A Survey of Tomato and Potato Fields in Florida Reveals Unique Genotypes of *Phytophthora infestans* between 2005 and 2007

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Abstract. Late blight, caused by *Phytophthora infestans*, affects tomatoes and potatoes in Florida during the winter–spring crop season. During the 2005 season, severe late blight epidemics were observed in Florida prompting our survey. Isolates from 2005 to 2007 were characterized phenotypically based on growth on three media, mating type, pathogenicity, and sensitivity to metalaxyl and genotypically based on two isozymes, mitochondrial DNA (mtDNA), and genomic profiling using the RG57 probe. Isolates collected in this survey were all A2, mtDNA Ia, and either 100/100 (2005), or 100/122 (2006/2007) at the *Gpi* locus, and homozygous 100 at the *Pep* locus. Novel genotypes infecting tomato in Florida were observed based on the *Gpi* locus and RG57 genomic profile. We propose US-20 for the collection of clonal isolates recovered during the 2005 season and US-21 for clones recovered during 2006 and 2007. In addition to these novel genotypes recovered from tomato, one isolate was recovered from potato representing the US-8 clonal lineage. The findings of the survey in south Florida and their implications are presented.

Late blight of potato and tomato caused by the stramenopile *Phytophthora infestans* is regarded as the world's most destructive crop disease and has been considered a threat to global food security (Birch and Whisson, 2001; Duncan, 1999; Grunwald and Flier, 2005; Judelson and Blanco, 2005; Mizubuti and Fry, 1998; Raman et al., 2000). The genus *Phytophthora* encompasses more than 80 species with *P. infestans* representing perhaps one of the more highly evolved

species (Blair et al., 2007; Göker et al., 2007; Kroon et al., 2004). This is evidenced by its host specialization, formation of haustoria, near-obligate tendencies, and aerial dispersal of asexual spores (Fry, 2008; Fry et al., 1993; Fry and Mizubuti, 1998). *Phytophthora infestans* is a heterothallic species requiring the presence of both the A1 and A2 mating types for production of sexually recombinant oospores and sexual recombination (Erwin and Ribeiro, 1996; Galindo and Gallegly, 1960; Smoot et al., 1958). Tomato and potato production is chronically plagued by late blight epidemics resulting from conditions during the growing season in south Florida. Late blight impacts Florida's winter tomato crop, a \$464 million industry that accounted for 36% of the national production of fresh tomatoes in 2007 (National Agricultural Statistic Service, USDA).

Early genetic investigations of *P. infestans* populations relied on the use of isozymes, namely *glucose-6-phosphate* isomerase [GPI,

electrical conductivity (EC) 5.3.1.9] and *peptidase* (PEP EC, 3.4.3.1) and DNA fingerprinting through Southern hybridization with the RG57 probe (Goodwin et al., 1992a, 1992b, 1994b; Grunwald et al., 2001). A second marker uses the maternally inherited mitochondrial genome (mtDNA) and has been applied to scenarios varying from population studies to typing herbarium samples from the Irish potato famine (Carter et al., 1990; Gomez-Alpizar et al., 2007; Griffith and Shaw, 1998; Ristaino et al., 2001). These molecular tools when used in combination with the phenotypes for virulence, mating type, and resistance or sensitivity to the phenylamide fungicide metalaxyl have provided a standardized framework for classifying *P. infestans* genotypes (Forbes et al., 1998; Goodwin et al., 1994a).

Before the 1970s, populations of *P. infestans* in the United States and many countries of the world were predominated by the A1 mating type and some derivative of the US-1 genotype (Andrison, 1996; Goodwin et al., 1994a). In Florida, late blight epidemics were infrequent until the early 1990s when conducive conditions likely allowed for the migration of new genotypes from Mexico (Goodwin and Drenth, 1997; Goodwin et al., 1995b). The first novel genotype observed in Florida was US-6 (A1 mating type) in 1991 (Goodwin et al., 1994a; Weingartner and Tombolato, 2004). Both US-6 and US-7 (A2 mating type) had been reported within a single field as early as 1993 (Goodwin et al., 1995b). In Florida, by 1994, the US-8 genotype had appeared and by 1996, the US-17 genotype and possible progeny of US-6 × US-8 was recovered from tomato (Goodwin et al., 1998). We suspect that novel genotypes may be responsible for severe late blight epidemics that have been occurring since 2005. The goal of the current study was to characterize the genotypes of *P. infestans* recovered from tomato and potato grown in production regions in southern and central Florida over multiple seasons.

