SAULO ALVES SANTOS DE OLIVEIRA

GENETIC STRUCTURE, AGGRESSIVENESS AND FUNGICIDE SENSITIVITY OF *Phytophthora infestans* POPULATIONS FROM THE SOUTH AND SOUTHEAST REGIONS OF BRAZIL

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae*.

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À Deus, senhor da minha vida.

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RESUMO

OLIVEIRA, Saulo Alves Santos de, D.Sc., Universidade Federal de Viçosa, novembro de 2010. **Estrutura genética, agressividade e sensibilidade a fungicidas de populações de *Phytophthora infestans* das regiões sul e sudeste do Brasil.** Orientador: Eduardo Seiti Gomide Mizubuti. Co-orientadores: Luiz Antonio Maffia e Sérgio Hermínio Brommonschenkel.

O conhecimento sobre a estrutura genética, agressividade e sensibilidade a fungicidas da população atual de *Phytophthora infestans* é importante para o delineamento de estratégias de manejo da requeima da batateira e do tomateiro. Coletaram-se 166 isolados, de seis estados de duas regiões brasileiras, em três períodos 1998/2000, 2003/2005 e 2008/2010. Os isolados foram caracterizados com marcadores microssatélites, haplótipo mitocondrial, além de *mating type* (A1 ou A2), agressividade às diferentes hospedeiras e sensibilidade aos fungicidas comumente empregados no controle da requeima. Detectaram-se mudanças na população brasileira de *P. infestans* ao longo dos anos, evidenciadas principalmente pela presença dos dois *mating types* em locais onde antes só havia isolados A2, além do surgimento de um novo haplótipo mitocondrial associado a alguns isolados A2 (mtDNA, padrão Ib). Sinal de recombinação sexual foi detectado somente para os isolados coletados entre 03/05. Para testar a hipótese de mudanças na agressividade, oito isolados, quatro de batateira (GB) e quatro de tomateiro (GT), foram comparados por meio de cinco variáveis epidemiológicas: período latente (PL), período de incubação (PI), área lesionada (AL), frequência de infecção (FI) e capacidade de esporulação (CE). Não houve diferença na agressividade entre os isolados dos dois grupos quando inoculados em folíolos.

Entretanto, os isolados diferiram quanto a AL e CE, quando inoculados em haste. Os valores mais altos de CE (1599,14 esporângios/cm^2) foram registrados para isolados GB inoculados em hastes de tomateiro. Avaliaram-se 68 isolados quanto à sensibilidade ao fungicida propamocarbe e 62 para os fungicidas mancozeb, clorotalonil, fenamidone, dimetomorfe e mandipropamide. Nenhum isolado foi insensível aos fungicidas mancozeb e clorotalonil, e os valores de EC_{50} para mancozeb e clorotalonil foram positivamente correlacionados. Os maiores valores de EC_{50} dentre os fungicidas sistêmicos foram detectados para propamocarbe sendo que os isolados menos sensíveis foram os da região Sul. Não há evidências de resistência aos demais fungicidas, nem resistência cruzada entre os diferentes grupos de fungicidas testados.

ABSTRACT

OLIVEIRA, Saulo Alves Santos de, D.Sc., Universidade Federal de Viçosa, November, 2010. **Genetic structure, aggressiveness and fungicide sensitivity of *Phytophthora infestans* populations from the south and southeast regions of Brazil.** Adviser: Eduardo Seiti Gomide Mizubuti. Co-advisers: Luiz Antonio Maffia and Sérgio Hermínio Brommonschenkel.

The knowledge about the genetic structure, aggressiveness and sensitivity to fungicides of the current population of *Phytophthora infestans* can be useful to delineate strategies for the management of potato and tomato late blight. A total of 166 isolates were collected from six states of Brazil, in three periods: 1998/2000, 2003/2005 and 2008/2010. The isolates were assayed with microsatellite markers, mitochondrial haplotype (mtDNA) and phenotypic traits such as mating type, differences in aggressiveness and sensitivity to commonly used fungicides. Temporal analysis revealed changes in the population across the years. The distribution of mating types changed and isolates of A1 and A2 were found in the same potato field in two locations. Additionally, a new mtDNA haplotype was found associated with A2 isolates. Recombination signal was detected only in 03/05. To test the hypothesis that genetic changes altered host specificity of the isolates, eight isolates, four from potato (PG) and four from tomato (TG) were characterized based on five epidemiological variables: incubation period (IP), latent period (LP), lesion area (LA), infection frequency (IF) and sporulation capacity (SC). In leaflets, no differences in aggressiveness were detected between PG and TG. However, differences in LA and SC were detected between PG and TG inoculated in potato and tomato stems. Highest values of LA (2.87 cm^2) were recorded for PG in both hosts and for SC values

when PG were inoculated in tomato stem (1599.14 sporangia/cm^2). Sensitivity to six fungicides was evaluated: mancozeb, chlorothalonil, propamocarb-HCl, fenamidone, dimetomorph and mandipropamid. Sixty eight isolates were accessed for propamocarb-HCl sensitivity and 62 isolates for mancozeb, chlorothalonil, fenamidone, dimethomorph and mandipropamid. No insensitivity was detected to mancozeb and chlorothalonil and it was observed a positive correlation between EC_{50} values of both fungicides. Among the systemic fungicides the highest EC_{50} values were observed for propamocarb-HCl, and the frequency of less sensitive isolates was highest in the South region. There was no evidence of resistance to the other systemic fungicides, and no evidence of cross-sensitivity among the different groups of fungicides.

INTRODUÇÃO GERAL

A batateira (*Solanum tuberosum* L.) e o tomateiro (*Solanum lycopersicum* L.) são as espécies olerícolas mais importantes para o Brasil. A maior parte da produção de batata e tomate está concentrada nos estados da região Sudeste e Sul, além da Bahia e Goiás nas regiões Nordeste e Centro-Oeste, respectivamente (IBGE 2008). Atualmente, a batata é a quarta cultura mais importante do mundo sendo que mais de um terço da produção global de batata é oriunda de países em desenvolvimento (CIP 2007). A produção mundial anual é de aproximadamente 300 milhões de toneladas enquanto a produção brasileira é de 3,1 milhões de toneladas (FAO 2006). Países como China e Rússia são os maiores produtores desta olerícola. A cultura do tomate, antes considerada atividade de pequenas propriedades, vem experimentando rápida expansão, especialmente na China e nos Estados Unidos (Kemmitt 2002). A produção mundial em 2006 chegou a mais de 123 milhões de toneladas e o Brasil é o 9º maior produtor, com aproximadamente 3,3 milhões de toneladas (FAO 2006).

Um dos fatores mais limitantes para as culturas da batata e do tomate é a suscetibilidade a doenças. Algumas doenças afetam a produção e a qualidade do produto. A requeima, uma das doenças mais destrutivas dessas culturas (Fry 2008), é causada pelo oomiceto *Phytophthora infestans* (Mont.) de Bary (Erwin & Ribeiro 1996). O patógeno coloniza grandes extensões de tecido de folhas e haste (batateira e tomateiro), frutos de tomateiro e tubérculos de batata. O controle da requeima é baseado na aplicação de fungicidas, uma vez que em grande parte dos casos as cultivares com boa aceitação de mercado possuem baixa resistência à doença (Fry 2008). Em casos extremos,

produtores de batata podem efetuar até 30 aplicações de agroquímicos para controlar doenças (Nazareno & Jaccound Filho 2003).

A espécie *P. infestans* pertence ao reino Stramenopila, filo Oomycota, classe Peronosporomycetidae, ordem Pythiales família Pythiaceae (Petersen & Rosendahl 2000; Kroon at al. 2004). A reprodução, quando em sua forma assexuada, ocorre com a produção de esporângios (zoosporângios) e zoósporos (Ristaino & Gumpertz 2000; Vargas et al. 2009). A reprodução sexuada resulta na produção de oósporos, entretanto, como a espécie é heterotálica, com dois grupos de compatibilidade (A1 e A2), a reprodução sexuada ocorre somente quando indivíduos de ambos os grupos acasalam (Erwin & Ribeiro 1996; Flier et al. 2003). Os oósporos são capazes de se manterem viáveis no solo na ausência de hospedeiro (Widmark et al. 2007).

Até o final da década de 1970 ou início de 1980 apenas no planalto central do México eram encontrados os dois grupos de compatibilidade de *P. infestans* (Gómez-Alpizar et al. 2007). No restante do mundo, a população do patógeno era dominada por uma linhagem clonal, US-1, cujos isolados apresentavam grupo de compatibilidade A1. Segundo Reis et al. (2003), no Brasil, os dois grupos de compatibilidade estão presentes, associados às duas linhagens clonais: US-1, que afeta tomateiro e é constituída por isolados do grupo de compatibilidade A1; e BR-1, encontrada associada à requeima em batateira e constituída por isolados do grupo A2. No entanto, observou-se no Rio Grande do Sul a ocorrência de isolados dos grupos A1 e A2 em um mesmo campo de batata (Santana 2006), o que pode favorecer a reprodução sexuada do patógeno. A recombinação pode trazer consequências epidemiológicas em função do aumento da variabilidade genética das populações de *P. infestans*, além da formação de oósporos que podem servir de inóculo para epidemias de requeima (Anderson 2007; Cooke et al. 2003).

