Mating type, mefenoxam sensitivity, and pathotype diversity in *Phytophthora infestans* isolates from tomato in Brazil

Bruno Eduardo Cardozo de Miranda[1], Nelson Dias Suassuna[2] and Ailton Reis[3]

[1]Universidade de Brasília, Departamento de Fitopatologia, Campos Universitário, CEP 70910-900 Brasília, DF, Brazil. E-mail: becmiranda@yahoo.com.br [2]Embrapa Algodão, Caixa Postal 174, CEP 58107-720 Campina Grande, PB, Brazil. E-mail: suassuna@cnpa.embrapa.br [3]Embrapa Hortaliças, Caixa Postal 218, CEP 70359-970 Brasília, DF, Brazil. E-mail: ailton@cnph.embrapa.br

Abstract – The objective of this work was to characterize 79 *Phytophthora infestans* isolates collected in tomato (*Solanum lycopersicum*) fields, as to mating type, mefenoxam sensitivity, and pathotype composition. The isolates were sampled in 2006 and 2007 in seven Brazilian states as well as in the Distrito Federal. They were characterised as to mating type (n=79), sensitivity to fungicide mefenoxam (n=79), and virulence to three major resistance genes *Ph-1*, *Ph-2*, and *Ph-3*/*Ph-4* (n=62). All isolates were of the mating type A1. Resistant isolates were detected in all sampled states, and its average frequency was superior to 50%. No difference was detected in pathotype diversity, neither between subpopulations collected in 2006 and 2007 nor between isolates grouped as resistant or intermediately sensitive to mefenoxam. All major resistance genes were overcome at different frequencies: *Ph-1*, 88.7%; *Ph-2*, 64.5%; and *Ph-3*/*Ph-4*, 25.8%. Isolates with virulence genes able to overcome all major resistance genes were detected at low frequencies. Tomato breeding programs in Brazil must avoid the development of cultivars with resistance based exclusively on major genes.

Index terms: *Lycopersicon esculentum*, *Solanum lycopersicum*, disease management, fungicide resistance, late blight, pathogen variability.

Grupo de compatibilidade, sensibilidade ao mefenoxam e diversidade de patótipos de isolados de *Phytophthora infestans* de tomate no Brasil

Resumo – O objetivo deste trabalho foi caracterizar 79 isolados de *Phytophthora infestans*, coletados em campos de tomate (*Solanum lycopersicum*), quanto ao grupo de compatibilidade, à sensibilidade ao mefenoxan, e à diversidade de patótipos. Os isolados foram obtidos em coletas realizadas nos anos de 2006 e 2007, em sete Estados do Brasil e no Distrito Federal. Os isolados foram usados para determinação do grupo de compatibilidade sexual (n=79), resistência ao fungicida mefenoxam (n=79) e espectro de virulência aos genes de efeito principal *Ph-1*, *Ph-2* e *Ph-3*/*Ph-4* (n=62). Todos os isolados foram classificados no grupo de compatibilidade A1. Isolados insensíveis ao fungicida mefenoxam foram detectados em todos os Estados amostrados, e apresentaram frequência média superior a 50%. Não houve diferença de diversidade de patótipos entre as subpopulações coletadas em 2006 e 2007, e nem entre os isolados agrupados como resistentes ou intermediariamente sensíveis ao mefenoxam. Os genes de resistência foram suplantados em diferentes frequências: *Ph-1*, 88,7%; *Ph-2*, 64,5%; e *Ph-3*/*Ph-4*, 25,8%. Isolados complexos capazes de suplantar a resistência dos quatro genes de resistência foram encontrados em baixa frequência. Programas de melhoramento de tomate no Brasil devem evitar o desenvolvimento de cultivares com resistência baseada exclusivamente em genes de efeito principal.

Termos para indexação: *Lycopersicon esculentum*, *Solanum lycopersicum*, manejo de doenças, resistência a fungicida, requeima, variabilidade do patógeno.

Introduction

Under cool and moist weather conditions, late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is a very damaging disease to tomato (*Solanum lycopersicum* L. Syn. *Lycopersicon esculentum* Mill.) in Brazil. The pathogen is heterothallic with two mating types: A1 and A2 (Erwin & Ribeiro, 1996). Isolates of the A2 mating type were not reported outside Central Mexico until 1984 (Hohl & Iselin, 1984). In Brazil, isolates of the mating type A2 were first reported in 1988 (Brommonschenkel, 1988). However, despite the presence of both A1 and A2 mating types, there is no evidence of recombination, and the Brazilian population of *P. infestans* comprises two clonal lineages: US-1 and BR-1.

The US-1, or some variants within this lineage, is associated with late blight in tomato crops. Its isolates have the A1 mating type, and the restriction pattern of mitochondrial DNA (mtDNA) is of the Ib type. The new lineage, BR-1, is associated with potato crops, and its isolates have the A2 mating type, and mtDNA IIa (Reis et al., 2002, 2003). The host specificity of clonal lineages is, at least in part, due to quantitave differences in aggressiveness components (Suassuna et al., 2004). Also, under higher temperatures, US-1 isolates are fitter on tomato than BR-1 isolates (Maziero et al., 2009). As recombination in *P. infestans* is dependent on the presence of both mating types, it is important to know if pathogen population on tomato remains composed only by the A1 mating type. Otherwise, if there is two mating types on the same host, strains can mate and generate a more virulent or a fungicide resistant offspring.

