hours at 37º C. Then 100 µl of bacterial culture were spread on LB agar plates containing 50 µg/ml kanamycin, and incubate overnight at 37º C. A single colony was selected and cultured in 4 ml of LB liquid media containing 50 µg/ml kanamycin. Isolation of plasmid DNA was performed using a kit from Fermentas.

DNA sequencing and clustering analysis

The cloned fragments in pENTR vector were sequenced in both directions by the Big Dye terminator method using a set of 10 primers and fluorescent dNTPs. DNA sequencing of the *RB*-orthologous genes was carried out at the Biotechnology Center of University of Wisconsin-Madison. Sequence analyses were performed as follows: ambiguous calls were checked against chromatograms using the program ContigExpress (Invitrogen, CA). The open reading frames of the 18 *RB* orthologous were translated into amino acid sequences the program Vector NTI Explorer (Invitrogen, CA); and multiple sequence alignment was performed using the MegAligment program.

Evaluation of transient expression of *RB*-orthologous genes with CaMV 35S promoter

Each individual *RB* orthologous gene was transferred from the pENTR vector to a pGateway Binary vector containing the promoter from cauliflower mosaic virus. Transfer of the genes were performed in 200 µl tube, in a volume of 5 µl reaction as follows: 1 µl (200-300 ng/µl) of plasmid pENTR carrying the *RB* orthologous gene, 1 µl (600 ng/µl) of pGateway1 binary vector, 1 µl of LR clonase II enzyme, and 2 µl of TE; the reactions were incubated overnight at 25º C. To stop the LR reaction, 1 µl of Proteinase K (2 µg/ µl) solution were added and then incubated for 10 minutes at 37º C. Transformation of *Agrobacterium tumefaciens* strain GV3101 cells with the pGateway binary vectors were performed using an electroporator Gene Pulser (Bio-Rad, IL). Electroporated cells were plated on LB agar media amended with 75 µg/ml Kanamycin and 100 µg/ml Hygromycin and incubated 48 h at 28º C. A single colony from each plate was selected and transferred to 5 ml of LB supplemented with 75 µg/ml Kanamycin and 100 µg/ml Hygromycin, following incubation at 28º C for 24 h and shaking at 180 rpm. *Agrobacterium tumefaciens* cells were harvested by centrifugation and re-suspended in MMA induction buffer (1 lt MMA = 1 g MS salts, 1.95 g MES, 20 g sucrose, 200 µM acetosyringone, pH 5.6). Cell suspensions in MMA induction buffer were subjected to a second process of centrifugation with the objective to eliminate any residual of LB media, supernatant was discarded, and cells were re-suspended again in MMA induction buffer. Bacterial concentration of each suspension were measured using a plate reader and adjusted to OD_{600} = 0.4. All suspensions were incubated at room temperature for 3 h prior to infiltration. As a positive control, the *RB* gene from *S. bulbocastanum* was used. For negative control *A. tumefaciens* carrying a green fluorescent protein was used. Evaluation of the expression of the *RB* orthologous genes was performed by infiltration of *A. tumefaciens* carrying the *RB*

orthologous genes from each wild potato species into *Nicotiana benthamiana* leaves. Leaves were wounded with a 5 µl micropipette tip to facilitate the process of infiltration, and 24 hours after, infiltration in the same infiltration spots were performed with *A. tumefaciens* carrying IPI-O4 or IPI-O1 effectors from *P. infestans*. Inoculated *N. benthamiana* plants were incubated in a growth chamber with conditions of 22º C day, 18º C night temperatures, and 16 hours of light. Visual evaluations were performed after four to seven days of infiltration; a hypersensitive reaction in regions infiltrated with *A. tumefaciens* with *RB* orthologous genes and IPI-O effectors is expected to observe when there was interaction of *RB* gene and IPI-O effector.

Evaluation of transient expression of *RB*-orthologous genes with native promoter from *Solanum bulbocastanum*

A binary vector pCLD04541 containing *RB* from *S. bulbocastanum* with its native promoter and its native 3'UTR, was provided by Dr. Jiming Jiang UW-Horticulture. The size of the regions containing the promoter region is 2,200 base pairs and the size of the 3'UTR is 1,800 base pairs. The *RB* gene was digested from the vector pCLD04541, using *BamHI*, and cloned in the pENTR vector. Two digestion site for Cla I enzyme were introduced by site directed mutagenesis, one digestion site was located two nucleotides before the start codon and the other one is located two nucleotides after the stop codon of the open reading frame of *RB* gene. A backbone of pENTR with the promoter region and 3'UTR from *S. bulbocastanum* was prepared by digesting the open reading frame of *RB* gene in pENTR with Cla I enzyme. The 18 *RB* orthologous genes from 7 wild species were cloned into the backbone of pENTR with promoter and 3'UTR from *S. bulbocastanum*. The *RB* orthologous genes with promoter and 3'UTR from

S. bulbocastanum were transferred from the pENTR vector to a pGateway Binary vector without promoter, using the protocol previously described.

To evaluate the ability of the 19 *RB* orthologs from wild potato species to recognize the IPI-O protein effectors from *P. infestans*, *N. benthamiana* leaves were infiltrated with *A. tumefaciens* strains carrying *RB* orthologous genes from each wild potato species, as previously described. For a positive control the *RB* gene from *S. bulbocastanum* was used. For the negative control, *A. tumefaciens* carrying a green fluorescent protein was used.

Results

Amplification of *RB* orthologous genes from genomic DNA of 18 wild *Solanum* species was performed using a set of primers specific for the *S. bulbocastanum RB* gene. PCR produced a single fragment of DNA ranging between 3300 to 4050 nucleotides from each wild *Solanum* species. Analysis of the DNA sequences revealed an open reading frame for each accession. Compared with the open reading frame of the *RB* gene from *S. bulbocastanum*, the *RB* orthologous have several insertion/deletion (indels). Based on the similarity of the DNA sequences obtained from the 18 wild *Solanum* species to the *RB* gene from *S. bulbocastanum,* all accessions encode full-length CC-NB-LRR domains. The predicted amino acid sequences of the 18 RB orthologs had 83% similarity to *RB blb* (Fig. 1).

Despite that *RB* orthologous were amplified from 7 *Solanum* species, the coiled coil (CC) domain was very conserved, with only 19 amino acids out of 165 were polymorphic (Fig.2). The putative NBS domain of *RB* orthologous genes contained three motifs kinase with same amino acids sequence as *RB blb,* reported by Song et al (26): kinase 1a or P-loop (positions 182-190),

kinase 2 (positions 255-264) and 3a (position 288-293) (Fig. 3). One amino acid in the second kinase of the accession 160221_1503-17 was different. Fourteen accessions encode potential proteins of 979 amino acids. Four accessions: 160221 1503-29, 160221 2374-7, 160221 1503-17, and 160221 1503-24 encode potentially proteins of 876 amino acids (Table 1). The group of fourteen orthologous genes encoding 977 amino acids, contain 21 leucine rich repeats (LRR), very similar to the LRR domain from *S. bulbocastanum*. This group has deletions of 3 and 6 amino acids in the LRR No. 9 and 13 respectively and an insertion of 20 amino acids in the LRR No. 13. The remaining four accessions encoding 863 amino acids have a deletion of 103 amino acids in the LRR domain, the missing amino acid correspond to full LRR repeat No. 4, 5, 6, and 7, and a fraction of LRR domains No. 3 and 9 (Fig. 4 to Fig. 14)

Clustering analysis of *RB* orthologous

To investigate phylogenetic relationship among proteins sequence obtained from the 18 *RB* orthologous, and *RB* from *S. bulbocastanum*, a phylogenetic analysis was performed using the neighbor-joining method (Fig. 15). In the phylogenetic three *RB* orthologous were clustered in three main branches. The *RB blb* did not cluster in any of the three branches of *RB* orthologs.

No hypersensitive response was induced by *RB*-orthologous genes with CaMV 35S promoter

For evaluation of *RB* orthologous genes expression, the promoter from cauliflower mosaic virus (CaMV 35S) was used, it is a powerful promoter and is not greatly influenced by environmental conditions and tissue types (10). Evaluation was performed by visual observation 4 to 7 days after infiltration. Hypersensitive response was observed only in the positive control,

HR was in the infiltrated area with *RB* from *S. bulbocastanum* and IPI-O1. None of the *RB* orthologous genes was able to recognize the IPI-O1 and IPI-O4 effectors from *P. infestans*.

No hypersensitive response was induce by *RB*-orthologous genes with native promoter and 3'UTR from *Solanum bulbocastanum*

Evaluation of the expression of RB orthologous genes from 18 accession of wild *Solanum* species was performed by visual examination. No hypersensitive reaction was observed in all infiltrated areas, except in the infiltrate area with *RB blb* and IPI-O1.

Discussion

None of the *RB* orthologous genes from 6 wild *Solanum* species were able to recognize the IPI-O effector from *P. infestans,* even though a full-length CC-NB-LRR domain was observed in most of the genes with 83 % of similarity between the *RB* gene and the *RB* orthologous genes.

Protein resistance (R) genes with nucleotide binding site and leucine-rich repeat (NB-LRR) domain represent the highest percent of resistance genes in plant genome. This group of resistance genes has high gene duplication and amplification events (19). The NBS domains of plant resistant genes are involved in signaling and contain conserved and strictly ordered motifs (29). In the present research, three motifs kinase with similar amino acid sequences, as the functional *RB* blb, were present in the NBS domain of the 18 orthologous genes. The current hypothesis is that *RB blb* forms an active protein complex through self-association of the CC domain, which allows for recognition of IPI-O1. Direct interaction of the CC domain with the IPI-O4 effector leads to suppression of resistance (3). In this study the variation in amino acid

sequence observed in the CC domain, in some of them only 7 amino acids are different compared with the functional *RB* blb, could be in part responsible for the unsuccessful recognition of IPI-O effectors. Another explanation of the non-functionality of the *RB* orthologous genes could be the difference observed in the LRR domain. In published research it has been documented that expansion and contraction of LRR repeats are responsible for loss of functionality of genes (1,33). Usually LRR domains in resistance genes are very diverse, likely as a result of selective pressures and diversifying selection, that promote evolve of new pathogen-specificities for recognition of new effector or Avr proteins (4,19,20). In high percent the non-functional genes are characterized by large deletions in the LRR domain (18). In the genome of *S. tuberosum* group Phureja, the number of non-functional genes of the CCNBLRR group is 156 (17). A correlation of the loss of functionality of resistance gene *M* in flax, was associated with the deletion of a LRR unit (1). In the present study we found that 4 *RB* orthologous genes have a deletion of 103 amino acids corresponding to 4 complete LRR units, and likely the missing LRR units play a role in the function of the resistance. The resistant *RB* from *S. bulbocastaum* is a member of four-gene family, organized in cluster within a 40-kb region on chromosome 8, and only one allele out of five is functional and one of the non-functional gene contain 22 LRR units, (26). We found that in the group of 14 *RB* orthologous genes containing 21 LRR units, there is an insertion of 20 amino acids in the LRR unit No.13, which also could affect the functionality of the genes. The information generated in the present study and the sequence diversity between non-functional and the functional *RB* genes can be used for future research to identify regions of importance.