Materials and Methods

Stramenopile cultures. Isolates were obtained from leaf, stem, and fruit of tomato (85%) and potato (15%) plants in central and south Florida during the fall/spring (August/May) growing seasons spanning 3 years (2004–2005 = 2005; 2005–2006 = 2006; and 2006–2007 = 2007). Samples were obtained from southwest Florida, including Hendry, Collier, and Lee counties and from the west–central Florida county of Manatee and the east coast, Palm County. Infected leaf tissue was placed in a petri plate on a moistened sterile filter paper, wrapped with parafilm, and incubated at 20 °C in darkness. Fruit and stem samples were treated similarly, with the exception that plastic clam food boxes (14 × 14 × 7 cm; Dart Container Corp., Mason, MI) were used in place of petri plates. Tissue was monitored daily for sporulation, and sporangia were picked with the tip of a sterile scalpel and transferred onto

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agar plates of either Rye seed A agar (RSA) or barley potato dextrose agar (BPDA) amended with 150 µg·mL⁻¹ ampicillin (Caten and Jinks, 1967; Erwin and Ribeiro, 1996). Single-spore isolation was performed by picking sporangia from a sporulating lesion and placing in a 1.5-mL microcentrifuge tube containing 1 mL of 0.8% water agar amended with 300 µg·mL⁻¹ ampicillin warmed to 35 °C. The suspension was vortexed for 30 s and poured on BPDA amended with ampicillin. Plates were incubated at 20 °C for 24 h and when a single, germinating, sporangium was detected through a stereomicroscope, lifted with a bit of agar using the tip of a sterile scalpel, and placed either into pea broth (Wangsomboondee et al., 2002) or on BPDA.

We evaluated BPDA, RSA, and V8-juice agar (V8A) media to determine if there was a nutritional preference for two isolates representing US-20 (Pi052 and Pi058) and two representing US-21 (Pi0626 and Pi06339). The same media, also tested in the same experiments, were amended with 5% activated charcoal (AC) to test for growth enhancement because AC is reported to improve the growth of *P. infestans* without interfering with sporulation (Caten and Jinks, 1967; Erwin and Ribeiro, 1996). Three plates per treatment per isolate were inoculated with a 5.0-mm plug of mycelia taken from an actively growing colony of *P. infestans* and incubated at 20 °C in the dark. After 7 d, the colony diameter was measured in two orthogonal directions; these experiments were repeated four times. The data were analyzed using SAS (Version 9.1; Cary NC) using PROC GLM and the means were separated by Fisher's protected least significant difference.

Phenotypic characterization. Mating types were determined as has been described previously (Grunwald et al., 2001). Plugs of isolates representing 42 cultures were paired on RSA agar and V8A plates either with US940501 (A1) or Pi02-007 (A2) tester isolates. The plates were then sealed with parafilm, incubated at 15 °C in darkness for 3 to 4 weeks, and then examined microscopically for the presence of oospores.

Phenylamide sensitivity was assessed by placing a 7-mm agar plug from 1-week-old cultures on three 100 × 15-mm plates of RSA agar and three 100 × 15-mm plates of RSA amended with 100 µg·mL⁻¹ metalaxyl (Ridomil® 2E) (Deahl et al., 1995). Plates were incubated at 18 to 21 °C for 7 d, and colony diameters were measured once average growth diameters reached 15 mm on unamended plates and again 3 d later. Each isolate was tested at least twice. The percentage reduction in growth compared with growth on the unamended control plates was calculated. Isolates were designated as metalaxyl-resistant if growth was greater than 60% of the control, intermediate if growth was 10% to 60% of the control, or sensitive if growth was less than 10% of the control using previously described criteria (Shattock, 1988).