Several evolutionary mechanisms can act upon the pathogen population. Besides mutation, migration and recombination are often mentioned as the main evolutionary mechanisms that shape genetic variability in *P. infestans* populations (Fry, 2008). As the population of *P. infestans* from tomatoes in Brazil remains very uniform (Reis et al., 2002), migration and recombination are not acting on pathogen population, and mutation is the main source of variation. Despite the apparent genetic uniformity of the Brazilian population of *P. infestans*, it is expected that factors affecting disease management, such as fungicide insensitivity and virulence, must be variable, due to mutation.

Large amounts of protective and systemic fungicides are used to control tomato late blight. There have been complaints about a reduction in fungicide efficacy, mainly metalaxyl, in controlling the disease in Brazil (Reis et al., 2005). The widespread use of the phenylamide class of systemic fungicides soon after their release drastically increased the frequency of resistant isolates in Europe (Dowley & O'Sullivan, 1985) resulting in failures in disease control (Bradshaw & Vaughan, 1996). Selection of fungicide-insensitive individuals in populations of plant-pathogenic fungi is a common event and is reported more frequently for systemic fungicides. There are several reports of resistance to metalaxyl in *P. infestans* (Gisi & Cohen, 1996; Reis et al., 2005; Pérez et al., 2009). Thus, monitoring pathogen insensitivity, especially to systemic fungicides, is an important component of disease management (Gisi & Cohen, 1996).

Along with fungicide use, genetic control based on resistant cultivars is also important for disease management. The investigation of pathotype composition of *P. infestans* provides important information for tomato breeding programs aiming to obtain cultivars with disease resistance. Three race-specific late blight resistance genes (*Ph-1*, *Ph-2*, and *Ph-3*) have been introgressed into tomato from different accessions of *Solanum pimpinellifolium* in the past years (Moreau et al., 1998). Later, it was demonstrated that accession L3708 of *S. pimpinellifolium* carries at least one other late blight resistance gene in addition to *Ph-3* (Kim & Mutschler, 2005). This putative gene was named *Ph-4* (Chen et al., 2008). The status of the actual field efficacy of these major resistance genes has been scarcely investigated in Brazil. Knowledge about the dynamics of virulence genes in the pathogen population able to overcome resistance genes is helpful in tomato breeding programs for parental choice (resistance genes), breeding strategies, and efficacy of resistance genes (durability).

The objective of this work was to characterize *P. infestans* isolates collected in tomato fields during 2006 and 2007 as to mating type, mefenoxam (metalaxyl-m) sensitivity, and pathotype composition.

Materials and Methods

Seventy-nine isolates of *P. infestans* were obtained from infected leaf, stem, or fruit tissues of tomato plants during 2006 and 2007 from commercial growers' fields, home gardens or research plots in most of the fresh tomato producing areas in Brazil (Table 1). Isolation was performed directly (Erwin & Ribeiro, 1996) on Rye B culture medium (Caten & Jinks, 1968) amended with rifampicin (50 ppm). For isolation, several individual sporangia were taken from the esporangiophores and distributed on isolated points of the Rye B culture media in Petri dishes. After three days of incubation, the plates were observed under stereoscopic microscope, checking for germinated sporangia. One germinated sporangia was taken from each plate and transferred for another plate containing Rye B media amended with 50 ppm of Rifampicim. Therefore, each isolate was obtained from a single sporangium (monosporic).

Table 1. Isolate code, cultivar, site and year in which *Phytophthora infestans* isolates were collected and mefenoxam sensitivity and pathotype.