Com o aumento da área plantada destas duas olerícolas, bem como o plantio ao longo de quase todo o ano, especialmente na região Sudeste, a necessidade do uso de fungicidas é essencial como estratégia de controle da requeima. A intensificação da utilização de fungicidas, principalmente aqueles com modo de ação sítio-específico, gera pressão de seleção e aumento de frequência de indivíduos resistentes (Brent & Hollomon 2007). Diversos agricultores relataram a redução da eficiência de controle por fungicidas. Para o controle da requeima da batateira e do tomateiro, é comum a aplicação de

fungicidas de amplo espectro (protetores) como o oxicloreto de cobre, mancozeb e clorotalonil (Nazareno & Jaccoud 2003), além de fungicidas sistêmicos com ação específica contra *P. infestans*, como por exemplo, o mefenoxam, cymoxanil, dimetomorfe e propamocarbe (Reis et al. 2005; Pérez et al. 2009). No caso dos QoI's, além dos produtos liberados para o controle de *P. infestans* (famoxadone e fenamidone), existe também a pressão de seleção exercida na aplicação de estrobilurinas (azoxystrobin) para o controle de *Alternaria tomatophila* em tomateiro e *A. grandis* em batateira (Rodrigues et al. 2010), que também pode afetar *P. infestans*.

A primeira caracterização da sensibilidade de isolados brasileiros de *P. infestans* a fungicidas foi realizada entre os anos de 1998 e 2000 (Reis et al. 2005). Foi detectada alta frequência de indivíduos resistentes à mefenoxam, entretanto não foram detectados isolados resistentes aos fungicidas cymoxanil, clorotalonil e mancozeb (Reis et al. 2005).

A compreensão da dinâmica da variabilidade genética dos fitopatógenos é necessária para o entendimento de como as populações evoluem e as implicações desse processo na durabilidade de cultivares resistentes e no controle de doenças. De forma prática, esta compreensão pode ser traduzida na duração da efetividade da adoção de uma planta hospedeira resistente ou de um fungicida específico para o manejo da doença (Milgroom & Peever 2003). Segundo Lees et al. (2006), diversos trabalhos foram realizados em todo o mundo para caracterizar o grupo de compatibilidade e a resistência a fungicidas das populações de *P. infestans*, no entanto, os estudos relacionados à diversidade genética têm sido limitados pela sensibilidade dos marcadores genéticos utilizados. Este aspecto justifica a adoção de novos marcadores nas análises de biologia de populações de fitopatógenos, como o caso dos microssatélites, também conhecidos como sequências simples repetidas (*SSR*) (Cooke & Lees 2004).

Estudos de caracterização da população brasileira de *P. infestans* foram realizados com marcadores fenotípicos, como grupo de compatibilidade sexual, resistência a mefenoxam, além de marcadores moleculares como RFLP (com sonda RG_{57}) e haplótipos mitocondriais (mtDNA), juntamente com marcadores bioqímicos como as isoenzimas *Gpi* e *Pep* (Reis et al. 2003). Neste mesmo trabalho, os isolados foram caracterizados quanto ao padrão de virulência (patótipos) em série de plantas diferenciadoras. Recentemente, possíveis

mudanças na população foram relatadas em plantios de batata do Rio Grande do Sul (Santana, 2006), gerando a necessidade de uma nova caracterização das populações brasileiras com marcadores moleculares mais informativos, como os microssatélites. Faz-se necessário, também, nova avaliação de possíveis modificações na agressividade do patógeno, em relação às plantas hospedeiras, e na sensibilidade aos principais fungicidas empregados nas duas culturas. O entendimento da dinâmica populacional de *P. infestans* ao longo dos anos, permitirá melhor embasamento para o desenvolvimento e a adoção de práticas de manejo da doença, no auxílio ao desenvolvimento de cultivares resistentes à requeima e melhorar a eficiência do controle químico.

Este trabalho teve por objetivo a caracterização da população atual de *Phytophthora infestans* nas regiões Sul e Sudeste do Brasil. Foram estabelecidos os seguintes sub-objetivos:

i) Efetuar a caracterização fenotípica e molecular dos isolados de *Phytophthora infestans* coletados nos períodos de 1998-2000, 2003-2005 e 2008-2010 nas regiões Sul e Sudeste do Brasil

ii) Determinar a estrutura genética das populações analisadas por meio de técnicas de genética molecular de populações.

iii) Quantificar a agressividade de isolados de *Phytophthora infestans* oriundos de batateira e tomateiro quando inoculados no hospedeiro de origem ou de forma cruzada.

iv) Avaliar a sensibilidade de isolados brasileiros de *P. infestans* aos fungicidas protetores clorotalonil e mancozeb, e aos fungicidas sistêmicos dimetomorfe, propamocarbe, mandipropamide e fenamidone.

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1. Characterization and temporal analysis with SSR markers of *Phytophthora infestans* isolates from Brazil reveal population admixture, sexual recombination and bottleneck effects.

1.1. Abstract

A total of 166 isolates of *P. infestans* from six states of Brazil were collected in three distinct periods 1998/2000, 2003/2005 and 2008/2010. They were accessed with microsatellites markers, mitochondrial haplotype (mtDNA) and phenotypic traits such as mating type. Temporal analysis revealed changes in the population across the years. This changes were confirmed by the mating type distribution that changed from prevalence of the A2 mating type in potatoes fields, to the occurrence of both mating types in new locations. All tomato isolates were A1 mating type and mtDNA Ib, the same was noticed in a previous study in Brazil. However, now two mitochondrial haplotypes were detected in the A2 isolates associated with potato, IIa and Ib, the latter is a new variant of *P. infestans*. High levels of diversity within and among years were observed, based on ten microsatellite markers, in all periods, supporting the presence of an admixture population. Signal of sexual recombination was detected only in the 03/05 sample. Significant genetic differentiation was found across the years and geographic regions, but there was no evidence of temporal and/or spatial isolation. The recent Brazilian population of *P. infestans* seems to be shaped by constant gene flow and introduction of new variants by airborne sporangia, plus rare events of sexual recombination. Three important characteristics are reported for the first time: the occurrence of A1 mating type isolates in commercial potato fields in the Southeast region of Brazil; the presence of a new variant of the A2 mating type (mtDNA Ib); and the detection of a signal of sexual recombination in the Brazilian population of *P. infestans*.

1.2. Introduction

In the past 25 years major genetic changes in the populations of *Phytophthora infestans* (Mont.) de Bary, the oomycete that causes potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) late blight, have been recorded worldwide (Fry 2008). These changes impacted on yield of those crops and on management of this severe foliar disease that affects two of the most widely cultivated vegetable crops. In developing countries, crop losses due to late blight in potatoes alone are estimated to have reached more than 3 billion dollars (Bradshaw et al. 2006). Late blight epidemics are notoriously known for high progress rates. The pathogen can complete its life cycle in a short time span, under favorable conditions (moderate temperatures and high humidity) it can be as short as three days (Fry 2008). Two types of spores can be formed by *P. infestans* since this organism can reproduce both sexually and asexually. Sexual recombination can occur only when individuals of the opposite mating types, A1 and A2, are present in the same geographic area. Until 1984, except for the Toluca Valley in Mexico, where a recombining population was known to be present, populations worldwide were clonal and comprised of individuals of the A1 mating type. However, starting 1985 isolates of the A2 mating type were reported in areas outside the Toluca Valley (Fry et al. 1993).

Migrations resulted in the introduction of new genotypes and recombination thereafter are two evolutionary mechanisms likely to have contributed to change the genetic makeup of the populations. Isolates of both mating types are present in several countries and increased genotypic diversity is well documented in many areas (Day et al. 2004; Möller et al. 2009; Gisi et al. 2010). Previous analyses of the Brazilian population of *P. infestans* support the occurrence of migration events with the introduction of the A2 mating type, but there was no evidence of recombination and only two clonal lineages, BR-1 (A2 mating type), associated with late blight on potato, and US-1 (A1), predominantly found on tomato fields, were detected countrywide (Reis et al. 2003). A decade has passed since this study was conducted and potato and tomato growers have noticed more severe late blight epidemics with the increase in the occurrence of stem lesions. These facts raised the possibility that the genetic structure of the Brazilian populations of *P. infestans* could have changed.

Sexual recombination, mutation, and migration are important evolutionary mechanisms that are linked with the origin of individuals with higher aggressiveness in

a population (Miller & Johnson 2000). The presence of isolates of the A2 mating type and the establishment of a sexually recombining populations in some European countries resulted in new genotypes of the pathogen capable to overcome disease resistance genes in potato and tomato (Anderson 2007).

The increase in diversity and changes in the aggressiveness of the population are often linked with the introduction of new variants of the pathogen (Goodwin 1997; Linde et al. 2010). In Brazil the coexistence of individuals of both mating types could lead to changes in the genetic structure of *P. infestans* population as reported elsewhere (Cooke et al. 2006; Carlisle et al. 2002; Lehtinen et al. 2004; Lehtinen et al. 2009). Potatoes and tomatoes are contiguously and, depending on the region, continually cultivated in many areas, thus hosts are available all year round. It is also known that sporangia of *P. infestans* can be found as airborne inoculum at any season (Lima et al., 2009). From a practical perspective, the combination of host-inoculum availability makes late blight epidemics a constant threat, as is normally perceived by potato and tomato growers. From a population biology perspective, this could contribute to enhance the chances of recombination and the occurrence of many life cycles of the pathogen and the production of large amounts of propagules (sporangia) favoring the formation and distribution of new variants of the pathogen (McDonald & Linde 2002). The late blight context in Brazil seems to allow for major changes in the genetic structure of the population of *P. infestans* in a relatively short period of time. The present study was conducted aimed at (i) studying the current genetic structure of the Brazilian population of *P. infestans* and (ii) analyzing potential changes in the genetic composition of the population that occurred in the past 10 years. The main approach was to analyze three sets of isolates collected approximately five years apart using microsatellite markers and aiming to identify the evolutionary mechanisms that could have been acting on population.