Table 1. Wild *Solanum* species, accession numbers, and number of amino acids of the predicted RB protein.

No.	Species	Accession number	Amino acids of predicted RB protein
1	*S. stoloniferum*	161178_2075-88	979
2	*S. fendleri*	225661_1946-3	979
3	*S. hjertingii*	283103_2017-23	979
4	*S. stoloniferum*	161178_2017-5	979
5	*S. fendleri*	225661_2075-47	979
6	*S. fendleri*	225661_1985-8	979
7	*S. stoloniferum*	161178_2075-85	979
8	*S. demissum*	160221_1503-17	876
9	*S. demissum*	160221_2374-7	876
10	*S. demissum*	160221_1503-24	876
11	*S. demissum*	160221_1503-29	876
12	*S. trifidum*	283064_1985-61	979
13	*S. trifidum*	283064_1985-60	979
14	*S. pinnatisectum*	186553_2049-30	979
15	*S. pinnatisectum*	186553_2017-17	979
16	*S. polyadenium*	320342_2017-15	979
17	*S. polyadenium*	320342_2017-13	979
18	*S. polyadenium*	320342_2017-14	979
19	*S. bulbocastanum*	*RB_blb*	970

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		93.9	99.3	99.2	99.5	90.2	89.8	90.5	90.5	89.8	93.8	93.8	90.5	90.8	89.5	90.4	90.5	90.5	82.2	**1**	60221_1503-_24
2	6.3		94.2	94.3	94.4	93.8	92.8	93.4	93.4	93.0	99.2	99.3	93.2	93.4	92.8	92.2	92.3	92.3	82.5	**2**	61178_2075-85
3	0.7	6.1		99.4	99.8	90.4	90.1	90.8	90.8	90.1	94.1	94.1	90.8	91.0	89.7	90.6	90.8	90.8	82.4	**3**	160221_1503-17
4	0.8	5.9	0.6		99.7	90.5	90.2	90.9	90.9	90.2	94.1	94.2	90.9	91.1	89.8	90.6	90.8	90.8	82.6	**4**	160221_1503-29
5	0.5	5.8	0.2	0.3		90.6	90.3	91.0	91.0	90.3	94.3	94.3	91.0	91.2	90.0	90.9	91.0	91.0	82.7	**5**	160221_2374-7
6	10.5	6.5	10.3	10.2	10.0		99.1	93.1	93.1	98.6	93.3	93.4	92.3	92.5	98.1	91.1	91.2	91.2	83.7	**6**	161178_2017-5
7	10.9	7.5	10.7	10.5	10.4	0.9		92.3	92.3	99.5	92.5	92.6	91.6	91.8	98.4	90.4	90.5	90.5	83.5	**7**	161178_2075-88
8	10.2	7.0	9.9	9.8	9.6	7.3	8.1		100.0	92.4	93.2	93.3	97.7	97.9	92.2	95.8	95.9	95.9	83.7	**8**	186553_2017-17
9	10.2	7.0	9.9	9.8	9.6	7.3	8.1	0.0		92.4	93.2	93.3	97.7	97.9	92.2	95.8	95.9	95.9	83.7	**9**	186553_2049-30
10	10.9	7.4	10.7	10.5	10.4	1.4	0.5	8.0	8.0		92.6	92.7	91.7	91.9	98.3	90.5	90.6	90.6	83.5	**10**	225661_1946-3
11	6.4	0.8	6.2	6.2	5.9	7.1	7.9	7.2	7.2	7.8		99.9	93.1	93.3	92.5	92.2	92.3	92.3	82.2	**11**	225661_1985-8
12	6.4	0.7	6.2	6.1	5.9	7.0	7.8	7.1	7.1	7.6	0.1		93.2	93.4	92.6	92.2	92.3	92.3	82.3	**12**	225661_2075-47
13	10.2	7.2	9.9	9.8	9.6	8.1	8.9	2.4	2.4	8.8	7.3	7.2		99.8	91.5	95.7	95.8	95.8	82.9	**13**	283064_1985-60
14	9.9	7.0	9.6	9.5	9.4	7.9	8.7	2.2	2.2	8.6	7.1	7.0	0.2		91.7	95.9	96.0	96.0	83.1	**14**	283064_1985-61
15	11.3	7.5	11.1	10.9	10.8	2.0	1.7	8.2	8.2	1.8	7.9	7.8	9.0	8.8		90.3	90.4	90.4	82.9	**15**	283103_2017-23
16	10.3	8.2	10.0	10.0	9.8	9.5	10.3	4.3	4.3	10.2	8.2	8.2	4.4	4.2	10.4		99.9	99.9	82.5	**16**	320342_2017-13
17	10.2	8.1	9.9	9.9	9.6	9.4	10.2	4.2	4.2	10.1	8.1	8.1	4.3	4.1	10.3	0.1		100.0	82.7	**17**	320342_2017-14
18	10.2	8.1	9.9	9.9	9.6	9.4	10.2	4.2	4.2	10.1	8.1	8.1	4.3	4.1	10.3	0.1	0.0		82.7	**18**	320342_2017-15
19	20.4	19.9	20.1	19.9	19.8	18.4	18.7	18.4	18.4	18.7	20.3	20.2	19.5	19.2	19.5	19.9	19.8	19.8		**19**	RB blb
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**		

Figure 1. Pairwise comparison of amino acid identity and distance among *RB* orthologous and *RB blb* genes, based on the exon sequence.