Isolate	Cultivar	Collection site	Year	Mefenoxam sensibility	Pathotype
Pi – 128	Carmen	Rancho Queimado, SC	2006	Intermediate	-[(1)]
Pi – 129	Carmen	Rancho Queimado, SC	2006	Sensitive	-
Pi – 130	Carmen	Rancho Queimado, SC	2006	Intermediate	1
Pi – 131	Kadá	Tijucas, SC	2006	Intermediate	1,3
Pi – 132	Santa Clara	Florianópolis, SC	2006	Sensitive	1,2
Pi – 133	Carmen	Alfredo Wagner, SC	2006	Sensitive	1
Pi – 134	Carmen	Alfredo Wagner, SC	2006	Intermediate	1,2
Pi – 137	Indústria	Patos de Minas, MG	2006	Resistant	1,2
Pi – 138	Indústria	Patos de Minas, MG	2006	Resistant	1,2
Pi – 140	Alambra	Paty do Alferes, RJ	2006	Intermediate	1
Pi – 141	Alambra	Paty do Alferes, RJ	2006	Resistant	1,2,3
Pi – 142	Alambra	Paty do Alferes, RJ	2006	Resistant	1,2
Pi – 143	Alambra	São José de Ubá, RJ	2006	Resistant	-
Pi – 144	Alambra	São José de Ubá, RJ	2006	Resistant	1,2
Pi – 145	Processing tomato	Patos de Minas, MG	2006	Resistant	-
Pi – 146	Processing tomato	Patos de Minas, MG	2006	Resistant	-
Pi – 147	Processing tomato	Patos de Minas, MG	2006	Resistant	1,2
Pi – 148	Processing tomato	Patos de Minas, MG	2006	Resistant	1,3
Pi – 149	Processing tomato	Patos de Minas, MG	2006	Resistant	2,3
Pi – 150	Processing tomato	Patos de Minas, MG	2006	Resistant	2,3
Pi – 151	Processing tomato	Gama, DF	2006	Sensitive	1,2
Pi – 152	Processing tomato	Gama, DF	2006	Sensitive	1,2
Pi – 153	Santa Clara	Planaltina, DF	2006	Intermediate	1,2
Pi – 154	Santa Clara	Planaltina, DF	2006	Intermediate	-
Pi – 155	Carmen	Ibicoara, BA	2006	Resistant	1,3
Pi – 156	Carmen	Ibicoara, BA	2006	Resistant	1,2
Pi – 157	Carmen	Ibicoara, BA	2006	Intermediate	1,2
Pi – 158	Carmen	Ibicoara, BA	2006	Sensitive	1,2
Pi – 159	Carmen	Mucugê, BA	2006	Resistant	1,2
Pi – 160	Carmen	Mucugê, BA	2006	Resistant	-
Pi – 161	Itapitã	Mucugê, BA	2006	Resistant	1,3
Pi – 162	Itapitã	Mucugê, BA	2006	Resistant	1
Pi – 163	Itapitã	Mucugê, BA	2006	Intermediate	-
Pi – 164	Ty-Fanny	Irupi, ES	2006	Intermediate	1,3
Pi – 165	Ty-Fanny	Irupi, ES	2006	Intermediate	1,3
Pi – 166	Ty-Fanny	Irupi, ES	2006	Intermediate	1,3
Pi – 167	Miramar	Muniz Freire, ES	2006	Resistant	1,2
Pi – 168	Miramar	Muniz Freire, ES	2006	Resistant	1,2
Pi – 169	Alambra	Brazlândia, DF	2006	Resistant	2
Pi – 170	Alambra	Brazlândia, DF	2006	Resistant	1,2
Pi – 171	Carmen	Caçador, SC	2006	Resistant	2,3
Pi – 172	Carmen	Caçador, SC	2006	Resistant	1,2,3
Pi – 173	Carmen	Caçador, SC	2006	Resistant	-
Pi – 174	Carmen	Caçador, SC	2006	Sensitive	1,2,3
Pi – 175	Giovana	Caçador, SC	2006	Resistant	1
Pi – 176	Giovana	Caçador, SC	2006	Resistant	1
Pi – 177	Netta	Caçador, SC	2006	Resistant	1,2
Pi – 178	Netta	Caçador, SC	2006	Intermediate	1,2
Pi – 179	Netta	Caçador, SC	2006	Intermediate	-

Continue...

Table 1. Continuation...

Isolate	Cultivar	Collection site	Year	Mefenoxam sensibility	Pathotype
Pi – 180	Lineage CNPH	Gama, DF	2007	Sensitive	-
Pi – 181	Lineage CNPH	Gama, DF	2007	Intermediate	1
Pi – 182	L. hirsutum	Gama, DF	2007	Sensitive	1,2,3
Pi – 183	L. hirsutum	Gama, DF	2007	Sensitive	1,2,3
Pi – 184	Alambra	Padre Bernanrdo, GO	2007	Intermediate	1,2
Pi – 185	Alambra	Padre Bernanrdo, GO	2007	Sensitive	-
Pi – 186	Alambra	Padre Bernanrdo, GO	2007	Resistant	-
Pi – 187	Alambra	Padre Bernanrdo, GO	2007	Resistant	1,3
Pi – 188	San Vito	Brazlândia, DF	2007	Resistant	2
Pi – 189	San Vito	Brazlândia, DF	2007	Resistant	2
Pi – 190	Dominador	Goianápolis, GO	2007	Sensitive	1
Pi – 191	Dominador	Goianápolis, GO	2007	Sensitive	1,2
Pi – 192	Dominador	Goianápolis, GO	2007	Intermediate	1
Pi – 193	Processing tomato	Itaberaí, GO	2007	Resistant	-
Pi – 194	Processing tomato	Itaberaí, GO	2007	Resistant	-
Pi – 195	Processing tomato	Itaberaí, GO	2007	Resistant	1,2
Pi – 196	Processing tomato	Itaberaí, GO	2007	Resistant	1,2
Pi – 197	Nemoneta	Caxias do Sul, RS	2007	Resistant	1
Pi – 198	Nemoneta	Caxias do Sul, RS	2007	Intermediate	-
Pi – 199	Nemoneta	Caxias do Sul, RS	2007	Resistant	1,2
Pi – 200	Alambra	Caxias do Sul, RS	2007	Resistant	1
Pi – 201	Alambra	Caxias do Sul, RS	2007	Intermediate	-
Pi – 202	Dominador	Planaltina, DF	2007	Sensitive	1,2
Pi – 203	Dominador	Planaltina, DF	2007	Intermediate	1,2
Pi – 204	Processing tomato	Vianópolis, GO	2007	Intermediate	0
Pi – 205	Processing tomato	Vianópolis, GO	2007	Sensitive	1
Pi – 206	Dominador	Vassouras, RJ	2007	Sensitive	1,2
Pi – 207	Dominador	Vassouras, RJ	2007	Intermediate	1,2
Pi – 208	Dominador	Araguari, MG	2007	Resistant	1
Pi – 209	Dominador	Araguari, MG	2007	Intermediate	1,2

[(1)]Not tested.