1.3. Material and Methods

Sampling and isolates

Infected leaves, fruits or stems of potato and tomato with typical late blight symptoms were collected in the major producing regions (Fig. 1). Isolates were collected during three periods: 1998/2000 (by Reis 2001); 2003/2005 (by Santana 2006) and 2008/2010. For each sample, geographic coordinates were obtained with a portable GPS device. Three to ten leaflets or stems were collected from each sampled field and taken to the laboratory where they were transferred to moist chamber at 18°C to induce sporulation. Isolation procedure started by harvesting the sporangia from the lesions and transferring them to the V8-medium (1.0 g $CaCo_3$, 200 mL of V8-juice, 0.05 g of β-sitosterol), amended with ampicillin at 100 mg L^{-1}, rifampicin at 20 mg L^{-1}, pentachloronitrobenzene at 670 mg L^{-1} (PCNB) and benomyl 100 mg L^{-1}. Pure culture of single lesion isolates were grown on V8, Rye-B (Trout et al. 1997) or Pea-glucose-agar (150g of frozen peas, 5g of glucose, 15g of agar and 0.05g of β-sitosterol) culture media, at 18°C in the dark.

Mating type characterization

A total of 166 isolates (Table S1) were characterized based on the presence of coenocytic hyphae, shape of the sporangia, ramification of the sporangiophores with swellings at sites of sporangium formation, and presence of papillae. Pathogen structures were examined under a compound microscope (400X).

All isolates were paired with known A1 (US-1) and A2 (BR-1) tester isolates on pea-agar. A strip of the culture medium and mycelia (~ 4mm width x 6mm length) from 7 to 15-day-old colonies of each tester (A1 and A2) were placed equidistantly in a Petri dish containing the culture medium. A similar strip (same dimensions and age) of the unknown isolate was placed in between the strips of the testers. Two dishes per unknown isolate were prepared for mating type determination and kept for 3 to 4 weeks at 18°C in the dark. After incubation colonies were observed under the microscope for the presence of oospores. Isolates that produced oospores when paired with the A1 tester, but not with the A2 tester, were designated A2. Isolates that produced oospores when paired with the A2, but not with the A1, were designated A1.

DNA extraction

Isolates were grown for 8 to 15 days at 18°C in clear pea broth (Goodwin et al 1995). After colony development, the mycelium was dehydrated, frozen with liquid nitrogen, and ground in a mortar with a pestle. DNA extraction was accomplished using two protocols: (i) Quick & Easy protocol and a (ii) modified protocol from the Reader and Brodar method (Eucablight, <www.eucablight.org>).

Sequencing of the ITS region

Ten isolates were randomly selected for PCR amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA, with the ITS4 and ITS5 primers (White et al. 1990). The reaction was done in a 25 μL volume and consisted of 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl and 0.1 % of Triton X-100), 2 mM $MgCl_2$, 200 mM dNTPs, 0.2 mM each primer, 1.25 units *Taq* DNA polymerase, and 50 ηg DNA. The PCR products were purified with the High Pure PCR Cleanup Micro Kit (Roche Diagnostics) and sequenced in a MegaBace™ DNA analysis system (GE Healthcare). The forward and reverse sequences of each isolate were aligned and manually edited with the Staden Package software (Staden 1996) and a consensus sequence was generated. The consensus sequences of the ITS region of all isolates were aligned with Clustal-W (Thompson, Higgins, & Gibson 1994) and manually re-edited for a more precise alignment (MEGA 4.1, (Tamura et al. 2007)).

The *blastn* algorithm (BLAST - NCBI <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to assess the identity of the nucleotide sequence of all isolates. Additionally, four random sequences of *P. infestans* isolates were selected from the GenBank and aligned with the sequences generated in this study and a phylogenetic analysis was carried out with other *Phytophthora* species using the Neighbor-Joining method with the Kimura-2-parameter substitution model (Kimura 1980).

Mitochondrial haplotypes

For all isolates the mtDNA regions P2 (F2 = 5' – TTCCCTTTGTCCTCTACCG AT - 3' and R2 = 5' - TTACGGCGGTTTAGCACATACA -3') and P4 (F4 = 5'-TGGT

CATCCAGAGGTTTATGTT - 3' and R4 = 5'-CCGATACCGATACCAGCACCAA - 3') (Griffith & Shaw 1998) were amplified in a 100 µL reaction consisting of 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl and 0.1 % of Triton X-100), 2.75 mM $MgCl_2$, 200 mM dNTPs, 0.325 mM of each primer, 5 units *Taq* DNA polymerase, and 100 ηg DNA (Gavino & Fry 2002). The amplification was under with the following PCR conditions: one cycle of 90° C for 90s, followed by 40 cycles of 90° C for 30s, 55° C for 40s and 72° C for 2 minutes, plus a final extension period of 72° C for 10 min.

The expected sizes of the PCR products were 1.2 kb for P2 and 0.96 Kb for P4. The amplification products of the P2 and P4 mtDNA loci were digested with *Msp* I and *Eco* RI, respectively. Five µL of the digested product were stained with GelRed™ (Biotium, Inc.), separated by electrophoresis on 2.5% agarose gels, visualized using standard techniques, and haplotypes were determined as previously described (Griffth and Shaw 1998).

SSR amplification

A total of eleven SSR loci were chosen: Pi02, Pi04, Pi16, Pi33, $Pi56_{(Long)}$, $Pi63_{(Long)}$, Pi89, D13, Pi70 (Lees et al. 2006; Cooke 2009), Pi4B, and G11 (Knapova & Gisi 2002). To allow the analysis of several loci at the same time, the forward primer of each locus was labeled with one of the three fluorescent dyes: FAM (Pi02, $Pi63_{(Long)}$, D13 and Pi70), NED (Pi33, Pi89, and G11), and HEX (Pi16, Pi04, $Pi56_{(Long)}$ and Pi04B). The Multiplex PCR reaction was performed in a 25 µL reaction with the Type-it® Microsatellite Kit (QIAGEN, USA), according to the manufacturer's recommendation using 20 ηg of DNA template. The PCR conditions for all multiplex panels were: one cycle at 95° C for 5 min, followed by 28 cycles at 95° C for 30 s, 58° C for 90 s and 72° C for 30 s, plus a final extension period of 60° C for 30 min. Five µL of the PCR product were stained with GelRed™, separated by electrophoresis on 2.5 % agarose gels and visualized using standard techniques.

The concentration of the multiplex PCR product was measured in a spectrophotometer and adjusted to approximately 25 ηg per locus (i.e., a multiplex with 4 primer pairs was adjusted to 100 ηg). Ten µL-samples (8 µL of deionized formamide Hi-Di™ (Applied Biosystems, USA), 1 µL of 600 bp standard labeled with ROX dye, and 1 µL of the multiplexed PCR product) were loaded in an ABI Prism® 3100 capillarity system (Applied Biosystems, USA). SSR patterns were automatically sized

with the GeneMapper^{TM} 3.5 software, and the calibration of the allele sizes was done with reference isolates kindly provided by Dr. David E. L. Cooke of the Scottish Crop Research Institute (SCRI), UK (Lees et al. 2006). A subset of isolates was replicated three times with independent mycelial growth (solid and liquid medium) and DNA extraction. Independent DNA extraction, PCR and scores of the fragments sizes, were used to confirm the reproducibility of the data.

Statistical and temporal population analyses

Based on ten independent microsatellite loci different multilocus genotypes (MLGs) were identified. The polymorphism of each locus and the heterozygosity values (Nei 1978) were accessed using the Microsatellite toolkit (Park 2001). Hierarchical analysis of the *P. infestans* population were conducted by period (98/00, 03/05 and 08/10), by region, and based on host of origin when more than five isolates were available. To access the genotypic diversity measured by the richness, diversity and evenness, and to reduce the error due to different sample sizes, the genotypic richness $E(g_n)$ was estimated using rarefaction curves (Grünwald et al. 2003), and the number of genotypes in a population of $n = 16$, the smallest population observed, was estimated. To measure genotypic diversity the N_1 (Hill 1973) and the Stoddart and Taylor's G (Stoddart & Taylor 1988) indices were calculated. Confidence intervals for N_1 and G values were calculated from 1000 bootstrap re-sampling of the original MLG frequency with "vegan" and "vegetarian" packages in R (R Development Core Team 2008). Similarly, the genotypic evenness was assessed using the E_5 index = $[(G\text{-}1) / (N_1\text{-}1)]$ (Grünwald et al. 2003).

The number and percent of polymorphic loci, number of alleles, allele frequency for each locus, and the homogeneity test of Ewens-Watterson were calculated using POPGENE 1.32 (Yeh & Yang 1999). Gene diversity was characterized by allele richness calculated with the Microsatellite analyzer tool (Dieringer & Schlötterer 2003) and scaled correct (rarefaction curve) based on sample size of the smallest population. The genetic differentiation among populations and sampling years was compared using the Wright's F-statistics and a more powerful index to detect the differences between populations (D_{est}) (Ryman & Leimar 2009; Gerlach et al. 2010). This index is a bias-corrected estimator of G_{ST} [and its relative (F_{ST})], and takes into account the effective number of alleles. Bootstrap of 1000 re-sampling were used to assess significance with

the package ‘DEMEtics’ in R (R Development Core Team 2008). The D_{est} values were used to calculate the number of migrants per generation, by the formula Nm = (0.25x(1-D_{est}))/D_{est}. A distance matrix based on Nei’s genetic distance (Nei 1972) was used to generate an UPGMA (unweighted pair group method with arithmetic mean) dendrogram with POPGENE 1.32.

The hypothesis of random mating was tested using the index of association (I_A) and the R_D which corrects for the number of loci with the software Multilocus 1.3 (Agapow & Burt 2001). The estimates of linkage-disequilibrium (I_A and R_D) were compared with the expected distribution of the loci when in random association (null hypothesis). Deviation from the null hypothesis (no linkage-disequilibrium) was accessed by 1000 permutation of the alleles between individuals for each locus.

To investigate the population structure, a bayesian clustering approach to genetic mixture analyses was performed using the software Structure 2.3.3 (Pritchard et al. 2000). The entire dataset was analyzed using the admixture model. The number of Monte-Carlo Markov Chains (MCMC) was 100,000 replicates with burn-in of 20,000. All other parameters were set at the default values. The number of underlying groups (*k*) varied from 1 to 10 and the analysis was conducted five independent times. The posterior probability of the likelihood [Ln P(D)] was used to choose cluster number (*k*) for the entire dataset. Analysis of molecular variance (AMOVA) was conducted in Arlequin ver. 3.5.1.2 (Excoffier & Lischer 2010) to test for significant variation among years, regions, and MLG. The default set of 10,000 permutations was used. Finally, the intensity of genetic drift was estimated with the software Bottleneck v.1.2.02 (Cornuet & Luikart 1996).