In-planta assays. Tomato plants of cultivar FL-47 were used for all pathogenicity

experiments. Plants were maintained in growth chambers at 25 °C receiving 14 h light before inoculation. Two US-20 tomato isolates (Pi052, Pi058) and two US-21 (Pi06268 and Pi06339) were used in pathogenicity assays. Inoculum was prepared by growing cultures on RSA for 2 weeks at 18 °C in darkness, then flooding plates with 10 mL of 4 °C H₂O, and rubbing to dislodge sporangia. The suspension was then filtered through four layers of cheesecloth. Sporangia were counted with a hemacytometer, and distilled water was used to adjust the suspension to 1 × 10⁴ sporangia/mL. In detached leaf assays, two leaflets were placed abaxial side up in a 100 × 15-mm plate with moistened filter paper. A 10-µL aliquot of the sporangial suspension was spotted onto each leaf. Inoculated leaflets were maintained in an incubator at 18 °C and a 14/10-h light/dark cycle. Lesion size was estimated as the percentage of the leaf area exhibiting symptoms. Detached leaf assays were repeated three times.

For intact plant assays, tomatoes were grown in 10-cm pots containing Metro-Mix 3000 with 6 g Scotts Miracle Gro Osmocote 14N-4.2P-11.6K in the greenhouse. At 4 weeks of age, plants were moved 3 d before inoculation to a growth chamber set at 20/15 °C with a 14/10-h light/dark cycle. Five plants per isolate were sprayed until runoff using a handheld aerosol mister with the sporangial suspension prepared as described previously. Untreated plants were sprayed with distilled water. After inoculation, plants were placed in clear plastic bags that had been sprayed with distilled water and returned to the growth chamber. Symptoms were evaluated 5 d after inoculation, estimating the disease severity as the percentage of total tissue that was symptomatic. Intact plant assays were repeated three times. The mean difference in disease severity was analyzed using an unpaired Student's *t* test.

Genotypic characterization. Genotypes at two polymorphic allozyme loci, *Gpi-1* (GPI EC 5.3.1.9.) and *Pep-1* (PEP, EC 3.4.3.1.), were determined using protocols of described in Goodwin et al. (1995a). Similarly, genomic fingerprinting was conducted using the RG57 probe as described previously (Chen et al., 2009; Goodwin et al., 1992a). For ease of visualization, RG57 profile data were transformed to binary data and complied with those data of previously published US genotypes (Goodwin and Drenth, 1997; Goodwin et al., 1998; Wangsomboondee et al., 2002). Mitochondrial DNA haplotypes were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) according to Griffith and Shaw (1998). The P2 amplicon was generated with primers F2/R2 and the F4 amplicon was amplified with F4/R4; products were confirmed by agarose electrophoresis. The resulting P2 amplicon was digested with *MspI* and the F4 amplicon was digested with *EcoRI*. Restriction profiles were resolved on a 2% agarose gel (Griffith and Shaw, 1998). Characterization of genotypes followed the

Phytophthora infestans global marker database (Forbes et al., 1998).

Results

Isolation and culture. Seventy-six isolates were recovered from tomato and potato during Fall 2004 through Spring 2007 representing three winter growing seasons. Significant differences were detected between the growth of the two genotypes ($P < 0.0001$) on all media. Isolates from 2005 grown on BPDA-AC and V8-AC had significantly greater colony diameters compared with 2006 isolates on these same media. At 20 °C, isolates from 2005 grew on all media but growth diameter was slightly increased with 5% AC amendment but not significantly (Fig. 1). The diameter of the colonies for isolates from 2006 was greatest on RSA, RSA-AC, and V8-AC, whereas growth was significantly reduced ($P < 0.0001$) on other media.

Detached leaf and intact plant assays. Both US-20 and US-21 isolates produced typical, large, blighted lesions with abundant sporulation. However, inoculation with US-20 isolates consistently resulted in a significantly higher percentage disease severity ($P < 0.0001$) when compared with the US-21 isolate (Fig. 2).