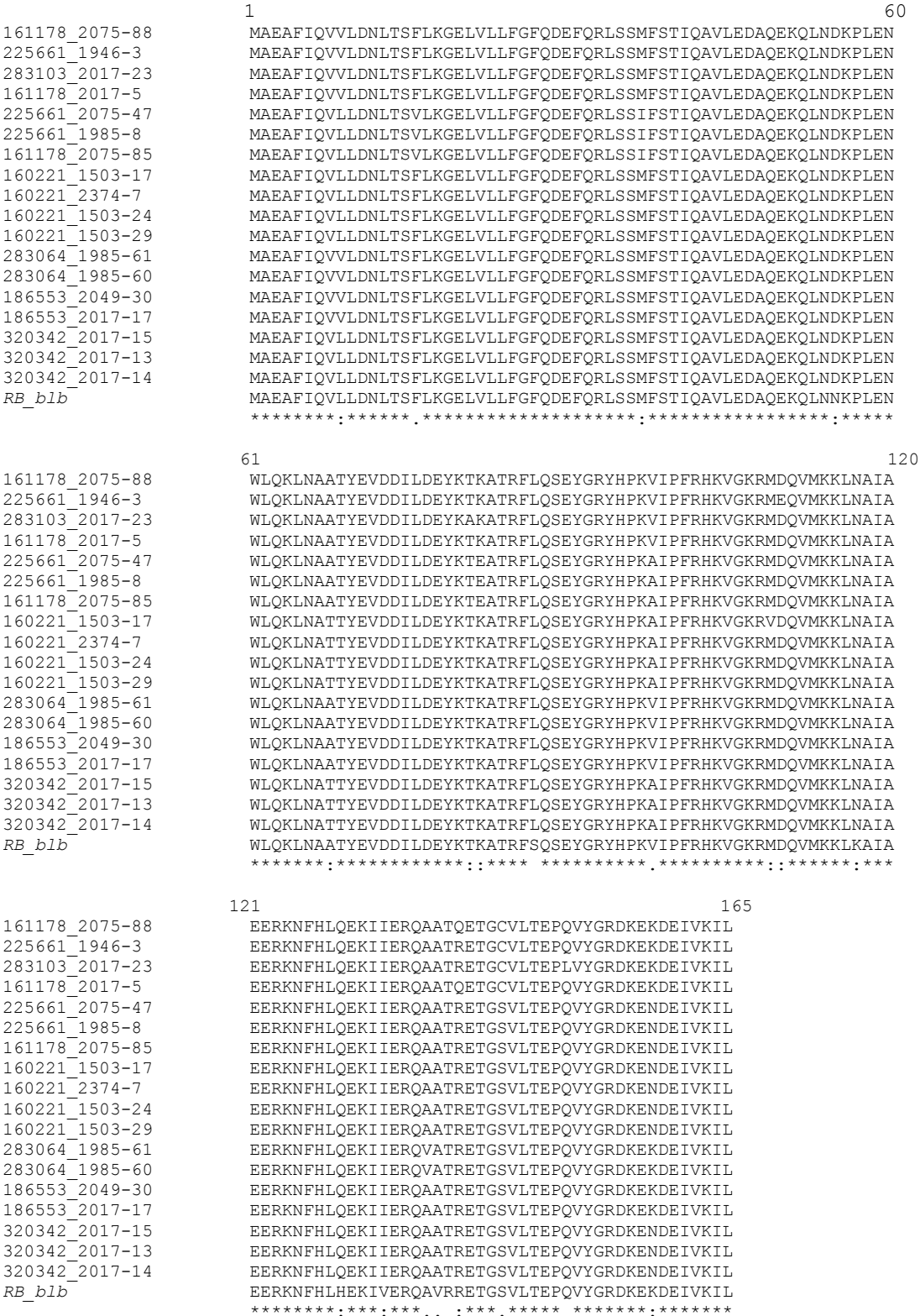

Figure 2. Predicted amino acid sequence of the coiled coil domain of the *RB* orthologous genes.

```
                    182       190                                                 242
161178_2075-88        LGMGGLGKTTLAQMVFNDQRVTERFYPKIWICVSDDFDEKRLIKAIVESIEGKSLS-DMD
225661_1946-3         LGMGGLGKTTLAQMVFNDQRVTERFYPKIWICVSDDFDEKRLIKAIVESIEGKSLS-DMD
283103_2017-23        LGMGGLGKTTLAQMVFNDQRVTERFYPKIWICVSDDFDQKRLIKAIVESIEGKSLS-DMN
161178_2017-5         LGMGGLGKTTLAQMVFNDQRVTERFYPKIWICVSDDFDEKRLIKAIVESIEGKSLS-DMD
225661_2075-47        LGMGGLGKTTLSQMVFNDQRVTEHFYPKLWICVSNDFDEKRLIKAIVESIEGKSLS-DMD
225661_1985-8         LGMGGLGKTTLSQMVFNDQRVTEHFYPKLWICVSNDFDEKRLIKAIVESIEGKSLS-DMD
161178_2075-85        LGMGGLGKTTLSQMVFNDQRVTEHFYPKLWICVSNDFDEKRLIKAIVESIEGKSLS-DMD
160221_1503-17        LGMGGLGKTTLSQMVFNDQRVTEHFYPKLWICVSDDFDEKRLIKAIVESIEGKSLS-DMD
160221_2374-7         LGMGGLGKTTLSQMVFNDQRVTEHFYPKLWICVSDDFDEKRLIKAIVESIEGKSLS-DMD
160221_1503-24        LGMGGLGKTTLSQMVFNDQRVTGHFYPKLWICVSDDFDEKRLIKAIVESIEGKSLS-DMD
160221_1503-29        LGMGGLGKTTLSQMVFNDQRVTEHFYPKLWICVSDDFDEKRLIKAIVESIEGKSLS-DMD
283064_1985-61        LGMGGLGKTTLSQMVFNDQTVTEHFYPKIWICVSDDFDEKRLIKAIVESIEGKSLS-NMD
283064_1985-60        LGMGGLGKTTLSQMVFNDQTVTEHFYPKIWICVSDDFDEKRLIKAIVESIEGKSLS-NMD
186553_2049-30        LGMGGLGKTTLSQMVFNDQRVTERFYPKIWICVSDDFDEKRLIKAIVESIEGKSLS-DMD
186553_2017-17        LGMGGLGKTTLSQMVFNDQRVTERFYPKIWICVSDDFDEKRLIKAIVESIEGKSLS-DMD
320342_2017-15        LGMGGLGKTTLSQMVFNDQRVTERFYPKIWICVSDDFDEKRLIKAIVESIEGKSLR-DMD
320342_2017-13        LGMGGLGKTTLSQMVFNDQRVTERFYPKIWICVSDDFDEKRLIKAIVESIEGKSLR-DMD
320342_2017-14        LGMGGLGKTTLSQMVFNDQRVTERFYPKIWICVSDDFDEKRLIKAIVESIEGKSLR-DMD
RB_blb                LGMGGLGKTTLAQMVFNDQRVTEHFHSKIWICVSEDFDEKRLIKAIVESIEGRPLLGEMD
                      ***********:******* ** :*:.*:*****:***:*************:.*  :*:

                    241           255       264                   288    293    300
161178_2075-88        LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
225661_1946-3         LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
283103_2017-23        LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
161178_2017-5         LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
225661_2075-47        LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGSFVLTTTRLEKVGSI
225661_1985-8         LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGSFVLTTTRLEKVGSI
161178_2075-85        LAPLQKKLQELQNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGSFVLTTTRLEKVGSI
160221_1503-17        LAPLQKKLQELLNGKGYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
160221_2374-7         LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
160221_1503-24        LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
160221_1503-29        LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
283064_1985-61        LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
283064_1985-60        LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
186553_2049-30        LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
186553_2017-17        LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
320342_2017-15        LAPLQKKLQELLNGKRYFLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
320342_2017-13        LAPLQKKLQELLNGKRYFLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
320342_2017-14        LAPLQKKLQELLNGKRYFLVLDDVWNEDQQKWANLRAVLKVGASGAFVLTTTRLEKVGSI
RB_blb                LAPLQKKLQELLNGKRYLLVLDDVWNEDQQKWANLRAVLKVGASGASVLTTTRLEKVGSI
                      *********** *** *:****************************: *************
```

Figure 3. Predicted amino acid sequence of the NBS domain, the three kinase motifs are in bold.

```
LRR No.1
Accession No.       522
161178_2075-88        LQKFVSLRVLNLRNSDLNQLPSS
225661_1946-3         LQKFVSLRVLNLRNSDLNQLPSS
283103_2017-23        LQKFVSLRVLNLRNSDLNQLPSS
161178_2017-5         LQKFVSLRVLNLRNSDLNQLPSS
225661_2075-47        LQKFVSLRVLNLRNSDLNQLPSS
225661_1985-8         LQKFVSLRVLNLRNSDLNQLPSS
161178_2075-85        LQKFVSLRVLNLRNSDLNQLPSS
160221_1503-17        VENFVSLRVLNLNYLEK-QLPSS
160221_2374-7         VENFVSLRVLNLNYLEK-QLPSS
160221_1503-24        VENFVSLRVLNLNYLEK-QLPSS
160221_1503-29        VENFVSLRVLNLNYLEK-QLPSS
283064_1985-61        LQNFVSLRVLNLRNSNLNQLPSS
283064_1985-60        LQNFVSLRVLNLRNSNLNQLPSS
186553_2049-30        LQKFVSLRVLNLRNSNLNQLPSS
186553_2017-17        LQKFVSLRVLNLRNSNLNQLPSS
320342_2017-15        LQKFVSLRVLNLRNSNLNQLPSS
320342_2017-13        LQKFVSLRVLNLRNSNLNQLPSS
320342_2017-14        LQKFVSLRVLNLRNSNLNQLPSS
RB_blb                LEKFISLRVLNLGDSTFNKLPSS
                      :::*:*******      :****


LRR No. 2

161178_2075-88        IGDLVHLRYLDLSHNIRIHSLPKR
225661_1946-3         IGDLVHLRYLDLSHNIRIRSLPKR
283103_2017-23        IGDLVHLRYLDLSHNIRIRSLPKR
161178_2017-5         IGDLVHLRYLDLSHNIRIHSLPKR
225661_2075-47        IGDLVHLRYLDLSDNIRIRSLPKR
225661_1985-8         IGDLVHLRYLDLSDNIRIRSLPKR
161178_2075-85        IGDLVHLRYLDLSDNIRIRSLPKR
160221_1503-17        IGDLVHLRYLDLSDNFRICSLPKQ
160221_2374-7         IGDLVHLRYLDLSDNFRICSLPKQ
160221_1503-24        IGDLVHLRYLDLSDNFRICSLPKQ
160221_1503-29        IGDLVHLRYLDLSDNFRICSLPKQ
283064_1985-61        IGDLVHLRYLDLSGNIRIRSLPKR
283064_1985-60        IGDLVHLRYLDLSGNIRIRSLPKR
186553_2049-30        IGDLVHLRYLDLSGNVRIRSLPKR
>186553_2017-17       IGDLVHLRYLDLSGNVRIRSLPKR
320342_2017-15        IGDLVHLRYLDLSGNIRIRSLPKR
320342_2017-13        IGDLVHLRYLDLSGNIRIRSLPKR
320342_2017-14        IGDLVHLRYLDLSGNIRIRSLPKR
RB_blb                IGDLVHLRYLNLYG-SGMRSLPKQ
                      **************:*     :
```

Figure 4. Amino acid sequence of the predicted No.1 and No. 2 LRR units from the 18 *RB* orthologous genes and *RB blb* gene

```
LRR No. 3

161178_2075-88          LCKLQNLQTLDLHNCYSLSCLPKQ
225661_1946-3           LCKLQNLQTLDLHNCYSLSCLPKQ
283103_2017-23          LCKLQNLQTLDLHNCYSLSCLPKQ
161178_2017-5           LCKLQNLQTLDLHNCYSLSCLPKQ
225661_2075-47          LCKLQNLQTLDLHNCYSLSCLPKQ
225661_1985-8           LCKLQNLQTLDLHNCYSLSCLPKQ
161178_2075-85          LCKLQNLQTLDLHNCYSLSCLPKQ
160221_1503-17          LCKLQNLLTLDLHGCKS-------
160221_2374-7           LCKLQNLLTLDLHGCKS-------
160221_1503-24          LCKLQNLLTLDLHGCKS-------
160221_1503-29          LCKLQNLLTLDLHGCKS-------
283064_1985-61          LCKLQNLQTLDLHYCDSLSCLPKQ
283064_1985-60          LCKLQNLQTLDLHYCDSLSCLPKQ
186553_2049-30          LCKLQNLQTLDLHYCDSLSCLPKQ
186553_2017-17          LCKLQNLQTLDLHYCDSLSCLPKQ
320342_2017-15          LCKLQNLQTLDLHYCDSLSCLPKQ
320342_2017-13          LCKLQNLQTLDLHYCDSLSCLPKQ
320342_2017-14          LCKLQNLQTLDLHYCDSLSCLPKQ
RB_blb                  LCKLQNLQTLDLQYCTKLCCLPKE
                        ******* ****: *



LRR No.4

161178_2075-88          TSKLGSLRNLLLDGC-SLMSMPPRIGL
225661_1946-3           TSKLGSLRNLLLDGC-SLMSMPPRIGL
283103_2017-23          TSKLGSLRNLLLDGC-SLMSMPPRIGL
161178_2017-5           TSKLGSLRNLLLDGC-SLTSTPPRIGL
225661_2075-47          TSKLGSLRNLLLDGC-SLTSTPPRIGL
225661_1985-8           TSKLGSLRNLLLDGC-SLTSTPPRIGL
161178_2075-85          TSKLGSLRNLLLDGC-SLTSTPPRIGL
160221_1503-17          ---------------------------
160221_2374-7           ---------------------------
160221_1503-24          ---------------------------
160221_1503-29          ---------------------------
283064_1985-61          TSKLGSLRNLLLGGC-SLTSTPPRIGL
283064_1985-60          TSKLGSLRNLLLGGC-SLTSTPPRIGL
186553_2049-30          TSKLGSLRNLLLGGC-SLTSTPPRIGL
>186553_2017-17         TSKLGSLRNLLLGGC-SLTSTPPRIGL
320342_2017-15          TSKLGSLRNLLLGGC-SLTSTPPRIGL
320342_2017-13          TSKLGSLRNLLLGGC-SLTSTPPRIGL
320342_2017-14          TSKLGSLRNLLLGGC-SLTSTPPRIGL
RB_blb                  TSKLGSLRNLLLDGSQSLTCMPPRIGS
```

Figure 5. Amino acid sequence of the predicted No.3 and No. 4 LRR units from the 18 *RB* orthologous genes and *RB blb* gene

```
LRR No.5

161178_2075-88        LTCLKSLSCFVIGKKN
225661_1946-3         LTCLKSLSCFVIGKKN
283103_2017-23        LTFLKSLSCFVIGKRN
161178_2017-5         LTCLKSLSCFVIGKRK
225661_2075-47        LTCLKSLSCFVIGKRK
225661_1985-8         LTCLKSLSCFVIGKRK
161178_2075-85        LTCLKSLSCFVIGKRK
160221_1503-17        ----------------
160221_2374-7         ----------------
160221_1503-24        ----------------
160221_1503-29        ----------------
283064_1985-61        LTCLKSLSCFVIGKRK
283064_1985-60        LTCLKSLSCFVIGKRK
186553_2049-30        LTCLKSLSCFVIGKRK
186553_2017-17        LTCLKSLSCFVIGKRK
320342_2017-15        LTCLKSLSCFVVGKRK
320342_2017-13        LTCLKSLSCFVVGKRK
320342_2017-14        LTCLKSLSCFVVGKRK
RB_blb                LTCLKTLGQFVVGRKK



LRR No.6

161178_2075-88        GYQLGELKNLNLYG
225661_1946-3         GYQLGELKNLNLYG
283103_2017-23        GYQLGELKNLNLYG
161178_2017-5         GYQLGELKNLNLYG
225661_2075-47        GYQLGELKNLNLYG
225661_1985-8         GYQLGELKNLNLYG
161178_2075-85        GYQLGELKNLNLYG
160221_1503-17        --------------
160221_2374-7         --------------
160221_1503-24        --------------
160221_1503-29        --------------
283064_1985-61        GYQLGELKNLNLYG
283064_1985-60        GYQLGELKNLNLYG
186553_2049-30        GYQLGELKNLNLYG
186553_2017-17        GYQLGELKNLNLYG
320342_2017-15        GYQLGELKNLNLYG
320342_2017-13        GYQLGELKNLNLYG
320342_2017-14        GYQLGELKNLNLYG
RB_blb                GYQLGELGNLNLYG
```