1.4. Results

Isolate morphology and mating type characterization

A total of 166 isolates from six states and 38 sample sites were obtained (Fig. 1). The morphological characteristics of all isolates were typical of *P. infestans*: coenocytic hyphae; ramification of the sporangiophores with swellings at successive sites of sporangium formation; and lemon-shaped sporangia with papillae (Erwin & Ribeiro 1996). Potato isolates comprised 57.8% of the total number of isolates and tomato isolates, 42.2%. Both groups of sexual compatibility were detected in all sampled years.

Regardless of collection date, all isolates from tomato were of A1 mating type (Table 1). Among the potato isolates (n = 99), 15 were of A1 and 84 of the A2 mating type. In the 98/00 period (n = 29), the number of potato isolates of the A1 and A2 mating types was 1 and 28, respectively. In the 08/10 period, the A1:A2 ratio in the Alto Paranaíba region of Minas Gerais State was 1:3, and for isolates collected in Vargem Grande do Sul, São Paulo State, the ratio was close to 1:1. For the 03/05 period Santana (2006) noticed a frequency close to 1:1 in the Rio Grande do Sul state. The isolates collected in 03/05 were part of the analysis of the present work.

ITS region and mitochondrial haplotypes

All sequences had approximately 795 bp and low polymorphisms were detected. The Blastn analysis revealed more than 98% identity with ITS sequences of *P. infestans* isolates deposited in the GenBank, with e-value <0.0001. All ten isolates of *P. infestans* grouped together with the GenBank sequences, confirming the *P. infestans* identity.

Among the four possible mitochondrial haplotypes: Ia, Ib, IIa and IIb (Griffth and Shaw 1998), only two, Ib and IIa, were detected among the isolates analyzed (Table 1). All A1 isolates had the Ib haplotype pattern. Two haplotypes were detected among the A2 mating type isolates: Ib and IIa. In the 08/10 period all isolates from Minas Gerais State (Southeast region) had the Ib haplotype, whereas 92.6% of the isolates from the South region had mtDNA haplotype IIa and 7.4% the Ib haplotype

Gene and genotypic diversity

All isolates (n = 166) were analyzed with 10 microsatellite markers and polymorphism was high (Figure S1). The amplification of the locus Pi4B was not reproducible and it was not included in the analysis, thus 10 SSR markers were used in this study.

All polymorphic loci were selectively neutral according to the Ewens-Watterson test for neutrality. There was no difference regarding the number of alleles among populations which varied from 3.4 to 5.5 across ten loci. The gene diversity values (unbiased Hz) and the observed heterozygosity (Obs Hz) was very similar across all populations, and the unbiased Hz values ranged from 0.46 to 0.47. The unbiased Hz and

Obs Hz did not differ among all populations. In sampled locations with sample size higher than 10 isolates, the highest values of allele number were found in Minas Gerais State (Table 2).

Across all years 157 multilocus genotypes (MLG's) were identified and there was no significant difference among years or host (potato and tomato) for genotype richness ($E_{(gn)}$). There was no significant difference of diversity and evenness among all periods (Table 3). There was no redundant MLG among the three periods. In the same year, MLGs were shared only among isolates sampled from the same State. Complementary analysis of pairwise comparison, conducted with the Microsatellite Toolkit, revealed 158 cases of more than 80% of shared alleles among different years (data not shown).

Linkage disequilibrium and population structure

According to the I_A and R_d tests, there was no evidence of random mating in the 98/00 and 08/10 periods. The 03/05 period was in linkage equilibrium and there was strong evidence of random mating in this period (Fig. 2).

Estimates of population differentiation based on fixation index (F_{ST}) and the differentiation index (D_{est}) support strong population differentiation among and within sampled years (Table 4). The D_{est} among the 1998-2003 was 0.24 (Nm=0.78) and low differentiation was found when 03/05 and 08/10 periods were compared (D_{est} = 0.15 and Nm = 1.42) (Table 5). Based on the AMOVA the high population differentiation was due to the great level of heterozigosity across population (52.7%, Table 6). Variation among periods was approximately 4% of the total variation. When the individual level was not included, the effect of periods was not significant (1.9%, P = 0.08), and differentiation was caused by the variation within collected regions (87.7%, $P < 0.001$). There was no correlation between genetic and geographic distance based on the Mantel's test (R_{xy} = 0.092; P = 0.19).

The cluster analysis based on Nei's genetic distance revealed that isolates were grouped according to the State of origin and region (South or Southeast). The isolates collected from the Southeast region were closely related (Fig. 3), but isolates of Santa Catarina (SC) State, from the South region, clustered in the Southeast group. The bayesian assignment analysis using Structure indicated that 6 groups (k = 6) are likely to occur in the Brazilian population (Fig. 4). According to the cluster distribution, all

sampled years shared part of each cluster, but the frequency changed from periods. An admixture population was detected across different periods; for instance the 03/05 periods represents part of the 98/00 period.

Evidence for population shifts and bottleneck effect

The allele frequency in each of three sampled periods differed (Table S2), and the homogeneity test was significant to all loci, thus an unequal distribution of the alleles was detected across the 10-year period. However, overall, none of these loci can be fit on Hardy-Weinberg equilibrium, indicating the presence of clonality (Table S2). Bottleneck results showed that all tested microsatellite loci evolved according to the infinite allele model (IAM). All sub-populations of each state within year were combined and three groups were formed. Signals of recent genetic drift could be detected based on the excess of heterozygosity (*H*) for all loci ($P < 0.01$) (Table 7). The same level of significance was observed with the standardized differences test (SDT), assuming that all loci are in mutation-drift equilibrium, and also with the Wilcox test (one tail for *H* excess) (data not shown).

The 98/00 period had a L-shaped distribution of alleles, suggesting that this subpopulation is in a mutation – drift equilibrium, without evidences of a recent population bottleneck (Fig. 5). For the 03/05 and 08/10 subpopulations, the distribution do not fit the mutation-drift equilibrium (shifted mode), and there was evidence of recent population bottlenecks. To exclude the effect of the smaller sample size of tomato isolates in 03/05 and 08/10, an additional analysis was conducted only with potato isolates. The majority of loci (9 of 10 loci) were in *H* excess, and only one was considered on *H* deficiency, but this difference was low and was assumed not to affect the results obtained with the overall data. Similarly to the whole population, the potato collection had an *H* excess with significant difference ($P < 0.01$) of the mutation-drift equilibrium, based on the Sign, SDT and Wilcox tests (data not shown).

1.5. Discussion

Based on the set of markers used in this study, there is strong evidence that genetic changes occurred in the population of *P. infestans* associated with potatoes, but not in those associated with tomato. The results of the analyses of three markers corroborate this claim: the mating type ratio; the detection of mtDNA haplotype Ib associated with individuals of the A2 mating type, and the different alleles found at the SSR loci. The presence of individuals of the A1 mating type in recent samples from potato fields in the Southeast region is noteworthy and can have important consequences to late blight management. On the other hand, the population of *P. infestans* associated with tomato late blight is comprised only by isolates of the A1 mating type, as previously reported (Reis et al. 2003). In this previous study, potato isolates were mostly A2 (82%) and only in an experimental field located in Viçosa, Minas Gerais State, were A1 isolates found in potato plots (Reis et al. 2003). The experimental field was located in an important tomato producing area where only A1 isolates were found to cause late blight in this crop. Thus, migration from the surrounding tomato fields was postulated to have contributed to the presence of A1 in potato fields.

In 2006, A1 isolates were reported to occur in potato fields in Rio Grande do Sul State, in the Southern region (Santana, 2006). The presence of both mating types in the same field in approximately 1:1 ratio was noticed one year later in the same state, reinforcing the hypothesis of sexual recombination (Gomes et al. 2007). Isolates of the A1 and of the A2 mating types are now reported be found for the first time in potato fields in the municipalities of São Gotardo, located in the Alto Paranaíba region of Minas Gerais State and Vargem Grande do Sul, São Paulo State. The consequence of this finding is the high chance of sexual reproduction to take place and the establishment of a recombining population as reported elsewhere (Widmark et al. 2007; Lehtinen et al. 2007). Nevertheless, to date, no genetic signal of a recombining population was detected in these fields. The lack of random mating signal, associated with the presence of both mating types in the same field, are indicatives of the recent introductions of the A1 mating type in those fields. Some possible explanations for the lack of recombination signal in this period are described. First, the sexual recombination could be not occurring on these populations or, if it takes place, the oospores formed are not viable. Another explanation could be due to the fact that the number of random mating versus the number of asexual cycles after the A1 introduction was not sufficient

to break the linkage-disequilibrium, or that the frequent population expansion during the epidemics can be more favorable to more closely related individuals, resulting in dominance of some alleles and maintaining the linkage-disequilibrium (Smith et al. 2000; Groenewald et al. 2008). More intense sampling in these areas with an increased sample size is required to address this issue.

If random mating is occurring in the field then increased variability in the population of *P. infestans* and the potential role of oospores in the epidemics of late blight are anticipated to implicate in the adjustment of the disease management. Pathogens with mixed reproduction models, like *P. infestans*, pose the highest risk of evolution, because during the sexual cycle new allelic combinations are formed and the more fitted genotypes generated can predominate in the population due the asexual reproduction (McDonald & Linde, 2002). Late blight is a polycyclic disease and in Brazil the main inoculum for epidemics are the asexual structures (sporangia). In countries where sexual reproduction commonly takes place, the oospores contribute significantly to initiate late blight epidemics (Goodwin 1997; Fry et al. 2009; Deahl et al. 2009; Widmark et al. 2007). Earlier development of late blight epidemics in potato fields are frequently reported by growers. However, at this point no study was conducted to assess if the initial lesions were originated from oospore germination or by the "constant supply" of airborne sporangia. Intensive sampling in potato fields where A1 and A2 isolates were found and the genetic characterization of the isolates with SSR markers should help elucidate this issue.