Subsequently, the isolates were maintained in Rye B agar slants filled with mineral oil, in test tubes.

All 79 isolates were analyzed for mating type through pairing them with known A1 (PiEH-102) and A2 (PiEH-86) tester isolates, on Petri dishes containing 20 mL of Rye B medium. Mycelial plugs (12 mm in diameter) from an active growing colony of a known A1 or A2 isolate were placed on one side of the dish. Another mycelial plug was removed from an 8- to 14-day-old colony of the unknown isolate and placed on the opposite side (3.5-4 cm apart) of the plug of the known isolate. The process was repeated for another tester. The plates were kept in an incubator at 18°C. After incubation for 2 to 3 weeks in the dark, plates were checked microscopically for the presence of oospores where mycelia of the known and unknown isolates intermingled. Isolates that produced oospores when paired with the A1 tester isolate but did not produce oospores with the A2 isolate were designated A2. Isolates that formed oospores when paired with the A2 tester and did not form oospores when paired with the A1 isolate were designated A1.

The sensitivity of the 79 isolates to mefenoxam (metalaxyl-m) was assessed based on radial growth on mefenoxam-amended agar medium, as described elsewhere (Matuszak et al., 1994; Reis et al., 2003, 2005). A 12 mm-diameter mycelial plug from a 12 day-old colony was placed in the center of a Petri dish containing 10% clarified V8 juice agar (Miller, 1955) amended with metalaxyl suspension, prepared from mefenoxam (Technical grade), to a final concentration of 0, 5, or 100 μg mL^{-1}. For each

isolate and mefenoxam concentration, two plates (replicates) were used. The plates were kept at 18ºC in the dark. After 12 to 15 days, depending on the growth of the colony on controls, without fungicide, the colony diameter of all isolates was measured in two perpendicular directions.

Final radial growth was corrected by subtracting 12 mm of the mycelial plug from the measured colony diameter. Mean colony diameters in culture medium amended with mefenoxam at 5 or 100 μg mL^{-1} were divided by the mean colony diameter on the control plates and multiplied by 100 to determine relative growth. Isolates were rated as: sensitive, when relative growth was less than 40%, both for metalaxyl at 5 or 100 μg mL^{-1}; intermediately sensitive, when relative growth was more than 40% at 5 μg mL^{-1} and less than 40% in 100 μg mL^{-1}; or insensitive, when relative growth was more than 40% in both 5 and 100 μg mL^{-1} (Matuszak et al., 1994). Each isolate was tested twice and the mean of both trials was used in the analyses. In case of divergent results, a third test was performed. Chi-square tests were carried out each year to compare frequencies of resistant isolates and to compare if isolates sensitive, intermediately sensitive and resistant occurs at same frequency.

The virulence of 62 isolates was assessed by the detached leaflet test (Tooley et al., 1986) on tomato differential plants with different major resistance genes to late blight. The sources of major resistance genes used were CNPH-1538 (*L. esculentum* cultivar New Yorker, possessing *Ph-1* gene), CNPH-1523 (*L. esculentum* cultivar Moboline, possessing *Ph-2* gene), and CNPH-1124 [*L. pinpinellifolium* (Just.) Mill., accession L03708, possessing *Ph-3* gene and the putative *Ph-4* gene] (Kim & Mutschler, 2005). The tomato cultivar IPA-5, which was previously found to be susceptible to all isolates, was used as control. Inoculum production was performed on detached lateral leaflets from tomato cv. IPA-5 leaves, from the middle third of 6- to 9-week-old plants grown in the glasshouse. Three leaflets were placed with the abaxial side up inside disinfested square plastic boxes (11-cm area and 3.5-cm depth) lined with wet paper towel to produce a moist chamber.

Mycelium fragments were removed from cultures grown on Rye B media and deposited on each leaflet. Boxes were kept at 19°C with a 16 h day length. After 6–7 days, lesions were cut and washed in distilled water to obtain sporangial suspensions to be used in experiments or reinoculated on new tomato leaflets in order to maintain viable inoculum. For virulence tests, three leaflets of each tomato differential cultivar were kept in a plastic box as described above. Each leaflet was inoculated on the abaxial side with 30-μL drop of a suspension with 2×10^4 sporangia mL^{-1}. Every isolate was assayed in each differential plant in three replicate plastic boxes. The boxes were kept in an incubator at 18±2°C with a 16 h day length. After seven days, the leaflets were scored for presence or absence of *P. infestans* sporulation under a stereoscope. Every isolate was assayed at least twice, and the isolate was considered virulent on respective *R* gene only if there was abundant sporulation in at least two of three leaflets. If there was neither large lesion nor profuse sporulation on control cultivar (IPA-5), the virulence test was not considered successful and it was repeated. The number of virulence genes of each isolate was determined based on the number of compatible interactions between *P. infestans* isolates and differential cultivars (Tooley et al., 1986).