Metalaxyl sensitivity. Eighty percent of isolates ($n = 15$) from 2005, proposed as US-20, were sensitive to metalaxyl and 20% exhibited intermediate resistance (Table 1). Proposed US-21 isolates ($n = 16$) from 2006 were 38% sensitive, 31% intermediate, and 31% resistant and US-21 isolates ($n = 14$) recovered in 2007 were 50:50 sensitive and intermediate.

Mating type. All of the isolates ($n = 45$) were the A2 mating type regardless of host, geographical location, or year of isolation. The sole exception was the US-8 potato isolate (Pi0626) recovered in 2006, which appeared self-fertile (Table 1).

Genotypic characterization. Isolates recovered in 2005, designated as US-20, were homozygous (100/100) at the *Gpi* locus and have the RG57 profile presented in Table 1. We propose US-21 for tomato isolates from 2006 ($n = 23$) and 2007 ($n = 35$), which were heterozygous 100/122 at the *Gpi* locus and are polymorphic from the US-20 in the RG57 profile (Table 2). One isolate recovered in 2006 from potato was observed to be 100/111/122 at the *Gpi* locus and had a RG57 profile identical to that of US-8. All isolates recovered were homozygous (100/100) at the *Pep* locus. Similarly, PCR-RFLP of mtDNA indicated that all of the isolates from 2005 to 2007 ($n = 30$) were the Ia haplotype. Consistent with the shift from the 100/100 *Gpi* profile to 100/122, isolates representing US-21 exhibited a unique RG57 profile different from US-20 isolates.

Discussion

Our analysis of *P. infestans* isolates recovered from tomato and potato in Florida

Fig. 1. Mean diameter growth of three US-20 and three US-21 genotypes of *P. infestans* colonies on different media (BPDA= barley-potato dextrose agar; V8 = V8 juice agar; RSA= rye seed agar; AC= 5% activated carbon). Statistical differences detected by genotype (A, B) and media within genotype (a, b) at $P < 0.0001$.

Fig. 2. Disease severity (% of infected tissue) of two US-20 (Pi058 and Pi052) isolates and two US-21 isolates (Pi0662 and Pi0628) inoculated on FL 47 tomato (mean of two experiments). Columns with different letters (a, b) are statistically different at $P < 0.0001$.

Table 1. Metalaxyl sensitivity of *Phytophthora infestans* isolates collected in Florida from 2005 to 2007.

Yr	Number of isolates			
	Total	Sensitive	Intermediate	Resistant
2005	15	12	3	0
2006	16	6	5	5
2007	14	7	7	0

through growing seasons 2005 to 2007 identified unique genotypes not previously described within the United States. In 2005, isolates were similar to the US-13 genotype based on mating type, mtDNA, and isozyme

profiles but could not be identified conclusively because a RG57 profile for US-13 genotype could not be obtained from the literature. Lacking a complete reference profile for US-13, we propose to recognize the 2005 isolates from tomato in Florida as US-20 following the suggestions put forth previously (Forbes et al., 1998; Goodwin et al., 1994a). In 2006 to 2007, the US-20 clonal lineage was essentially displaced by a new genotype, and we propose the name US-21 for the clonal lineage collected in 2006 and 2007 in Florida. In addition to the US-21 genotype, we also observed the historic US-8 genotype, which has been recovered from

potato in Florida for over 12 years (Goodwin et al., 1998). It would appear that the US-8 clonal lineage is endemic in Florida's potato production.

Isolates of *P. infestans* have been observed to be resistant to the phenylamide metalaxyl since the 1990s. Phenylamide resistance is related to a single dominant gene and levels of sensitivity may involve minor genes (Fabritius et al., 1997; Lee et al., 1999). Isolates of the same genotype in Florida varied in sensitivity to phenylamide, similar to reports elsewhere (Fraser et al., 1999; Goodwin et al., 1998). Metalaxyl-resistant isolates have higher fitness compared with sensitive isolates, and this might contribute to the increased difficulty in managing the novel isolates (Goodwin et al., 1996; Kadish and Cohen, 1988, 1992).