The occurrence of mtDNA Ib, an haplotype not previously reported to be associated with isolates of the A2 mating type, support the changes in the population that affects potato. Previous studies involving the Brazilian population of *P. infestans* always pointed to the presence of two clonal lineages, BR-1 associated with potato and US-1 on tomato (Reis et al. 2002; 2003). The BR-1 isolates were A2 and of the IIa mtDNA haplotype, whereas US-1 isolates were A1 and of the haplotype Ib. The detection of the Ib mtDNA haplotype indicates that the current A2 isolates are genetically more diverse than those previously described (Reis et al. 2002, 2003). The association of the Ib mtDNA haplotype with A2 isolates could have resulted from sexual recombination among isolates within an area or the immigration of new genotypes of *P. infestans*. The latter is the most likely hypothesis giving the easiness of introduction via infected seed tubers (migration). Potato growers heavily depend on seed tubers supplies from wholesalers, which in turn commonly import their stock materials from abroad (mainly from The Netherlands, France, Germany, Canada, and United States). Using infected

seed tubers could have contributed to introduce new genotypes of *P. infestans*. Tomato growers in Brazil do not use their own seeds and also purchase from several companies. However, transmission of late blight by tomato seeds is not as likely as by seed tubers (Baker & Smith 1966).

The higher resolution of the SSR markers used in the current study revealed a more variable population of *P. infestans* in Brazil then previously reported using RFLP. The multilocus genotype system based on these SSR markers has been useful in assessing the genetic structure of *P. infestans* (Lees et al. 2006). The SSR markers are well-known for being highly polymorphic and codominant (Queller et al. 1993; Lees et al. 2006). These features make them a powerful tool to detect population changes caused by sexual recombination, population expansion or genetic bottlenecks (Linde et al. 2010). In the present analysis, higher gene and genotypic diversity among all sampled years were detected when compared to previous estimates (Reis et al. 2003). The presence of a more variable population since 1998 can be partly due to the use of a more polymorphic marker and/or the presence of variants of the two dominant clonal lineages. Despite the prevalence of US-1 on tomatoes, variants of this lineage were reported to be present in the Brazilian population of *P. infestans* (Reis 2001). This could explain the diversity noticed in this work, when the 98/00 period was analyzed. However, no signal of random mating was found based on the linkage-disequilibrium analysis. The high diversity in this subpopulation could be explained by the presence of an admixture population or presence of variants of a same clonal lineage. A more recent study with isolates collected from 03/05 in the South region already pointed to a change in the population. New variants were sampled that did not fit in any of the known clonal lineages of *P. infestans* (Santana 2006). The current data allowed more reliable inferences regarding the occurrence of sexual recombination. Variation in the allele frequency and the introduction of alleles that were not present in the 98/00 period also support the change in the population.

The analysis based on $k = 6$ clusters, revealed a shift in composition of the subpopulations, and dominance of some cluster across years. Clusters are the constituent of the structure and are admixed in individuals, representing different populations within a region or a sample year, or indicating a common source population (Linde et al. 2010). Frequent changes apparently occur in the Brazilian population of *P. infestans* resulting in a different composition along the years. No evidence of recombination was found when the overall data of the 08/10 period were analyzed. This fact strengthens the hypotheses that frequent introductions lead to shifts in gene

frequency and the maintenance of high variability. The linkage-disequilibrium in the 08/10 period agrees with the fact that recombination in Brazil can occur at low frequency and the epidemic is predominantly caused by asexual inoculum. However, the most likely explanation for not detecting recombination signal in the current population (08/10) is sampling artifact. Since that the sensitivity on random mating detection is connected with the frequency of recombination events. According to Weiss and Clark (2002), the sample size must be proportional to the epidemiological and evolutionary questions. Hence the linkage-disequilibrium is influenced by the substructures existent in the sampled population. The sample size must be great enough to overcome this background effect and excludes the presence of artifacts.

The distribution of both mating types close to a 1:1 ratio, high levels of gene and genotypic diversity, and linkage equilibrium in some populations provide evidence for the occurrence of randomly mating populations (Milgroom 1996; Milgroom & Peever 2003; McDonald & Linde 2002; Montarry et al. 2010). However, clonality is common in heterothallic oomycetes, even when and both mating types are present (Vercauteren et al. 2010). A study with isolates from a single field in southwest Sweden showed relatively low diversity even when sexual recombination occurs, but no direct comparison could be made because only 6 SSR marker were used and this considerably reduces probability of detecting polymorphisms (Widmark et al. 2007).

Migration rates increased over the years but some genotypes persist from year to year. The estimates of *Nm* based on D_{est} among different years increased from 98/00 to 03/05 (*Nm* = 0.776), and from 03/05 to 08/10 (*Nm* = 1.42). Despite the shift in gene frequency, the actual population is influenced to some extent by the previous population. The increase in the number of migrants across the years can be explained by differences in fitness of some isolates, and also with directional selection imposed by resistance genes (Lebreton et al. 1999) and fungicides sprays (Young et al. 2009). The increase in the *Nm* across years can also be linked with the increase in the cultivated area, plus the maintenance of susceptible host for all seasons in the year, plus the uniformity of the potato varieties, across different regions, plus the excessive usage of some fungicides, mainly mefenoxam (MFX) and possibly propamocarb (PMC). Previous work with Brazilian population has noticed the presence of high frequency of insensitive and intermediately sensitive isolates to MFX (Reis et al. 2005; Miranda et al. 2010). Despite the high usage of this compound in Brazilian fields, the contribution of the more fitted isolates increased with time. To PMC, no previous work was carried out in Brazil, but based on changes in North American and European populations, it is

feasibly that changes in PMC sensitivity are occurring, noted as presence and/or an increase in the frequency of isolates capable to tolerating more than 10 ppm of this compound (Grünwald et al. 2006; Lehtinen et al. 2008). These two works has noticed the ability of some isolates of grow in culture medium or leaflet treated with more than 100 ppm of PMC. The consequences of this finding, if happening in Brazil are unknown, but insensitive isolates can become more prevalent across the years.

The sub-populations of the Southeast are genetically more similar across the years while more variation from year to year was observed for the South sub-population. Genetic distance was reduced in more than ten years, and agree with the increase in the gene flow in Brazilian population. Despite gene flow, differences are maintained in the population probably due to genetic drift and local selection events, as differences on climate conditions and fungicides applications. Evidence of sexual recombination was only recorded in the South Region. In the Southeast region diversity is maintained as an admixture of lineages and this fact are favorable to population differentiation by dominance of more fitted genotypes to local conditions.

Despite the abundance of airborne sporangia of *P. infestans* and its importance as inoculum, sporangia are very sensitive to the environmental conditions (temperature and humidity), and can be rapidly inactivated by the solar irradiance (Mizubuti et al. 2000; Fry et al. 2008; Lima et al. 2009). Dispersion by itself does not guarantee the survival and the contribution of the inoculum to the next generation (Pariaud et al. 2009). The occurrence of unfavorable environmental conditions can cause population bottlenecks even in presence of hosts.

Many Brazilian farmers produces their potato seeds, and the usage of infected tuber or the transport of infested soil (with oospores) adsorbed in the tuber, can be a dangerous source of pathogen dispersion. Maybe the most reliable and economically feasible could be the crop rotation with non host plants with good market acceptance (i.e. maize, soybean,). Tomato growers in Brazil do not use their own seeds, but buy them from seed companies. Potato growers import seed from other countries from Europe (mainly from The Netherlands and France) (Reis 2005). This could be an important source of introduction of new variants of the pathogens, or the dispersion among Brazilian fields. Different to what happen in Europe, the oospores in tropical and sub-tropical conditions are postulated to be not so important as inoculum for late blight epidemics. Susceptible hosts are available almost all year round, and airborne sporangia are present in all seasons in high density (Lima et al. 2009). However, under tropical and subtropical conditions, oospores may be more important due to the probability to

increase variability and the contribution to the emergence of new variants of the pathogens that could lead to more aggressive isolates and/or isolates resistant to commonly used fungicides, mainly the site-specific products. The results of characterization of the population are of great interest to plant pathologist. Because knowledge about the changes that are occurring in the population are important to understanding the evolutionary potential of the population, genetic changes in the pathogen population can lead to the reassessment of management strategies for controlling the late blight.

More intense intra-region sampling is needed in addition to frequent analysis of the population. From an epidemiological perspective, the identification of prevalent MLG or recombinant genotypes with altered aggressiveness is of great interest. The estimates of aggressiveness of the new populations can shed light into the epidemiology of late blight and explain the alleged increase in the severity of late blight epidemics. In a previous work host specificity of the two dominant clonal lineages (BR-1 on potato and US-1 on tomato) was demonstrated (Suassuna et al. 2004), but changes in the population and the presence of new variants could lead to a different scenario. Likewise, the assessment of the sensitivity of current population to the commonly used fungicides is required. Further investigations of these topics are urgently warranted in Brazil.