Pathotype diversity was estimated for each subpopulation: sensitivity to mefenoxam (resistant and intermediately sensitive isolates) and year of sampling (2006 and 2007). Pathotype diversity analysis for each population distinguished richness, evenness, and diversity as described previously (Grünwald et al., 2003). Pathotype diversity was calculated as Hill´s and genotypic diversity indices: N_1 and G. For Hill's index, $N_1 = e^{H'}$, where H' refers to Shannon-Wiener's index (Shannon & Weaver, 1949). This index represents the number of equally common genotypes which would produce the same diversity as H'. The genotypic diversity index was calculated according to Stoddart & Taylor (1988): $G = 1/p_i^2$, where p_i is the observed frequency of the ith pathotype. Both N_1 and G measure how effectively proportional abundances are distributed among the different genotypes (Grünwald et al., 2003). G weighs the number of abundant genotypes more strongly, whereas N_1 weighs rarer genotypes more strongly. N_1 generally falls between the number of genotypes observed (g) and G. The evenness index (Ludwig & Reynolds, 1988) was calculated as $E_5 = (G-1)/(N_1-1)$. Diversity indexes were estimated using rarefaction curves based on the sample size of the smallest population. This calculation was made using the package Vegan for R, the open source language and

environment for statistical computing and graphics (R Development Core Team, 2008). To make a possible comparison between subpopulations, a bootstrapping of pathotypes observed within subpopulations was done (SAS Institute, 2010) to calculate indices of diversity and evenness and their respective confidence intervals (Grünwald et al., 2003). Bootstrapping was done using 2,000 resamples at a confidence interval of 95%, using the normal "standard" confidence interval option without bias correction, with the option Size adjusted to the sample size of the smallest subpopulation.

Results and Discussion

All 79 isolates were A1 mating type and, based on earlier findings (Reis et al., 2003), they probably belong to the US-1 clonal lineage. Due to the host specificity of clonal lineages of *P. infestans* in Brazil, only isolates of the mating type A1 have been found associated with tomato plants. Isolates of the mating type A2 (BR-1 clonal lineage), and only sporadically A1, are associated with potato plants (Reis et al., 2002, 2003). As only isolates of the mating type A1 remain associated with tomato plants, there is no risk at all or low risk of sexual reproduction of *P. infestans* on this host.

Nevertheless, the hypothesis of sexual recombination cannot be utterly rejected. Spatial separation of mating types by host (tomato and potato plants), as is the case in Ecuador (Oyarzun et al., 1998), makes rare the chance of both mating types occurring together. It is also possible that if both A1 and A2 are present and mate, the oospores are not viable, as observed in Japan (Mosa et al., 1993). The absence of sexual reproduction minimizes the possibility of new *P. infestans* genotypes arising that are able to overcome host resistance or that are fungicide insensitive (Sujkowski et al., 1994), and excludes oospores as initial inoculum (Turkensteen et al., 2000).

Isolates of *P. infestans* insensitive to mefenoxam were detected in all sampled Brazilian states, with mean frequency of 50.6% (Table 2). Out of 49 isolates collected in 2006, 28 (57.1 %) were resistant and the frequency of resistant isolates was higher (χ^2 = 14,15, p = 0.0008) than the expected with the null hypothesis, in which equal frequencies of sensitive, intermediately sensitive and resistant isolates should be observed. Nevertheless, out of 30 isolates collected in 2007, only 12 (40 %) were resistant. In this year the null hypothesis could not be rejected (χ^2 = 0,60, p = 0.74). There was a reduction in percentage of resistant isolates over time. The frequency of 57,1% in 2006 was different from 40% detected in 2007 (χ^2 = 3.60, p = 0.05).

One reason for this observation was the gradual substitution of mefenoxam by other chemicals to control *P. infestans* in Brazil, such as cymoxanil, dimetomorph, propamocarb and some strobilurins. Sampling error could also have contributed for the differences observed. The frequency of resistant isolates to mefenoxam appears to have altered over the last years, since the mean frequency of resistant isolates collected from tomato in 1998 to 2000 was 36.1 % (Reis et al., 2005). However, those isolates were obtained only from South and Southeast regions of Brazil.

Originally, US-1 isolates were sensitive to mefenoxam (metalaxyl-m), and migration contributed to introducing insensitive isolates into new areas (Goodwin et al., 1996). In a recent survey carried out in Peru, all *P. infestans* isolates tested were resistant to metalaxyl-m (Pérez et al., 2009). The isolates belonged to the clonal lineage EC-1, a recently introduced one which is dominant on potato in Peru and Ecuador. This may not be the case in Brazil, where there is no evidence of introduction of insensitive isolates from a founder population (Reis et al., 2005). It is possible that the unique factor shaping Brazilian *P. infestans* population resistance to mefenoxam is mutation and subsequent selection, resulting in variation in mefenoxam sensitivity.