Figure 6. Amino acid sequence of the predicted No.5 and No. 6 LRR units from the 18 *RB* orthologous genes and *RB blb* gene

```
LRR No.7

161178_2075-88        SISITKLERVKKGRDAKEAN
225661_1946-3         SISITKLERVKKGRDAKEAN
283103_2017-23        SISITKLERVKKGRDAKEAN
161178_2017-5         SISITKLERVKKGRDAKEAN
225661_2075-47        SISITKLERVKKGRDAKEAN
225661_1985-8         SISITKLERVKKGRDAKEAN
161178_2075-85        SISITKLERVKKGRDAKEAN
160221_1503-17        --------------------
160221_2374-7         --------------------
160221_1503-24        --------------------
160221_1503-29        --------------------
283064_1985-61        SISITKLERVKKGRDAKEAN
283064_1985-60        SISITKLERVKKGRDAKEAN
186553_2049-30        SISITKLDRVKKGRDAKEAN
186553_2017-17        SISITKLDRVKKGRDAKEAN
320342_2017-15        SIAITKLDRVKKGRDAKEAN
320342_2017-13        SIAITKLDRVKKGRDAKEAN
320342_2017-14        SIAITKLDRVKKGRDAKEAN
RB_blb                SIKISHLERVKNDMDAKEAN



LRR No. 8

161178_2075-88        ISVKANLHSLSLSWDFDGTRRYE
225661_1946-3         ISVKANLHSLSLSWDFDGTRRYE
283103_2017-23        ISVKANLHSLSLSWDFDGTRRYE
161178_2017-5         ISVKANLHSLSLSWDFDGTHRYE
225661_2075-47        ISVKANLHSLSLSWDFDGTHRYE
225661_1985-8         ISVKANLHSLSLSWDFDGTHRYE
161178_2075-85        ISVKANLHSLSLSWDFDGTHRYE
160221_1503-17        -------------------HRYE
160221_2374-7          -------------------HRYE
160221_1503-_24        -------------------HRYE
160221_1503-29         -------------------HRYE
283064_1985-61        LSAKANLHSLSLSWDLDGKHRYD
283064_1985-60        LSAKANLHSLSLSWDLDGKHRYD
186553_2049-30        LSAKANLHSLSLSWDFDGKHRYD
186553_2017-17        LSAKANLHSLSLSWDFDGKHRYD
320342_2017-15        LSAKANLHSLSLSWDLDGKHRYD
320342_2017-13        LSAKANLHSLSLSWDLDGKHRYD
320342_2017-14        LSAKANLHSLSLSWDLDGKHRYD
RB_blb                LSAKGNLHSLSMSWNNFGPHIYE
                                      : *:
```

Figure 7. Amino acid sequence of the predicted No.7 and No. 8 LRR units from the 18 *RB* orthologous genes and *RB blb* gene

```
LRR No. 9

161178_2075-88        S---EVLEALKPHSNLK
225661_1946-3         S---EVLEALKPHSNLK
283103_2017-23        S---EVLEALKPHSNLK
161178_2017-5         S---EVLEALKPHSNLK
225661_2075-47        S---EVLEALKPHSNLK
225661_1985-8         S---EVLEALKPHSNLK
161178_2075-85        S---EVLEALKPHSNLK
160221_1503-17        S---EVLKALKPHSNLK
160221_2374-7         S---EVLKALKPHSNLK
160221_1503-24        S---EVLKALKPHSNLK
160221_1503-29        S---EVLKALKPHSNLK
283064_1985-61        S---EVLEALKPHSNLK
283064_1985-60        S---EVLEALKPHSNLK
186553_2049-30        S---EVLEALKPHSNLT
186553_2017-17        S---EVLEALKPHSNLT
320342_2017-15        S---EVLEALKPHSNLK
320342_2017-13        S---EVLEALKPHSNLK
320342_2017-14        S---EVLEALKPHSNLK
RB_blb                SEEVKVLEALKPHSNLT
                      *   :**:********.


LRR No. 10

161178_2075-88        HLEIIGFRGIRLPDWMN
225661_1946-3         HLEIIGFRGIRLPDWMN
283103_2017-23        YLEIIGFRGIRLPDWMN
161178_2017-5         YLEIIGFRGIHLPDWMN
225661_2075-47        YLEIIGFRGIRLPDWMN
225661_1985-8         YLEIIGFRGIRLPDWMN
161178_2075-85        YLEIIGFRGIHLPDWMN
160221_1503-17        YLEIIGFRGIRLPDWMN
160221_2374-7         YLEIIGFRGIRLPDWMN
160221_1503-24        YLEIIGFRGIRLPDWMN
160221_1503-29        YLEIIGFRGIRLPDWMN
283064_1985-61        YVEINGFGGIRLPDWMN
283064_1985-60        YVEINGFGGIRLPDWMN
186553_2049-30        YLEINGFGGIRLPDWMN
186553_2017-17        YLEINGFGGIRLPDWMN
320342_2017-15        YLEINDFGGIRLPDWMN
320342_2017-13        YLEINDFGGIRLPDWMN
320342_2017-14        YLEINDFGGIRLPDWMN
RB_blb                SLKIYGFRGIHLPEWMN
                       ::* .* **:**:***
```

Figure 8. Amino acid sequence of the predicted No.9 and No. 10 LRR units from the 18 *RB* orthologous genes and *RB blb* gene

```
LRR No. 11

161178_2075-88        QSVLRNIVSITIRGCENCSCLPP
225661_1946-3         QSVLRNIVSITIRGCENCSCLPP
283103_2017-23        QSVLRNVVSITIRGCENCSCLPP
161178_2017-5         QSVLKNVVSITIRGCENCSCLPP
225661_2075-47        QSVLKNVVSITIRGCENCSCLPP
225661_1985-8         QSVLKNVVSITIRGCENCSCLPP
161178_2075-85        QSVLKNVVSITIRGCENCSCLPP
160221_1503-17        QSVLKNVVSITIRGCENCSCLPP
160221_2374-7         QSVLKNVVSITIRGCENCSCLPP
160221_1503-24        QSVLKNVVSITIRGCENCSCLPP
160221_1503-29        QSVLKNVVSITIRGCENCSCLPP
283064_1985-61        QSVLKNVVSIRIRGCENCSCLPP
283064_1985-60        QSVLKNVVSIRIRGCENCSCLPP
186553_2049-30        QSVLKNVVSIRIRGCKNCSCLPP
186553_2017-17        QSVLKNVVSIRIRGCKNCSCLPP
320342_2017-15        QSVLKNVVSIRIKGCENCSCLPP
320342_2017-13        QSVLKNVVSIRIKGCENCSCLPP
320342_2017-14        QSVLKNVVSIRIKGCENCSCLPP
RB_blb                HSVLKNIVSILISNFRNCSCLPP
                      :***:*:*** * . .*******


LRR No. 12

161178_2075-88        FGELPNLESLELHTG
225661_1946-3         FGELPNLESLELHTG
283103_2017-23        FGELPNLESLELHTG
161178_2017-5         FGELPNLESLELHTG
225661_2075-47        FGELPCLESLELQDG
225661_1985-8         FGELPCLESLELQDG
161178_2075-85        FGELPSLESLELHTG
160221_1503-17        FGELPSLESLELHTG
160221_2374-7-        FGELPSLESLELHTG
160221_1503-_24       FGELPSLESLELHTG
160221_1503-29        FGELPSLESLELHTG
283064_1985-61        FGELPCLESLELHTG
283064_1985-60        FGELPCLESLELHTG
186553_2049-30        FGELPCLESLELHTG
186553_2017-17        FGELPCLESLELHTG
320342_2017-15        FGELPCLESLELYTG
320342_2017-13        FGELPCLESLELYTG
320342_2017-14        FGELPCLESLELYTG
RB_blb                FGDLPCLESLELHWG
                      **:** ******  *
```

Figure 9. Amino acid sequence of the predicted No.11 and No.12 LRR units from the 18 *RB* orthologous genes and *RB blb* gene

```
LRR No. 13

161178_2075-88        SAEVEYVEE---DDHPG---
225661_1946-3         SAEVEYVEE---DDHPG---
283103_2017-23        SAEVEYVEE---DVHPG---
161178_2017-5         SAEVEYVEE---DDHPG---
225661_2075-47        SVEVEYVEE---NAHPG---
225661_1985-8         SVEVEYVEE---NAHPG---
161178_2075-85        SAEVEYVEE---NAHPG---
160221_1503-17        SVEVEYVEE---NAHPG---
160221_2374-7-        SVEVEYVEE---NAHPG---
160221_1503-24        SVEVEYVEE---NAHPG---
160221_1503-29        SVEVEYVEE---NAHPG---
283064_1985-61        SAEVEYVED---NVHPG---
283064_1985-60        SAEVEYVED---NVHPG---
186553_2049-30        SAEVEYVED---NVHPG---
186553_2017-17        SAEVEYVED---NVHPG---
320342_2017-15        SAEVEYVED---NVHPG---
320342_2017-13        SAEVEYVED---NVHPG---
320342_2017-14        SAEVEYVED---NVHPG---
RB_blb                SADVEYVEEVDIDVHSGFPT
                      *.:*****:   : *.*


LRR No.14

161178_2075-88        --RFPSLRKLVIWDFSNLKGLLKKEG
225661_1946-3         --RFPSLRKLVIWDFSNLKGLLKKEG
283103_2017-23        --RFPSLRKLVIWDFSNLKGLLKKEG
161178_2017-5         --RFPSLRKLVIWDFSNLKGLLKKEG
225661_2075-47        --RFPSLRKLVICDFGNLKGLLKKEG
225661_1985-8         --RFPSLRKLVICDFGNLKGLLKKEG
161178_2075-85        --RFPSLRKLVICDFGNLKGLLKKEG
160221_1503-17        --RFPSLRKLVICDFGNLKGLLKKEG
160221_2374-7         --RFPSLRKLVICDFGNLKGLLKKEG
160221_1503-24        --RFPSLRKLVICDFGNLKGLLKKEG
160221_1503-29        --RFPSLRKLVICDFGNLKGLLKKEG
283064_1985-61        --RFPSLRKLVIWDFSNLKGLLKKEG
283064_1985-60        --RFPSLRKLVIWDFSNLKGLLKKEG
186553_2049-30        --RFPSLRKLVIWDFSNLKGLLKKEG
186553_2017-17        --RFPSLRKLVIWDFSNLKGLLKKEG
320342_2017-15        --RFPSLRKLIIWDFSNLKGLLKKEG
320342_2017-13        --RFPSLRKLIIWDFSNLKGLLKKGG
320342_2017-14        --RFPSLRKLIIWDFSNLKGLLKKEG
RB_blb                RIRFPSLRKLDIWDFGSLKGLLKKEG
                        ******** * **..******* *
```