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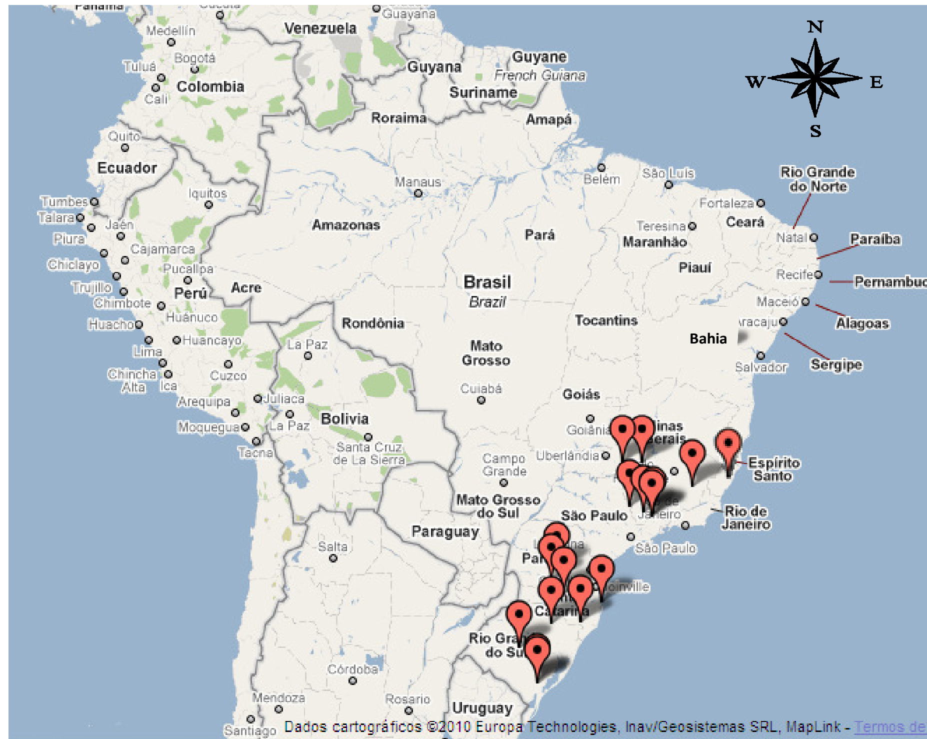

Figure 1. Map showing the sampled areas located in the the different regions of Brazil. Brazilian states names are in bold.

Table 1. Summary description of the collection of isolates of *Phytophthora infestans* used in this study according to the geographic region, year of collection, host crop, mating type (A1 or A2), and mtDNA haplotype.

Region			Potato					Tomato		
Southeast	Period	N[a]	n_p[b]	A1	mtDNA[c]	A2	mtDNA	n_t[d]	A1	mtDNA
MG	98/00	66	**17**	1	Ib	16	IIa	**49**	49	Ib
	08/10	53	**50**	9	Ib	41	Ib	**3**	3	Ib
SP	98/00	3	**0**	0	0	0	0	**3**	3	0
	08/10	5	**5**	2	Ib	3	IIa	0	0	0
ES	98/00	3	**1**	0	0	1	IIa	**2**	2	Ib
	08/10	2	**0**	0	0	0	0	**2**	2	Ib
South										
RS	98/00	1	**1**	0	0	1	IIa	**0**	0	0
	03/05	11	**9**	2	Ib	7	IIa	**2**	2	Ib
	08/10	2	**1**	0	0	1	Ib	**1**	1	Ib
SC	98/00	12	**10**	0	0	10	IIa	**2**	2	Ib
	03/05	4	**2**	1	Ib	1	IIa	**2**	2	Ib
	08/10	1	**0**	0	0	0	0	**1**	1	Ib
PR	98/00	1	**1**	0	0	1	IIa	**0**	0	0
	03/05	1	**1**	0	0	1	IIa	**0**	0	0
	08/10	1	**1**	0	0	1	Ib	**0**	0	0

MG = Minas Gerais, SP = São Paulo, ES = Espírito Santo, PR = Paraná, SC = Santa Catarina, and RS = Rio Grande do Sul State.
[a]N = sample size; [b]n_p = sample size of isolates from potato; [c]mtDNA = mitochondrial haplotype; [d]n_t = sample size of isolates from tomato.

Table 2. Population statistics with the heterozygosity, plus standard error, by period and Brazilian state collected.

Population	Sample size	Loci typed	Unbiased Hz	Unbiased Hz SD	Obs Hz	Obs Hz SD	No Alleles	No Alleles SD
98/00								
MG	66	10	0.47	0.07	0.28	0.02	5.20	3.61
SP	3	10	0.48	0.09	0.53	0.09	2.00	0.82
ES	3	10	0.50	0.10	0.10	0.05	2.20	0.79
PR	1	10	0.00	0.00	0.00	0.00	1.00	0.00
RS	1	10	0.10	0.10	0.10	0.09	1.10	0.32
SC	12	10	0.39	0.09	0.16	0.03	2.50	1.27
Total	86	10	0.47	0.07	0.26	0.01	5.50	3.75
03/05								
PR	1	10	0.40	0.16	0.40	0.15	1.40	0.52
RS	11	10	0.48	0.05	0.38	0.04	2.90	0.74
SC	4	10	0.40	0.09	0.30	0.07	2.10	0.88
Total	16	10	0.47	0.05	0.33	0.04	3.40	1.07
08/10								
MG	53	10	0.44	0.07	0.25	0.02	4.70	2.98
SP	5	10	0.54	0.07	0.44	0.07	2.60	0.97
ES	2	10	0.43	0.10	0.40	0.11	1.90	0.74
PR	1	10	0.20	0.13	0.20	0.13	1.20	0.42
RS	2	10	0.30	0.10	0.30	0.10	1.50	0.53
SC	1	10	0.20	0.13	0.20	0.13	1.20	0.42
Total	64	10	0.46	0.07	0.27	0.02	4.90	3.03

Unbiased Hz = Unbiased Nei gene diversity; Unbiased Hz SD = standard error of Unbiased Nei gene diversity; Obs Hz = Observed heterozygosity; Obs Hz SD = Standard error of Observed heterozygosity; No Alleles = Mean number of alleles per locus; No Alleles SD = Standard error of the Mean number of alleles per locus

MG = Minas Gerais, SP = São Paulo, ES = Espírito Santo, PR = Paraná, SC = Santa Catarina and RS = Rio Grande do Sul.

Table 3. Indices of richness, evenness and diversity for the three sampling period of isolates of *Phytophthora infestans* obtained period from 98/00, 03/05, and 08/10, from tomato and potato fields.

Statistic	98/00			03/05			08/10		
Sample size	Potato	Tomato	Total	Potato	Tomato	Total	Potato	Tomato	Total
n^a	29	57	86	10	6	16	57	7	64
Indices of Richness									
$g_{obs}{}^b$	28	56	81	10	6	16	53	7	60
$E_{(gn)}$ for smallest population (n=6 or 16)[c]	5.96	5.99	15.84	6	6	16	5.96	6	15.76
Indices of diversity									
$N_1{}^d$	27.65	55.63	79.34	10	6	16.00	51.71	7.00	58.69
	(24.0 – 31.3)[g]	(50.7 – 60.6)	(73.3 -85.4)	(8.0 – 12.0)	(4.4 – 75.6)	(13.48 – 18.52)	(46.8 – 56.7)	(5.3 – 8.7)	(53.5 – 63.9)
G^e	28.02	55.07	77.04	10	6	16.00	49.98	7.00	56.89
	(24.3 – 31.8)	(49.8 – 60.4)	(70.5 – 83.6)	(7.9 -12.0)	(4.4 – 7.6)	(13.39 – 18.61)	(44.9 – 55.1)	(5.3 – 8.7)	(51.3 – 62.5)
Indices of evenness									
$E_5{}^f$	1.01	0.99	0.97	1.00	1.00	1.00	0.97	1.00	0.97

[a]Number of sampled individuals; [b]Number of observed MLG's; [c]Number of expected genotype based on rarefaction curve for a sample with 16 individuals (among years) and 6 individual (among hosts); [d]Hill's index; [e]Stoddart & Taylor index; [f]E_5 index of evenness, calculated by $(G - 1)/(N_1 - 1)$; [g]Numbers in parentheses indicate confidence intervals calculated by the bootstrapping approach with 1000 replications.

Table 4. Population differentiation analysis by the Fisher (Fst) and the Dest tests of differentiation. Data were arranged by period and estimates were obtained for each SSR locus.

	98/00					03/05					08/10					Overall data (1998 – 2003 – 2008)				
Locus	N	Fst	Nm*	Dest	P-value	N	Fst	Nm*	Dest	P-value	N	Fst	Nm*	Dest	P-value	N	Fst	Nm*	Dest	P-value
Pi02	86	0.38	0.41	0.26	0.05	16	0.07	3.13	-0.28	0.78	64	0.40	0.38	0.31	0.06	166	0.38	0.41	0.26	0.01
Pi04	86	0.17	1.19	0.00	0.45	16	0.24	0.79	-0.01	0.61	64	0.00	568.57	0.33	0.00	166	0.13	1.63	0.13	0.00
Pi89	86	0.19	1.10	-0.05	0.61	16	0.11	2.03	0.01	0.48	64	0.42	0.35	-0.60	0.73	166	0.32	0.53	-0.23	0.35
Pi70	86	0.16	1.35	-0.01	0.37	16	0.10	2.18	-0.05	0.82	64	0.13	1.71	0.21	0.11	166	0.28	0.65	0.08	0.54
Pi63	86	0.25	0.76	-0.01	0.38	16	0.05	5.37	-0.12	0.54	64	0.36	0.45	-0.02	0.63	166	0.42	0.35	0.03	0.18
Pi56	86	0.64	0.14	0.04	0.26	16	0.28	0.66	-0.61	0.99	64	0.27	0.68	0.22	0.10	166	0.53	0.22	0.33	0.00
G11	86	0.37	0.43	0.34	0.01	16	0.17	1.25	0.06	0.17	64	0.43	0.33	0.09	0.07	166	0.45	0.31	0.17	0.02
D13	86	0.46	0.29	0.56	0.01	16	0.61	0.16	0.01	0.30	64	0.23	0.84	0.53	0.02	166	0.51	0.24	0.58	0.00
Pi16	86	0.59	0.17	0.62	0.02	16	0.75	0.08	0.70	0.00	64	0.17	1.23	-0.01	0.83	166	0.58	0.18	0.54	0.01
Pi33	86	0.02	16.67	0.48	0.04	16	0.06	3.75	0.66	0.01	64	0.46	0.30	-0.04	0.73	166	0.40	0.38	0.36	0.03
All locus	**86**	**0.36**	**0.45**	**0.22**	**0.00**	**16**	**0.23**	**0.85**	**0.04**	**0.20**	**64**	**0.26**	**0.63**	**0.10**	**0.01**	**166**	**0.40**	**0.37**	**0.15**	**0.00**

N = sample size; *Nm = gene flow; P-value refers to bootstrap test of significance by 1000 resamplings.