Table 2. Mefenoxam sensitivity[1] of 79 *Phytophthora infestans* isolates from tomato.

Parameter	Sensitive	Intermediate	Resistant
		Year 2006	
Sample size	7	14	28
Frequency (%)	14.3	28.6	57.1
		Year 2007	
Sample size	9	9	12
Frequency (%)	30.0	30.0	40.0
		Total	
Sample size	16	23	40
Frequency (%)	20.3	29.1	50.6

[1]Sensitive, growth less than 40% of control on both 5 and 100 μg mL^{-1} mefenoxam concentrations; intermediate, growth 40% or more of control on 5 μg mL^{-1}, but less than 40% on 100 μg mL^{-1}; resistant, growth 40% or more of control on both 5 and 100 μg mL^{-1} mefenoxam concentrations.

The appearance of mefenoxam insensitive isolates may be due to errors in fungicide management, such as use of sub-dosages, failure in plant coverage, and use of super-dosages, which may exert a greater selection pressure in selecting intermediate or insensitive isolates. Many isolates were found to be intermediately sensitive to mefenoxam (growth inhibition at 100 mg L^{-1} of mefenoxam), implying inefficiency of this fungicide when used at sub-dosages for control of tomato late blight.

All isolates tested were able to cause disease on tomato IPA-5 cultivar, which has not any known major resistance genes. Most isolates were able to sporulate on 'New Yorker', which possess the resistance gene *Ph-1* (88.71%), and on Moboline cultivar, which possess the resistance gene *Ph-2* (64.52%). However, few isolates (25.81%) were able to cause disease in the CNPH-1124 accession (Table 1). The investigation of race composition in the population of *P. infestans* provides important information for tomato breeding programs targeting late blight resistance. The virulence alleles for overcoming resistance conferred by *Ph-1* and *Ph-2* occurs at high frequency in the pathogen population. Similar results were reported in the USA and Ecuador (Goodwin et al., 1995; Oyarzun et al., 1998). These results explain the low exploitation of these sources of resistance in tomato breeding programs. Despite the lower frequencies of the alleles for virulence to the genes *Ph-3*/*Ph-4*, it is possible that resistance conferred by these genes would overcome rapidly if they were used in commercial cultivars, since the virulent strains are distributed in almost all of the Brazilian states sampled.

Considering both years of sampling (2006 and 2007), seven out of a 16-total possible pathotypes were detected, ranging from simple to complex types, occurring at different frequencies. Out of 62 isolates, only one was unable to overcome at least one major resistance gene. On the other hand, five isolates were able to overcome all three resistance genes tested. The occurrence of *P. infestans* complex pathotypes in 2006 and 2007, distributed in three different Brazilian regions (South, Southeast, and Central Western), renders the use of major resistance genes in cultivars unfeasible as the main disease management tactic. Early examples of unsuccessful use of genetic resistance were the potato cultivars Pentland Dell and Maris Peer, with resistance to late blight based on simple combinations of *R* genes, which were overcome as the frequency of matching virulence genes in the *P. infestans* population increased (Malcolmson, 1969). This increase is a direct result of the selection pressure imposed upon the pathogen population by the cultivation of these cultivars (Shattock et al., 1977) and illustrates the potential problems of relying on host resistance for disease control without proper consideration of how the pathogen population may respond to its deployment.

Richness, evenness, and diversity indices were similar for both subpopulations (Table 3). There was no difference in pathotype diversity in subpopulations collected in 2006 and 2007. Temporal subpopulations

Table 3. Pathotype diversity in different populations of *Phytophthora infestans* collected from tomato.

Statistic	Resistance to mefenoxam[1]		Year		Total
	Resistant	Intermediate	2006	2007	
Sample size	32	30	39	23	62
Indices of richness					
g_{obs}	6	5	6	6	7
g_{max}	16	16	16	16	
Indices of diversity					
N_1	4.724	3.664	4.200	4.1437	4.526
	(3.555–5.893)[2]	(2.778–4.549)	(2.934–5.467)	(2.865–5.422)	
G	3.793	3.000	3.271	3.3270	3.438
	(2.441–5.145)	(2.044–3.956)	(1.958–4.584)	(2.134–4.520)	
Index of evenness					
E_5	0.750	0.751	0.710	0.740	0.692
	(0.583–0.917)	(0.589–0.912)	(0.523–0.897)	(0.597–0.883)	

[1]Intermediate, growth 40% or more of control on 5 µg mL^{-1}, but less than 40% on 100 µg mL^{-1}; resistant, growth 40% or more of control on both 5 and 100 µg mL^{-1} mefenoxam concentrations. [2]Confidence intervals calculated by bootstrapping approach for the common sample size of the smallest population. g_{obs}, number of unique pathotypes observed; g_{max}, maximum number of expected pathotypes; $N_1 = e^{H'}$, where H' refers to Shannon-Wiener's index; $G = 1/p_i^2$ where p_i is the observed frequency of the ith pathotype; $E_5 = (G-1)/(N_1-1)$.