Figure 10. Amino acid sequence of the predicted No.13 and No. 14 LRR units from the 18 *RB* orthologous genes and *RB blb* gene

```
LRR No. 15

161178_2075-88        EKQFPVLEEMTIHWCPMFVI
225661_1946-3         EKQFPVLEEMTIHWCPMFVI
283103_2017-23        EKQFPVLEEMTIHWCPMFVI
161178_2017-5         EKQFPVLEEMTIHWCPMFVI
225661_2075-47        EEQFPVLEEMTIHGCPMFVI
225661_1985-8         EEQFPVLEEMTIHGCPMFVI
161178_2075-85        EEQFPVLEEMTIHGCPMFVI
160221_1503-17        EEQFPVLEEMTIHGCPMFVI
160221_2374-7         EEQFPVLEEMTIHGCPMFVI
160221_1503-24        EEQFPVLEEMTIHGCPMFVI
160221_1503-29        EEQFPVLEEMTIHGCPMFVI
283064_1985-61        EKQFPVLEEMTFYWCPMFVI
283064_1985-60        EKQFPVLEEMTFYWCPMFVI
186553_2049-30        EKQFPVLEEMTFYWCPMFVI
186553_2017-17        EKQFPVLEEMTFYWCPMFVI
320342_2017-15        EKQFPVLEEMTFYWCPMFVI
320342_2017-13        EKQFPVLEEMTFYWCPMFVI
320342_2017-14        EKQFPVLEEMTFYWCPMFVI
RB_blb                EEQFPVLEEMIIHECPFLTL
                      **:******** :: **::.


LRR No. 16

161178_2075-88        TLSSVKTLKFYVTYATVLMSISNFRALTSLYIRDNTKATLLQEEM
225661_1946-3         TLSSVKTLKVYVTYATVLMSISNFRALTSLYIRDNTKATLLQEEM
283103_2017-23        TLSSVKTLKFYVTYATVLMSISNFRALTSLYIRDNTKATLLLEEM
161178_2017-5         TLSSVKTLKFYVTYATVLMSISNFRALTSLYIRDNTKATLLQEEM
225661_2075-47        TLSSVKTLKVDVTDATVLRSISNLRALTSLDISSNYEATSLPEEM
225661_1985-8         TLSSVKTLKVDVTDATVLRSISNLRALTSLDISSNYEATSLPEEM
161178_2075-85        TLSSVKTLKVDVTDATVLRSISNLRALTSLDISSNYEATSLPEEM
160221_1503-17        TLSSVKTLKVDVTDATVLRSISNLRALTSLDISSNYEATSLPEEM
160221_2374-7         TLSSVKTLKVDVTDATVLRSISNLRALTSLDISSNYEATSLPEEM
160221_1503-24        TLSSVKTLKVDVTDATVLRSISNLRALTSLDISSNYEAASLPEEM
160221_1503-29        TLSSVKTLKVDVTDATVLRSISNLRALTSLDISSNYEATSLPEEM
283064_1985-61        TLSSVKTLKVIVTDATVLRSISNLRALTSLDISDNVEATSLPEEM
283064_1985-60        TLSSVKTLKVIVTDATVLRSISNLRALTSLDISDNVEATSLPEEM
186553_2049-30        TLSSVKTLKVIVTDATVLRSISNLRALTSLDISNNIEATSLPEEM
186553_2017-17        TLSSVKTLKVIVTDATVLRSISNLRALTSLDISNNIEATSLPEEM
320342_2017-15        TLSSVKTLKVIVTDATVLRSISNLRALTSLDITNNVEATSFPEEM
320342_2017-13        TLSSVKTLKVIVTDATVLRSISNLRALTSLDITNNVEATSFPEEM
320342_2017-14        TLSSVKTLKVIVTDATVLRSISNLRALTSLDITNNVEATSFPEEM
RB_blb                S--------------------SNLRALTSLRICYNKVATSFPEEM
                                           **:****** *  *  *: : ***
```

Figure 11. Amino acid sequence of the predicted No.15 and No. 16 LRR units from the 18 *RB* orthologous genes and *RB blb* gene

```
LRR No. 17

161178_2075-88        FKNLANLKDLTISGFKNLKELPTC
225661_1946-3         FKNLANLKDLTISGFKNLKELPTC
283103_2017-23        FKNLANLKDLTISGFKNLKELPTC
161178_2017-5         FKNLANLKDLTISGFKNLKELPTC
225661_2075-47        FKNLANLKDLTISDFKNLKELPTC
225661_1985-8         FKNLANLKDLTISDFKNLKELPTC
161178_2075-85        FKNLANLKDLTISDFKNLKELPTC
160221_1503-17        FKNLANLKDLTISDFKNLKELPTC
160221_2374-7         FKNLANLKDLTISDFKNLKELPTC
160221_1503-24        FKNLANLKDLTISDFKNLKELPTC
160221_1503-29        FKNLANLKDLTISDFKNLKELPTC
283064_1985-61        FKSLANLKYLNISFFRNLKELPTS
283064_1985-60        FKSLANLKYLNISFFRNLKELPTS
186553_2049-30        FKSLANLKYLNISFFRNLKELPTS
186553_2017-17        FKSLANLKYLNISFFRNLKELPTS
320342_2017-15        FKNLANLKYLKISLFDNLKELPTS
320342_2017-13        FKNLANLKYLKISLFDNLKELPTS
320342_2017-14        FKNLANLKYLKISLFDNLKELPTS
RB_blb                FKNLANLKYLTISRCNNLKELPTS
                      **.***** *.**   *******.


LRR No. 18

161178_2075-88        LASLNALNSLEIEYCDALESLPDEG
225661_1946-3         LASLNALNSLEIEYCDALESLPDEG
283103_2017-23        LASLNALNSLEIEYCDALESLPDEG
161178_2017-5         LASLNALNSLEIEYCDALESLPDEG
225661_2075-47        LASLNALNSLQIEYCDALESLPEEG
225661_1985-8         LASLNALNSLQIEYCDALESLPEEG
161178_2075-85        LASLNALNSLQIEYCDALESLPEEG
160221_1503-17        LASLNALNSLQIEYCDALESLPREG
160221_2374-7         LASLNALNSLQIEYCDALESLPREG
160221_1503-24        LASLNALNSLQIEYCDALESLPREG
160221_1503-29        LASLNALNNLQIEYCDALESLPREG
283064_1985-61        LASLNALKSLKFEFCDALESLPEEG
283064_1985-60        LASLNALKSLKFEFCDALESLPEEG
186553_2049-30        LASLNALKSLIFEFCDALESLPEEG
186553_2017-17        LASLNALKSLIFEFCDALESLPEEG
320342_2017-15        LASLNALESLKIEFCYALESLPEEG
320342_2017-13        LASLNALESLKIEFCYALESLPEEG
320342_2017-14        LASLNALESLKIEFCYALESLPEEG
RB_blb                LASLNALKSLKIQLCCALESLPEEG
                      *******:.* :: * ****** **
```

Figure 12. Amino acid sequence of the predicted No.17 and No. 18 LRR units from the 18 *RB* orthologous genes and *RB blb* gene

```
LRR No. 19

161178_2075-88        VKGLTSLTELSVSNCMMLKCLPEG
225661_1946-3         VKGLTSLTELSVSNCMMLKCLPEG
283103_2017-23        VKGLTSLTELSVSNCMMLKCLPEG
161178_2017-5         VKGLTSLTELSVSNCMMLKCLPEG
225661_2075-47        VKSLTSLTELSVSNCMTLKCLPEG
225661_1985-8         VKSLTSLTELSVSNCMTLKCLPEG
161178_2075-85        VKSLTSLTELSVSNCMTLKCLPEG
160221_1503-17        VKGLTSLTELSVSNCMTLKCLPEG
160221_2374-7         VKGLTSLTELSVSNCMTLKCLPEG
160221_1503-24        VKGLTSLTELSVSNCMTLKCLPEG
160221_1503-29        VKGLTSLTELSVSNCMTLKCLPEG
283064_1985-61        VKGLTSLTELSVSNCMMLKCLPEG
283064_1985-60        VKGLTSLTELSVSNCMMLKCLPEG
186553_2049-30        VKGLTSLTELFVSNCMMLKCLPEG
186553_2017-17        VKGLTSLTELFVSNCMMLKCLPEG
320342_2017-15        VKGLTSLTELSVSNCMMLKCLPEG
320342_2017-13        VKGLTSLTELSVSNCMMLKCLPEG
320342_2017-14        VKGLTSLTELSVSNCMMLKCLPEG
RB_blb                LEGLSSLTELFVEHCNMLKCLPEG
                      :::*:***** *.:*  *******


LRR No. 20

161178_2075-88        LQHLTALTTLTITECPIVFKRCERGIGED
225661_1946-3         LQHLTALTTLTITECPIVFKRCERGIGED
283103_2017-23        LQHLTALTTLTITECPIVFKRCERGIGED
161178_2017-5         LQHLTALTTLTITECPIVFKRCERGIGED
225661_2075-47        LQHLTALTTLIITQCPIVFKRCERGIGED
225661_1985-8         LQHLTALTTLIITQCPIVFKRCERGIGED
161178_2075-85        LQHLTALTTLIITQCPIVFKRCERGIGED
160221_1503-17        LQQLTALTTLIITQCPIVFKRCERGIGEE
160221_2374-7         LQQLTALTTLIITQCPIVFKRCERGIGEE
160221_1503-24        LQQLTALTTLIITQCPIVFKRCERGIGEE
160221_1503-29        LQQLTALTTLIITQCPIVFKRCERGIGEE
283064_1985-61        LQHLTALTTLIITQCPIVFKRCERGIGED
283064_1985-60        LQHLTALTTLIITQCPIVFKRCERGIGED
186553_2049-30        LQHLTALTTLTITECPIVFKRCEWGIGED
186553_2017-17        LQHLTALTTLTITECPIVFKRCEWGIGED
320342_2017-15        LQHLTALTTLIITQCPIVFKRCERGIGED
320342_2017-13        LQHLTALTTLIITQCPIVFKRCERGIGED
320342_2017-14        LQHLTALTTLIITQCPIVFKRCERGIGED
RB_blb                LQHLTTLTSLKIRGCPQLIKRCEKGIGED
                      **:**:**:* **.**   **** ****
```