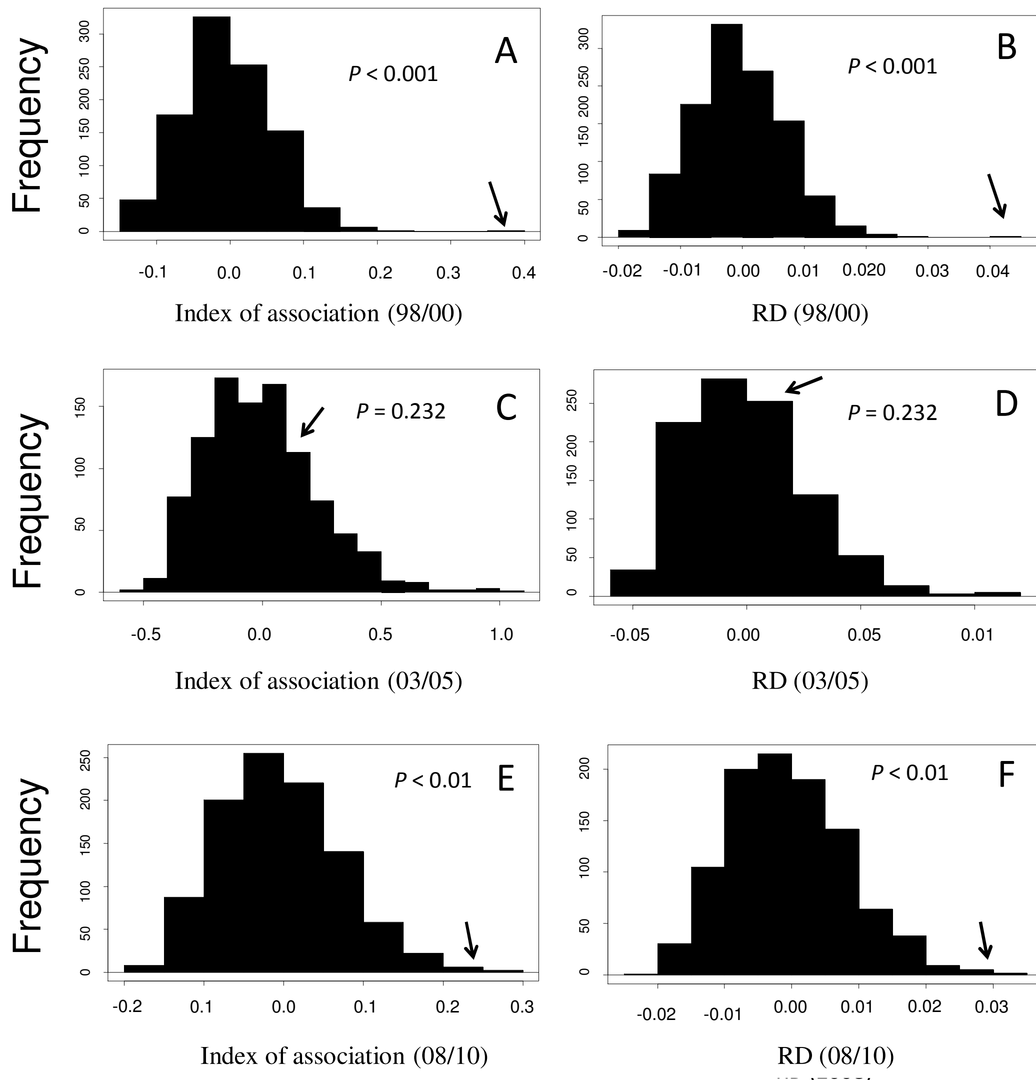

Fig 2. Histograms representing the expected distribution for the *Phytophthora infestans* subpopulations in linkage-equilibrium. Panels A, C, and E refer to index of association. Panels B, D, and F, represent the analysis of Rd. Data from A and B are for the 98/00, C and D for the 2003-2008, and E and F for the 08/10 periods. The observed values of the index of association (I_A) and R_D for the three different periods are indicated by the arrows. The P-value of each estimate is depicted above each panel.

Table 5. Genetic differentiation estimated by the D_{est} index (above diagonals) and gene flow (below diagonals, Nm) for the three sampling periods 1998/2000, 2003/2005 and 2008/2010.

	98/00	03/05	08/10
98/00	---	0.244*	0.217*
03/05	0.776	---	0.150*
08/10	0.900	1.416	---

*P <0.01, P – values obtained with 1000 randomization.
Nm = Gene flow estimated from $D_{est} = 0.25(1 - D_{est})/D_{est}$.

Table 6. Analysis of molecular variance (AMOVA) of *Phytophthora infestans* isolates collected in 1998/2000, 2003/2005 and 2008/2010.

AMOVA (with individual level)

SV	**df**	**SS**	**VC**	**Variation**	**F**[a]	***P – Value***
Among years	2	45.73	0.1	3.96%	0.04	<0.05
Among regions within year	12	58.62	0.17	6.56%	0.07	<0.001
Within regions	151	484.41	0.93	36.74%	0.41	<0.001
Heterozygosity	166	222.5	1.34	52.74%	0.47	<0.001

SV = source of variation; df = degrees of freedom, SS = sum of squares;
VC = variation components
[a]F = Fixation indices

AMOVA (without individual level)

SV	**df**	**SQ**	**VC**	**Variation**	**F**[a]	***P – Value***
Among years	2	42.73	0.05	1.94%	0.02[ns]	0.08
Among regions within year	12	58.62	0.26	10.37%	0.11	<0.001
Within regions	317	706.91	2.23	87.69%	0.12	<0.001

SV = source of variation; df = degrees of freedom, SS = sum of squares;
VC = variation components
[a]F = Fixation indices

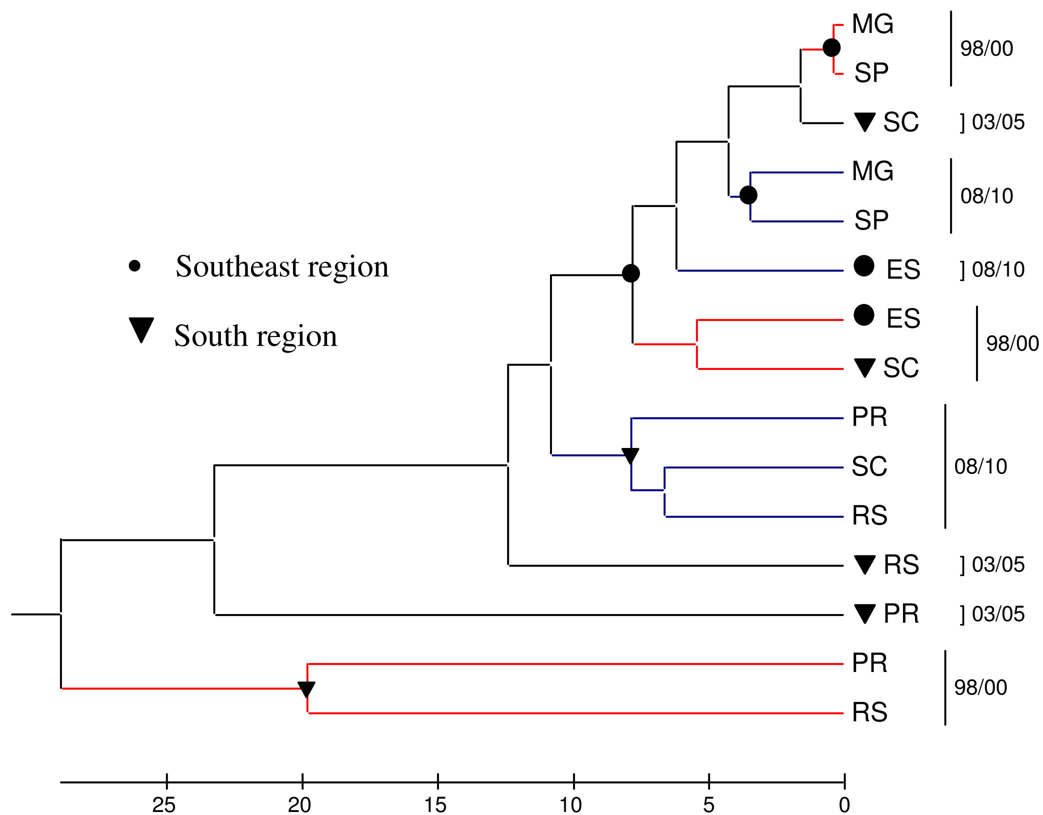

Fig 3. An UPGMA dendrogram of SSR MLG's of *Phytophthora infestans* collected from potato and tomato in 98/00 and 08/10 in Minas Gerais (MG), São Paulo (SP) and Espírito Santo (ES), and in 98/00, 03/05 and 08/10 in Rio Grande do Sul (RS), Paraná (PR) and Santa Catarina (SC). The horizontal axis represents values of Nei's genetic distance (Nei 1978).