(2006 and 2007) had similar richness (estimate of the number of genotypes contained in a population) and evenness (how genotypes are distributed in a sample), indicating that the avirulence alleles probably are not under selection. There was no difference in pathotype diversity in subpopulations with different sensitivity to mefenoxam, and there was no relation with pathotype complexity and mefenoxam resistance. Consistent with this, no linkage was found between the number and composition of virulence genes to potato clones carrying *R* genes, and the sensitivity profile on *P. infestans* in Europe (Gisi & Cohen, 1996). On the other hand, the *P. infestans* race structure in the United States seems to be composed of isolates with few avirulence genes (0–4) and more complex isolates, with 6–9 avirulence genes (Goodwin et al., 1995). The least complex races were mostly sensitive to phenylamide fungicides.

Few investigations were performed with *P. infestans* in order to determine pathotypes based on *Ph* major genes (Oyarzun et al., 1998; Chen et al., 2008). Besides major genes *Ph-1*, *Ph-2*, and *Ph-3*/*Ph-4* used in this study, there is some evidence of other putative major genes, namely *Ph*5 from *S. habrochaites* (accession LA1033). However, in Taiwan, *P. infestans* strains able to overcome all five major genes were observed (Chen et al., 2008). The use of resistant cultivars with one of the four major genes tested, probably, will not imply successful disease management because of the relatively high frequency of pathotypes able to overcome these genes already present in the population examined. Nevertheless, it is possible that pyramiding all four genes in a tomato genotype having some quantitative trait loci to resistance to late blight would result in tomato progenies with high levels of resistance. Hence, tomato breeding programs in Brazil must look for quantitative resistance to tomato late blight. In the short term, the use of fungicides is necessary to manage tomato late blight, mainly when environmental conditions favour disease outbreaks, but genetic quantitative resistance can be useful in reducing fungicide foliar applications (Mizubuti, 2001).

Conclusions

1. In all sampled Brazilian states, only *Phytophthora infestans* isolates of the A1 mating type are present on tomatoes.

2. *Phytophthora infestans* isolates resistant to mefenoxan are found in all sampled Brazilian states.

3. Brazilian *Phytophthora infestans* isolates from tomato are able to overcome all major tomato resistance genes at different frequencies.

References

BRADSHAW, N.J.; VAUGHAN, T.B. The effect of phenylamide fungicides on the control of potato late-blight (*Phytophthora infestans*) in England and Wales from 1978 to 1992. **Plant Pathology**, v.45, p.249-269, 1996.

BROMMONSCHENKEL, S.H. **Patogenicidade, compatibilidade, citogenética e padrões izoenzimáticos de isolados de *Phytophthora infestans* (Mont.) De Bary do Brasil**. 1988. 82P. Dissertação (Mestrado) - Universidade Federal de Viçosa, Viçosa.

CATEN, C.E.; JINKS, J.L. Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. **Canadian Journal of Botany**, v.46, p.329-348, 1968.

CHEN, C.H.; SHEU, Z.M.; WANG, T.C. Host specificity and tomato-related race composition of *Phytophthora infestans* isolates in Taiwan during 2004 and 2005. **Plant Disease**, v.92, p.751-755, 2008.

DOWLEY, L.J.; O'SULLIVAN, E. Monitoring metalaxyl resistance in populations of *Phytophthora infestans*. **Potato Research**, v.28, p.531-534, 1985.

ERWIN, D.C.; RIBEIRO, O.K. ***Phytophthora* diseases worldwide**. St. Paul: American Phytopathological Society, 1996. 562p.

FRY, W.E. *Phytophthora infestans*: the plant (and R gene) destroyer. **Molecular Plant Pathology**, v.9, p.385-402, 2008.

GISI, U.; COHEN, Y. Resistance to phenylamide fungicides: a case study with *Phytophthora infestans* involving mating type and race structure. **Annual Review of Phytopathology**, v.34, p.549-572, 1996.

GOODWIN, S.B.; SUJKOWSKI, L.S.; DYER, A.T.; FRY, B.A.; FRY, W.E. Direct detection of gene flow and probable sexual reproduction of *Phytophthora infestans* in Northern North America. **Phytopathology**, v.85, p.473-479, 1995.

GOODWIN, S.B.; SUJKOWSKI, L.S.; FRY, W.E. Widespread distribution and probable origin of resistance to metalaxyl in clonal genotypes of *Phytophthora infestans* in the United States and Western Canada. **Phytopathology**, v.86, p.793-800, 1996.

GRÜNWALD, N.J.; GOODWIN, S.B.; MILGROOM, M.G.; FRY, W.E. Analysis of genotypic diversity data for populations of microorganisms. **Phytopathology**, v.93, p.738-746, 2003.

HOHL, H.R.; ISELIN, K. Strains of *Phytophthora infestans* from Switzerland with A2 mating type behavior. **Transactions of the British Mycological Society**, v.83, p.529-530, 1984.

KIM, M.J.; MUTSCHLER, M.A. Transfer to processing tomato and characterization of late blight resistance derived from *Solanum pimpinellifolium* L. L3708. **Journal of the American Society for Horticultural Science**, v.130, p.877-884, 2005.

LUDWIG, J.A.; REYNOLDS, J.F. **Statistical ecology**: a primer on methods and computing. New York: John Wiley & Sons, 1988. 338p.