Figure 13. Amino acid sequence of the predicted No.19 and No. 20 LRR units from the 18 *RB* orthologous genes and *RB blb* gene

```
LRR No. 21

161178_2075-88        WHKISHIPNVNIYI
225661_1946-3         WHKISHIPNVNIYI
283103_2017-23        WHKISHIPNVNIYI
161178_2017-5         WHKISHIPNVNIYI
225661_2075-47        WHKISHIPNVNIYI
225661_1985-8         WHKISHIPNVNIYI
161178_2075-85        WHKISHIPNVNIYI
160221_1503-17        VHKISHIPNVNIYI
160221_2374-7         VHKISHIPNVNIYI
160221_1503-24        VHKISHIPNVNIYI
160221_1503-29        VHKISHIPNVNIYI
283064_1985-61        WHKISHIPNVNIYI
283064_1985-60        WHKISHIPNVNIYI
186553_2049-30        WHKISHIPNVNIYI
186553_2017-17        WHKISHIPNVNIYI
320342_2017-15        WHKISHIPNVNIYI
320342_2017-13        WHKISHIPNVNIYI
320342_2017-14        WHKISHIPNVNIYI
RB_blb                WHKISHIPNVNIYI
```

Figure 14. Amino acid sequence of the predicted No.21 LRR unit from the 18 *RB* orthologous genes and *RB blb* gene

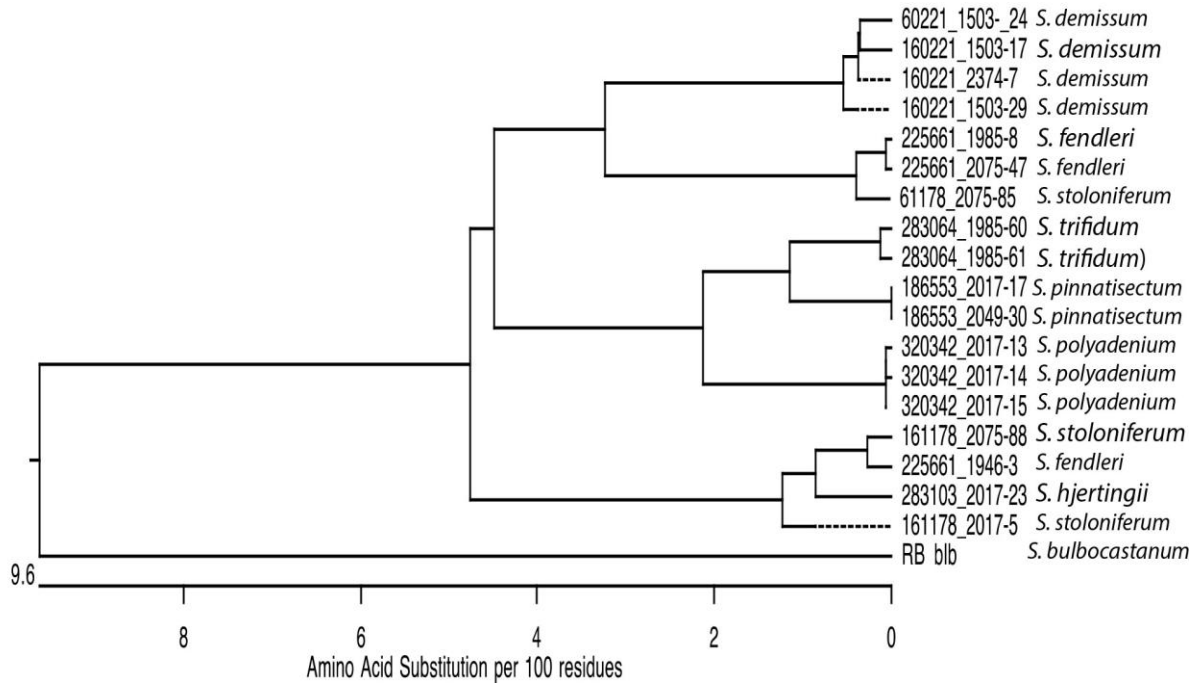

Fig. 15 Clustering analysis of amino acids of open reading frames of 18 *RB* orthologous genes from 7 wild *Solanum* species. *RB* amino acid sequence from *S. bulbocastanum* was included in the analysis.

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Chapter 6

Development of diploid potato clones with resistance to late blight, and early blight

Abstract

Late blight caused by the oomycete *Phytophthora infestans*, and early blight caused by *Alternaria solani*, result in serious losses in potato production worldwide. Host plant resistance is an important component of an integrated management for diseases, and it is safe for human health and the environment.

Resistance to late blight has been introgressed into five diploid hybrid clones. Two of these clones are also resistant to early blight. The wild potato species *Solanum verrucosum* and *S. commersonii* were the source of resistance to late blight and early blight, respectively. The clones with resistance to late blight were named LB1, LB2, and LB3. The clones with resistance to early blight and late blight were named ELB1 and ELB2. All five clones have acceptable tuber yield, do not produce 2n pollen grains, and produce viable pollen. These clones will be useful for introgressing early blight and late blight resistance into breeding lines.

Introduction

Late blight caused by the heterothallic oomycete *Phytophthora infestans* (Mont) de Bary, and early blight caused by the fungus *Alternaria solani* Sorauer, are two serious diseases of potato *Solanum tuberosum* L. Both diseases occur worldwide and are prevalent wherever susceptible potato plants are cultivated (10,13,24). While late blight is a disease that can infect and destroy potato plants at any phenological stage, early blight occurs on older leaves as they senesce; from the oldest leaves, the disease spreads to other foliage in the plant canopy (11). *P. infestans* can infect any tissue of the potato plant. Symptoms of late blight include leaf lesions beginning as olive green areas that quickly enlarge to become brown-black and water-soaked in appearance. Sporulation of the pathogen on leaves has white-gray fuzzy growth (12). Symptoms

of early blight are characterized by small and brown lesions with a concentric ring pattern mainly in old senescing plants with heavy fruit set (11). Integrated management of both diseases is essential to avoid severe losses. Currently most potato cultivars are susceptible to late blight and early blight, and the most common method to control both diseases is through the heavy use of fungicides (15,19). Application of fungicides may occur as frequently as every 5 days. However, even with this schedule, fungicide application is not always effective, especially under high disease pressure and during rainy periods.

The use of resistant cultivars is a convenient method to manage both diseases, and the development of potato cultivars with resistance to late blight is one of the main objectives in many potato breeding programs. Genetic resistance to late blight and early blight has been identified in wild *Solanum* species. High genetic diversity can be found in the 110 wild relatives of potato, approximately 70% of which are diploid (2n = 2x = 24). However the cultivated potato, *S. tuberosum*, is tetraploid (2n = 4x = 48). Most diploid *Solanum* species can be crossed with dihaploids (2n = 2x = 24) of the cultivated potato (16,17), providing an excellent way to broaden the genetic base of breeding populations. Tuber-bearing *Solanum* species have been used as a source of resistance to late blight disease, and other economically important traits such as resistance to fungus, insects, viruses, nematodes, frost hardiness, cold-induced sweetening, tuber dormancy, flesh color, uniformity and tuber shape (1,4,5,18,27,28). In the 1950s, the wild species *S. demissum* was the source of 11 race-specific genes for resistance to late blight in potato (30). These resistance genes were overcome by more virulent clonal lineages of *P. infestans* in the 1960s after their release in commercial potato cultivars (6,21,23,27). In 2003 the late blight partial resistance *RB* gene was isolated from *S. bulbocastanum.* This gene confers broad-spectrum resistance to multiple clonal lineages of *P. infestans* including US-1, US-1.7,

US-8, US-10, and US-14 (29). In 2005, an orthologous *RB* gene was identified in the wild diploid potato species *S. verrucosum*. This gene was cloned and introduced into a susceptible potato cultivar via transformation (20).

The production of numerically unreduced (2n) gametes is the result of meiotic mutation occurring during micro – and mega–sporogenesis and is a natural phenomenon that occurs in *Solanum* species (3,26). Potato breeders are taking advantage of this phenomenon for the introduction of resistance from diploid *Solanum* species to the tetraploid cultivated potato (31,33). Diploid plants can be screened for the presence of 2n pollen by looking for large pollen grains under a microscope (8). They can be evaluated for 2n egg production by crossing as females to tetraploid plants. If seeds are produced, then 2n eggs are present (25). Wild *Solanum* species can influence the production of male sterility in the progeny between dihaploid *S. tuberosum* and wild *Solanum* species, probably due the presence of a dominant gene of male sterility (MS) from the wild species (2,14). The species integrity in wild potato relatives is achieved by several mechanisms, such pollen-pistil incompatibility, endosperm/embryo barriers and nuclear cytoplasmic and male sterility (2,9,32). It is necessary to overcome these mechanisms when introgressing genes from wild *Solanum* species into cultivated *S. tuberosum.*

The objectives of this study were: a) to evaluate resistance to late blight of progeny from (*S. verrucosum* x dihaploid *S. tuberosum*) x (*S. tuberosum* x *S. chacoense*), and b) to evaluate resistance to late blight and early blight of progeny from [(*S. verrucosum* x *S. commersonii*) x dihaploid *S. tuberosum*)] x (*S. tuberosum* x *S. chacoense*).

Methods:

Population development:

For pollination, flower buds on female plants were emasculated before anthers dehisced. Pollen grains were collected from male plants with a mechanical buzzer (32). The diploid *S. verrucosum* accession 275260 was used as a source of resistance to late blight, while *S. commersonii* accession 472839 was the source of resistance to early blight. The diploid hybrid M9 was developed by crossing a late blight resistant *S. verrucosum* clone as a female to an early blight resistant *S. commersonii* clone. The diploid potato breeding line XD3 was developed by crossing the dihaploid *S. tuberosum* US- W4 as a female to a clone of *S. chacoense* accession 275138. The hybrids M9 and XD3 were developed in previous research by Dr. Shelley Jansky in the USDA-ARS Madison potato germplasm enhancement program.

Development of clones resistant to late blight

In a previous study, a clone of *S. verrucosum* PI275260, named ver 4 showed resistance to late blight caused by *P. infestans* genotypes US-1, US-8, and US-11 (12). A population of 150 ver 4 x US-W4 seedlings was grown and transplanted to individual pots containing 200 g of soil. Resistant ver 4, susceptible US-W4, and the susceptible potato cultivar Katahdin were included as controls. Plants were grown in a late blight-free greenhouse at 24°C, 80% relative humidity with a 16 hour photoperiod for late blight resistance screening (described below). Flowers of the selected resistant plants were pollinated with tetraploid *S. tuberosum* cultivars: Mega Chip and White Pearl, and the diploid breeding lines XD3 and hap-chc (dihaploid US-W973 x *S. chacoense)*. Seeds were formed only in the crosses of (*S. verrucosum* x US-W4) x (XD3). Parents and 174 plants progeny from this cross were screened for resistance to late blight using

the method of whole plant inoculation with a mixture of three clonal lineages of *P. infestans* as described below. A total of 12 plants were selected as resistant to late blight. These were evaluated in the field at the Hancock Agricultural Research Station, Wisconsin, (HARS) in 2013 for agronomic quality traits such as overall disease resistance, tuber yield, and number of tubers. Determination of pollen viability and screening for 2n pollen was performed, to identify the genetic barrier for the failure of hybridization with tetraploid cultivars. After evaluation in the field, three clones were selected as resistant to late blight.