Mitochondrial haplotypes Ia and IIa are associated with the migration of *P. infestans* populations into Europe around 1976, whereas haplotype IIb was limited to North America (Day and Shattock, 1997; Griffith and Shaw, 1998). All our isolates are of the mtDNA Ia haplotype, which agrees with the findings of others that the "old" genotype isolates representing those present before the identification of A2 mating types outside Mexico have been or are being displaced by "new" genotypes (Day and Shattock, 1997; Fry et al., 1993; Goodwin et al., 1995c; Spielman et al., 1991).

Late blight has been documented infecting potatoes in Florida as early as 1937 (Eddins, 1945). Likewise, *P. infestans* has been a persistent problem for tomato and potato growers Florida since 1993. This work was initiated as a result of an outbreak of late blight that occurred in the 2004–2005 (2005) growing season. This outbreak was unusual because the disease was particularly destructive to tomato, extremely difficult to control despite intensive spray programs, and it persisted through the end of the season. South Florida growers typically encounter late blight only during the coldest months of the season (December to March) and can generally achieve good control with fungicides. The growth chamber inoculations demonstrated that the US-20 isolates from 2005 caused statistically significant more disease severity on tomato under the same conditions when compared with US-21 isolates from 2006 (Fig. 2). All of the isolates produced symptoms on tomato, although the potato isolate Pi0639 caused the least disease severity in this test.

The observation of the potato isolate resembling US-8 and its production of oospores in single culture prompts inquiry as to whether these sexual structures are occurring in natural settings and playing a role in the next year's epidemics. Oospores in a single culture of *P. infestans* have been reported in the literature throughout the years (Gallegly and Galindo, 1958; Groves, 2002; Smart et al., 1998; Vartanian and Endo, 1985). Oospore formation is driven by pheromones produced and perceived by the opposite mating type; however, factors such as

Table 2. Summary of *P. infestans* genotypes of based on mating type, metalaxyl sensitivity, *Gpi*, and *Pep*, mtDNA haplotype, and RG57 fingerprint from tomato and potato in South Florida from 2005 to 2007.

Genotype	Isolate ID	Yr	Host	MT	MS	<i>Gpi</i>	<i>Pep</i>	mtDNA	RG57
US-20	Pi052	2005	Tomato	A2	I	100/100	100/100	Ia	1010101001001101010110011
US-20	Pi058	2005	Tomato	A2	S	100/100	100/100	Ia	1010101001001101010110011
US-8	Pi0626	2006	Potato	A2 ^c	R	100/111/122	100/100	Ia	1001100001001101000110111
US-21	Pi0639	2006	Tomato	A2	S	100/122	100/100	Ia	1000100001001100010110011
US-21	Pi07009	2007	Tomato	A2	S	100/122	100/100	Ia	1000100001001100000110111
US-21	Pi07026	2007	Tomato	A2	I	100/122	100/100	Ia	1000100001001100000110111

^cSelf-fertile in a single culture.

MT = mating type; MS = metalaxyl sensitivity; S = Sensitive; I = Intermediate; R = Resistant.

genotype, growth medium, presence of fungicides, and age of the culture influence the health and abundance of oospores observed (Smart et al., 1998). The presence of a self-fertile isolate or of both mating types does not guarantee the production of viable oospores because abortion, sexual incompatibility, and lethal factors have been reported in *P. infestans* (Erwin and Ribeiro, 1996; Flier et al., 2001). Additionally, oospore production and fecundity depend on the particular combination of parental genotypes (Flier et al., 2001). The detection of a single isolate that may be self-fertile may have implications for field survival in the field in south Florida.

Factors important to late blight epidemics such as the origin of inoculum, rate and pattern of spread, and mechanisms of survival between seasons by “overwintering” are still unknown in Florida. Although our observations are based on a survey, intensive sampling throughout the season would likely provide insight on the source(s) of inoculum and survival mechanisms in Florida and perhaps other production regions in the United States. Future studies may allow for the elucidation of migration pathways responsible for the introduction of novel *P. infestans* genotypes into Florida and possibly the United States.

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