MALCOLMSON, J.F. Races of *Phytophthora infestans* occurring in Great Britain. **Transactions of the British Mycological Society**, v.53, p.417-423, 1969.

MATUSZAK, J.M.; FERNANDEZ-ELQUEZABAL, J.; VILLARREAL-GONZALEZ, M.; FRY, W.E. Sensitivity of *Phytophthora infestans* populations to metalaxyl in Mexico: distribution and dynamics. **Plant Disease**, v.78, p.911-916, 1994.

MAZIERO, J.M.N.; MAFFIA, L.A.; MIZUBUTI, E.S.G. Effects of temperature on events in the infection cycle of two clonal lineages of *Phytophthora infestans* causing late blight on tomato and potato in Brazil. **Plant Disease**, v.93, p.459-466, 2009.

MILLER, P.M. V-8 juice agar as a general-purpose medium for fungi and bacteria. **Phytopathology**, v.45, p.461-462, 1955.

MIZUBUTI, E.S.G. Requeima ou mela da batata e do tomate. In: LUZ, E.D.M.N.; SANTOS, A.F. dos; MATSUOKA, K.; BEZERRA, J.L. (Ed.). **Doenças causadas por *Phytophthora* no Brasil**. Campinas: Rural, 2001. p.100-174.

MOREAU, P.; THOQUET, P.; OLIVIER, J.; LATERROT, H.; GRIMSLEY, N. Genetic mapping of *Ph-2*, a single locus controlling partial resistance to *Phytophthora infestans* in tomato. **Molecular Plant-Microbe Interactions**, v.11, p.259-269, 1998.

MOSA, A.A.; KOBAYASHI, K.; OGOSHI, A.; KATO, M.; SATO, N. Isoenzyme polymorphism and segregation in isolates of *Phytophthora infestans* from Japan. **Plant Pathology**, v.42, p.26-34, 1993.

OYARZUN, P.J.; POZO, A.; ORDOÑEZ, M.E.; DOUCETT, K.; FORBES, G.A. Host specificity o*f Phytophthora infesta*ns on tomato and potato in Ecuador. **Phytopathology**, v.88, p.265-271, 1998.

PÉREZ, W.; LARA, J.; FORBES, G.A. Resistance to metalaxyl-M and cymoxanil in a dominant clonal lineage of *Phytophthora infestans* in Huánuco, Peru, an area of continuous potato production. **European Journal of Plant Pathology**, v.125, p.87-95, 2009.

R DEVELOPMENT CORE TEAM. **R**: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing, 2008.

REIS, A.; RIBEIRO, F.H.S.; MAFFIA, L.A.; MIZUBUTI, E.S.G. Sensitivity of Brazilian isolates of *Phytophthora infestans* to commonly used fungicides in tomato and potato crops. **Plant Disease**, v.89, p.1279-1284, 2005.

REIS, A.; SMART, C.D.; FRY, W.E.; MAFFIA, L.A.; MIZUBUTI; E.S.G. Characterization of isolates of *Phytophthora infestans* from Southern and Southeastern Brazil from 1998 to 2000. **Plant Disease**, v.87, p.896-900, 2003.

REIS, A.; SUASSUNA, N.D.; ALFENAS, A.C.; MIZUBUTI, E.S.G. Monitoramento da população de *Phytophthora infestans* na região da Zona da Mata de Minas Gerais de 1998 a 2000. **Fitopatologia Brasileira**, v.27, p.614-620, 2002.

SAS INSTITUTE. **SAS customer suport**. Available at: <http://support.sas.com/>. Accessed on: 02 Aug. 2010.

SHANNON, C.E.; WEAVER, W. **The mathematical theory of communication**. Urbana: University of Illinois, 1949. 117p.

SHATTOCK, R.C.; JANSSEN, B.D.; WHITBREAD, R.; SHAW, D.S. An interpretation of the frequencies of host specific phenotypes of *Phytophthora infestans* in North Wales. **Annals of Applied Biology**, v.86, p.249-260, 1977.

STODDART, J.A.; TAYLOR, J.F. Genotypic diversity: estimation and prediction in samples. **Genetics**, v.118, p.705-711, 1988.

SUASSUNA, N.D.; MAFFIA, L.A.; MIZUBUTI, E.S.G. Aggressiveness and host specificity of Brazilian isolates of *Phytophthora infestans*. **Plant Pathology**, v.53, p.405-413, 2004.

SUJKOWSKI, L.S.; GOODWIN, S.B.; DYER, A.T.; FRY, W.E. Increased genotypic diversity via migration and possible occurrence of sexual reproduction of *Phytophthora infestans* in Poland. **Phytopathology**, v.84, p.201-207, 1994.

TOOLEY, P.W.; SWEIGARD, J.A.; FRY, W.E. Fitness and virulence of *Phytophthora infestans* isolates from sexual and asexual populations. **Phytopathology**, v.76, p.1209-1212, 1986.

TURKENSTEEN, L.J.; FLIER, W.G.; WANNINGEN, R.; MULDER, A. Production, survival and infectivity of oospores of *Phytophthora infestans*. **Plant Pathology**, v.49, p.688-696, 2000.

Received on September 15, 2009 and accepted on June 28, 2010