Development of clones resistant to early blight and late blight

In previous research, a diploid breeding line named M9 that is resistance to late blight and early blight was developed. The M9 was generated by crossing a late blight resistant clone from the diploid *S. verrucosum* accession 275260 as a female to an early blight resistant clone of diploid *S. commersonii*. The hybrid diploid clone M9 (*S. verrucosum* x *S. commersonii*) was pollinated with dihaploid *S. tuberosum* US-W4. The parents and 280 progeny were inoculated with *A. solani* using the method of whole plant inoculation as described below. A total of 16 plants were selected as resistant to early blight. Selected plants were inoculated with mixture of three clonal lineages of *P. infestans*. After visual evaluation, four plants were selected as resistant to late blight. Flowers of the selected plants were pollinated with the diploid breeding line XD3. Another set of crosses was attempted using tetraploid *S.tuberosum* cultivars Yukon Gold, Russet Burbank, and Superior as male parents. Successful crosses were obtained only with the diploid breeding line XD3. A total of 150 progeny from this cross were screened with a mixture of three clonal lineages of *P. infestans*, for resistance to late blight. Selected plants were screened for resistance to early blight by inoculating with *A. solani*, as described below. A total

of 12 plants were selected as resistant to early blight and late blight and they were evaluated in the field at the HARS in 2013, for agronomic quality traits such as overall disease resistance, yield, and tuber size. After evaluation in the field, two clones were selected.

Disease resistance screening:

Each plant was grown in a pot containing 200 g peat moss-vermiculite, in a late blight-free greenhouse at 26°C, for six weeks. On the day of inoculation, plants were moved to a mist chamber with 100% relative humidity, a temperature of 18°C, and natural lighting.

For late blight resistance screening, a whole plant disease resistance assay was used. Sporangial inoculum was generated from a single-zoospore culture of each *P. infestans* isolate. Cultures were grown on Rye A agar in 90-mm diameter petri plates at 18°C for 21 days in complete darkness. Sporangia were collected from these cultures by adding 5 ml sterile water to each plate and gently scraping the agar surface with a glass spreading rod. A mixture of three *P. infestans* genotypes collected in Wisconsin, US-22, US-23, and US-24 was used in equal proportions. Sporangial concentrations were calculated using a hemocytometer and adjusted to a concentration of 50,000 sporangia per ml. The sporangial suspensions were incubated for 2 h at 12°C under darkness to promote the release of zoospores. Zoospore inoculum in a 3-ml-volume total suspension was applied to both abaxial and adaxial surfaces of all leaflets of each plant using a spray bottle. Disease reactions to *P. infestans* inoculation were evaluated at eight days after inoculation (dai) with a categorical visual rating for each plant based on percent symptomatic tissue: 1: > 90%; 2: 81-90%; 3: 71-80%; 4: 61-70%; 5: 41-60%; 6: 26-40%; 7:11-25%; and 8 < 10% (7). Plants with scores > 7 were selected as resistance to late blight.

For early blight resistance screening, a whole plant disease resistance assay was also used. Inoculum was generated from a single conidia culture of a virulent isolate of *A. solani* cultivated on clarified V8 agar on 90-mm diameter petri plates at 22°C for 21 days in continuous light. Conidia were collected from cultures by adding 5 ml of sterile water to each plate and gently scraping the agar surface with a glass spreading rod. Conidial concentrations were calculated using a hemocytometer and adjusted to a concentration of 50,000 spores per ml. A three ml conidial suspension was applied to both abaxial and adaxial surfaces of all leaflets of each plant using a spray bottle. Disease reactions to *A. solani* inoculation were evaluated at eight days after inoculation (dai) with a categorical visual ranging for each plant that was based on percent symptomatic tissue, as follow: susceptible >20% and resistant <20%.

Field trial

A field trial was carried out in 2013 at the HARS. The trial was planted in a randomized block design with three replications, and each replication consisted of five plants. Entries included the five test clones and the susceptible check cultivar Russet Burbank. Tubers were planted on April 22, at a distance of 30 cm between each plant and 1 m between rows. The experimental plots were bordered by other experimental plots that were not treated against early blight and late blight. All plants were fertilized and received standard production practices and no fungicide application. Field trials were naturally infected by *A. solani*; first symptoms of early blight in the susceptible check plants were observed in the second week of July. There were no reports of late blight at the HARS during the growing season 2013. Vines were killed on September 4th, and all tubers in each replication were harvested on September 26th.

Disease rating:

Early blight scores were based on the percent early blight symptoms observed on each plant in the field at HARS during the 2013 growing season. Plots were scored for early blight three times in the season: July 12, August 5 and August 26. A categorical visual ranging for each plot was based on percent of symptomatic tissue, as follows: susceptible >20% and resistant <20%. All plots were also monitored for symptoms of late blight.

Tuber yield:

Tubers were dug with a tractor-mounted single row potato digger. Tubers from each replication were collected by hand and kept separate in plastic bags. Tuber weight from each replication was determined.

Determination of pollen viability and 2n pollen

Pollen grains were collected by shaking the flowers with a mechanical buzzer. Collected pollen grains were stained with 1% acetocarmine and observed at 100x magnification (22). A total of 100 pollen grains from each clone were counted from each of two samples. Viable pollen grains were estimated based on the percent those that were pink-red stained; non-stained pollen was counted as non-viable. Percent 2n pollen was determined based in the diameter of the pollen grains, since 2n pollen grains in *Solanum* species have larger diameter, approximately 1.26 times that of n-pollen (8).

Fertility evaluation

Evaluation of female fertility and 2n egg production of the five selected clones was performed on plants growing in a greenhouse. Flowers were emasculated before anthers

dehisced and pollen grains from the tetraploid potato cultivars Yukon Gold, Russet Burbank, Superior, and the diploid breeding line M6 was applied.

Data Analysis

Statistical analysis was performed using the software SigmaPlot (Systat Software, Inc. San Jose, CA, USA). After analysis of the data from the field trials, five clones were selected based on early blight resistance scores and tuber yield.

Results and discussion

The resistance to late blight from the diploid species *S. verrucosum* was introgressed into five diploid clones. Two clones, named ELB1 and ELB2, with resistance to early blight and late blight, and three clones named LB1, LB2, and LB3 with resistance to late blight were selected. Clones were selected based on early blight and late blight resistance observed in a mist chamber, resistance to early blight in the field, and tuber yield in the field in the 2013 growing season in Wisconsin. The *S. verrucosum* clone used in this study contains the *RB* orthologous gene for resistance to late blight.

Late blight resistance

For selections of plant progeny with resistance to late blight, screening was performed using mist chamber inoculations of whole plants. Selected clones were resistant to inoculations with a mixture of the three most prevalent clonal lineages in the U.S., US-22, US-23, and US-24. Each selected individual had a score >7, similar to the parental *S. verrucosum* clone. The

selected clones showed small water soaking lesion and chlorosis on the lower leaves. No sporulation was observed on the lesions that developed on the selected clones. The mean resistance score of the selected clones was 7.0 (20% symptomatic tissue), and the susceptible check was 2.0 (85% symptomatic tissue) (Fig. 1). There was a statistically significant difference (P=0.05) between the mean value of the selected clones and the susceptible check. Due to no natural infection of late blight in the HARS during the 2013 growing season, it was not possible to confirm the resistance to late blight in the field.

Early blight resistance

Evaluations of early blight resistance were initially made on plants in a mist chamber. Whole plant inoculations were carried out in the mist chamber, which maintained the relative humidity around 90% and a temperature of 18°C. Plants were characterized as either resistant or susceptible. Selected clones had >20% symptomatic leaves and symptoms were located only in the lower leaves. Resistance observed in the mist chamber experiment was confirmed in the field trial at the HARS. Heavy natural infection occurred in the susceptible check potato variety Russet Burbank. The first symptoms of infection occurred on the lower leaves on the second week of July, 2013. During the following weeks, early blight symptoms rapidly increased and spread onto higher leaves in the plant canopy. At the end of the season, five hybrid clones showed high resistance, with scores between 18 and 20% symptomatic leaves (Fig. 2).

Yield assessment

The tuber yield of each replication of the 24 diploid clones was calculated. There was a high variation in the yield from each clone, ranging from 0.2 kg/plot to 1.4 kg/5-hill plot. The

criteria for selection were acceptable yield and resistance to disease. Three clones named LB1, LB2, LB3, were selected for resistance to late blight; mean tuber yields were 837, 1066, and 922 g, respectively (Fig. 3). Two clones, named ELB1 and ELB2, with resistance to early blight and late blight were selected. The mean tuber yields per plot of those selected clones were 1481 and 1113 g, respectively (Fig.4).

Pollen viability, 2n pollen, and fertility assessment

Acetocarmine staining was used as a measure of pollen viability in the selected clones. The five selected clones are moderate fertile, producing from 30 to 59% stainable plump pollen grains. Pollen diameter ranged from 22 to 25 µm, while that of a tetraploid cultivar was 31 µm, indicating that the five clones do not produce 2n pollen. This was the reason that all attempts to cross with tetraploid cultivars were unsuccessful, (Table l).

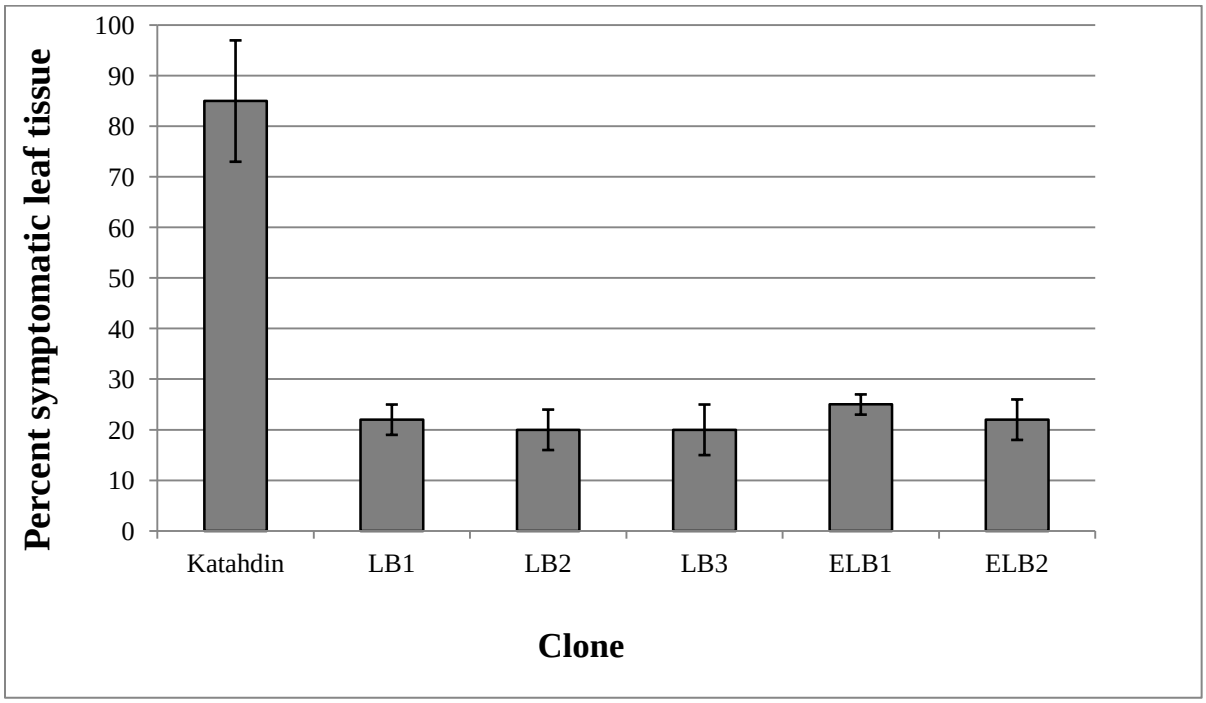

Figure 1. Percent symptomatic leaf tissue on five clones selected for resistance to late blight, eight days after inoculation with a mixture of US-22, US-23 and US-24 clonal lineages of *Phytophthora infestans*. Katahdin is the susceptible control. Error bar represents the standard deviation.

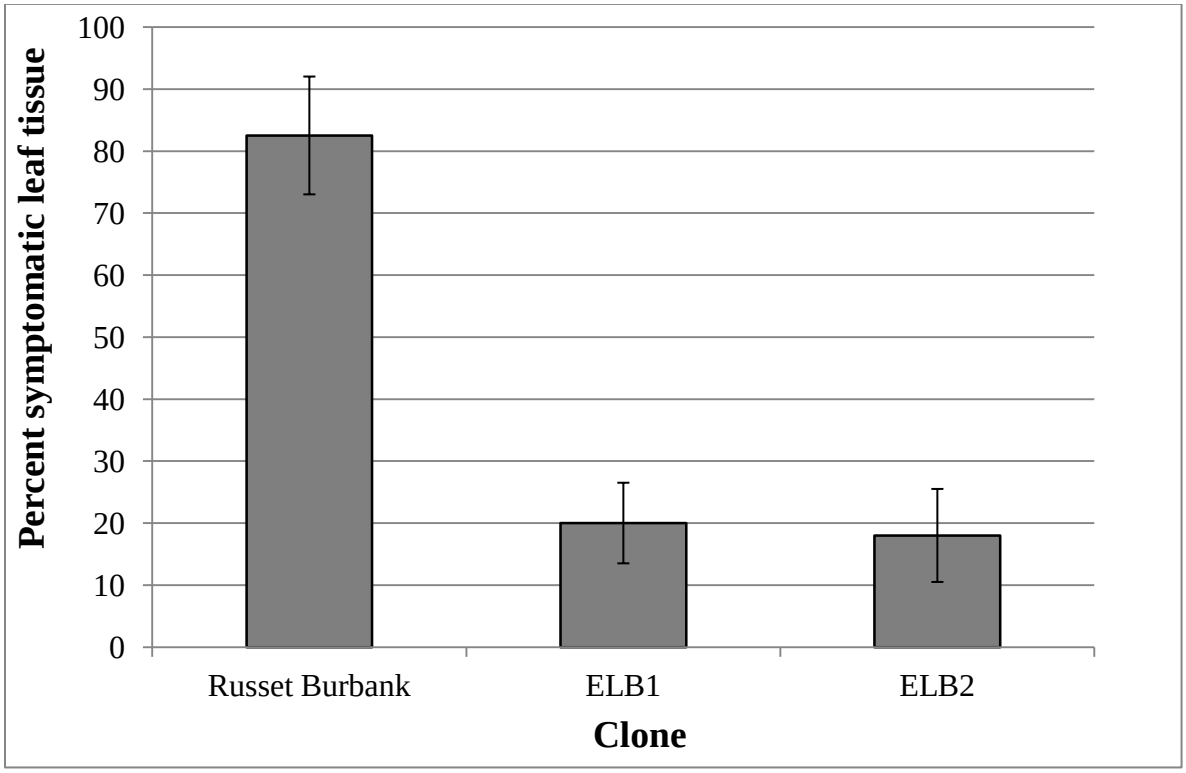

Figure 2. Percent early blight symptoms on clones with resistance to early blight, eight days after inoculation with *Alternaria solani*. Cultivar Russet Burbank is the susceptible control. Error bar represents the standard deviation.

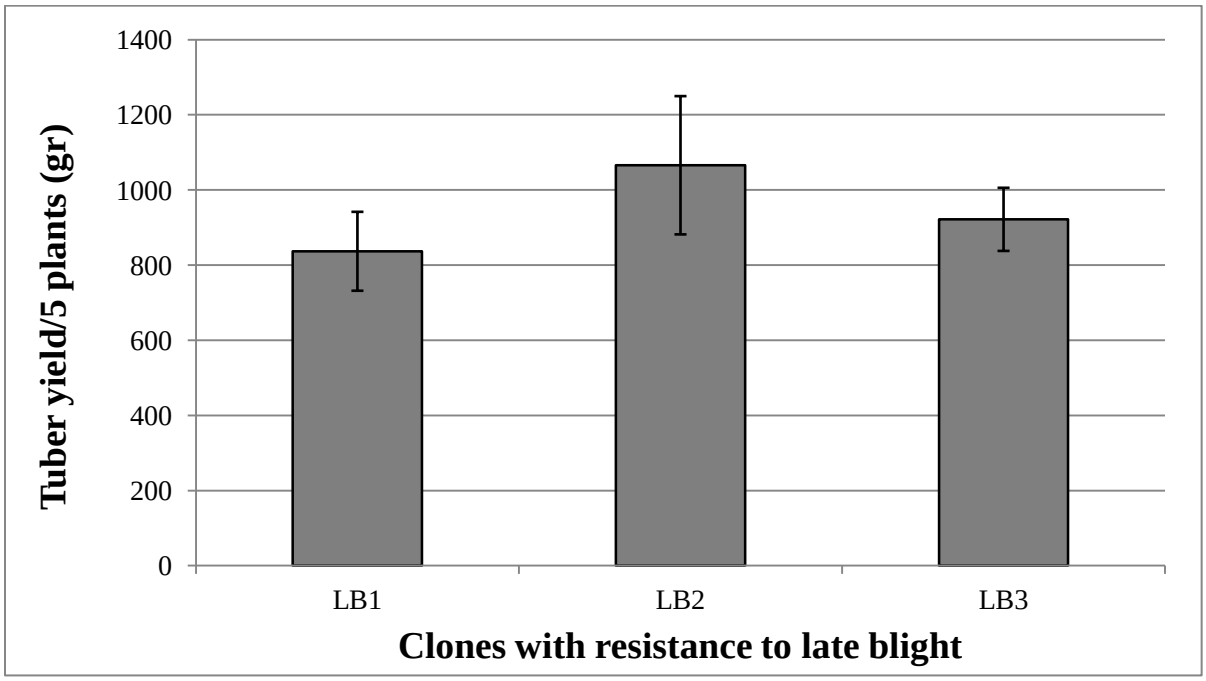

Figure 3. Mean tuber yield in g/five plants of three clones with resistance to late blight, harvested at the Hancock Agricultural Research Station in the 2013 growing season.

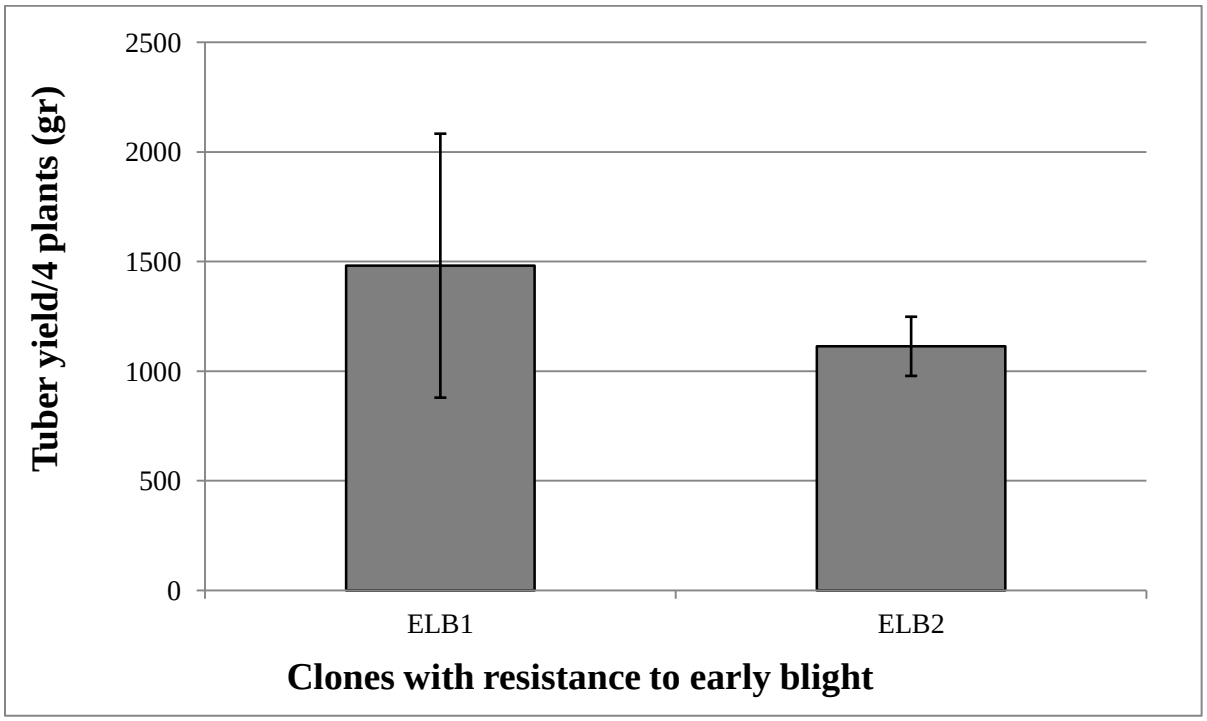

Figure 4. Mean tuber yield in g/five plants of two clones with resistance to early blight and late blight, harvested at the Hancock Agricultural Research Station in the 2013 growing season.

Table 1. Viability of pollen grains and production of 2n pollen of five diploid clones

No.	Clone	Stainability[a]
1.	LB1	35 ±9
2.	LB2	30 ±8
3.	LB3	36 ±11
4.	ELB1	59 ±14
5.	ELB2	51 ±13

[a] Percentage of pollen grains stained with acetocarmine; and its standard deviation.

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