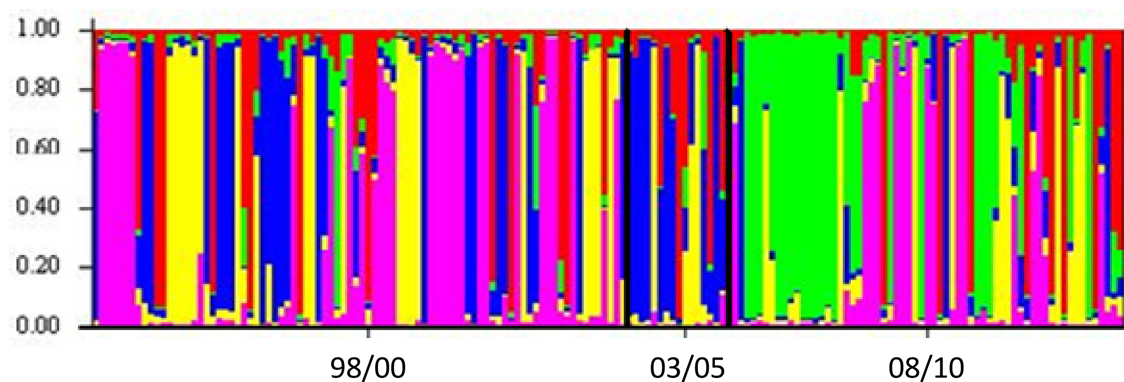

Fig 4. Cluster analysis of *Phytophthora infestans* from different periods 1998/2000, 2003/2005 and 2008/2010. Each bar in the figure represents an individual, divided into *k* colors where *k* is the number of cluster (assumed *k* = 6). The isolates are ordered by their probability of membership to the group.

Table 7. Calculation of the genetic bottleneck effect for the Brazilian population of *Phytophthora infestans* sampled in three different periods. The heterozygosity excess and the sign test were conducted assuming an infinite allele model (I.A.M).

	Observed			Under the I.A.M				
locus	n[a]	k_o[b]	He[c]	Heq[e]	SD[f]	DH/sd[g]	*P-Value*	Sign test
1998 – 2000								
Pi02	172	3	0.54	0.30	0.19	1.33	0.09	
Pi04	172	3	0.56	0.30	0.19	1.34	0.08	
Pi89	172	4	0.53	0.39	0.18	0.73	0.28	
Pi70	172	2	0.50	0.17	0.17	2.00	0.01	
Pi63	172	2	0.50	0.18	0.17	1.97	0.01	
Pi56	172	2	0.50	0.17	0.17	2.01	0.01	
G11	172	7	0.66	0.59	0.15	0.46	0.40	
D13	172	7	0.65	0.59	0.15	0.40	0.44	
Pi16	172	4	0.54	0.39	0.18	0.83	0.25	
Pi33	172	3	0.51	0.30	0.18	1.16	0.16	4.90***
2003 – 2005								
Pi02	32	3	0.64	0.40	0.17	1.41	0.06	
Pi04	32	3	0.60	0.41	0.17	1.12	0.14	
Pi89	32	4	0.60	0.53	0.14	0.47	0.39	
Pi70	32	2	0.52	0.24	0.16	1.76	0.01	
Pi63	32	2	0.52	0.25	0.16	1.67	0.01	
Pi56	32	2	0.52	0.24	0.16	1.71	0.01	
G11	32	3	0.63	0.41	0.16	1.34	0.07	
D13	32	5	0.62	0.61	0.12	0.09	0.45	
Pi16	32	3	0.55	0.41	0.16	0.85	0.24	
Pi33	32	2	0.52	0.25	0.16	1.64	0.02	5.12**
2008 – 2010								
Pi02	128	3	0.57	0.31	0.19	1.36	0.07	
Pi04	128	3	0.52	0.32	0.19	1.08	0.17	
Pi89	128	3	0.63	0.31	0.18	1.71	0.02	
Pi70	128	2	0.50	0.18	0.17	1.91	0.01	
Pi63	128	2	0.50	0.18	0.17	1.96	0.02	
Pi56	128	2	0.50	0.18	0.16	2.01	0.01	
G11	128	6	0.64	0.55	0.15	0.57	0.35	
D13	128	7	0.61	0.61	0.14	0.02	0.41	
Pi16	128	4	0.54	0.41	0.18	0.75	0.26	
Pi33	128	2	0.5	0.19	0.17	1.88	0.02	4.85***

$^{**}P < 0.01$, $^{***}P < 0.001$

[a]n = sample size, [b]k_o = observed number of alleles, [c]He = expected heterozygozity, [d]Heq = observed heterozygosity, [f]SD = standard deviation of the mutation-drift equilibrium, [g]DH/sd = standardized difference for each locus,

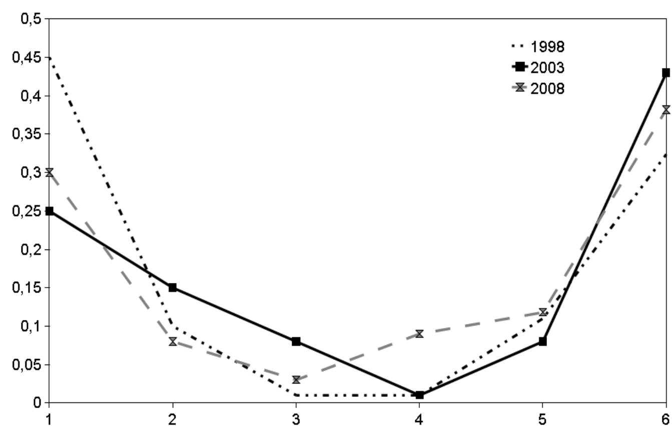

Fig 5. Graphic representation of allele distribution for each sampled period evidencing the shift in the allelic composition of the Brazilian population of *Phytophthora infestans*. The 1998/2000 period has a "L-shaped" distribution of alleles (expected in normal distribution). The 2003/2005 and 2008/2010 has a shifted-mode distribution (non-normal distribution).

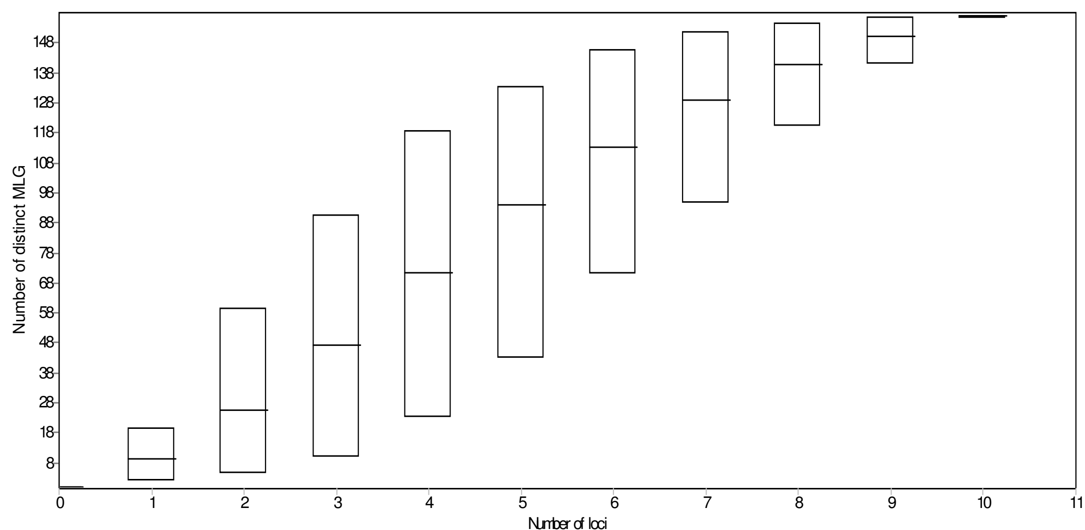

Fig S1. Number and distribution of multilocus genotypes (MLG's) detected by each one of the ten microsatellite markers: 1 = Pi02, 2 = Pi04, 3 = Pi89, 4 = Pi70, 5 = Pi63, 6 = Pi56, 7 = G11, 8 = D13, 9 = Pi16, and 10 = Pi33.

Table S1. Isolates code (cod.), host of origin (potato= POT; tomato = TOM), mating type (M.T.), mtDNA haplotype, city, state and period of collection (1998-2000; 2003-2005 or 2008-2010).

Cod.	Host	M.T.	mtDNA	City	State	Year	Cod.	Host	M.T.	mtDNA	City	State	Year
Pib-925	POT	A2	IIa	UNKNOW	PR	98/00.	Pib-99	POT	A1	Ib	VIÇOSA	MG	98/00.
Pib-140	TOM	A1	Ib	CAJURÍ	MG	98/00.	Pib-99	TOM	A1	Ib	VIÇOSA	MG	98/00.
Pib-54	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-220	TOM	A1	Ib	SÃO JOÃO BATISTA	SC	98/00.
Pib-1	POT	A2	IIa	URUBICI	SC	98/00.	Pib-880	POT	A1	Ib	MORRO REDONDO	RS	03/05.
Pib-10	POT	A2	IIa	SÃO JOAQUIM	SC	98/00.	Pib-881	POT	A2	IIa	UNKNOW	RS	03/05.
Pib-102	POT	A1	Ib	VIÇOSA	MG	98/00.	Pib-882	POT	A2	IIa	MORRO REDONDO	RS	03/05.
Pib-103	TOM	A1	Ib	COIMBRA	MG	98/00.	Pib-883	TOM	A2	IIa	MORRO REDONDO	RS	03/05.
Pib-104	TOM	A1	Ib	COIMBRA	MG	98/00.	Pib-884	TOM	A1	Ib	CRISTAL	RS	03/05.
Pib-111	TOM	A1	Ib	VIÇOSA	MG	98/00.	Pib-885	TOM	A1	Ib	CRISTAL	RS	03/05.
Pib-112	TOM	A1	Ib	VIÇOSA	MG	98/00.	Pib-886	TOM	A1	Ib	SANTA MARIA	RS	03/05.
Pib-121	POT	A2	IIa	PASSO FUNDO	RS	98/00.	Pib-887	POT	A1	Ib	CANGUÇU	RS	03/05.
Pib-14	TOM	A1	Ib	CARANDAÍ	MG	98/00.	Pib-891	TOM	A1	Ib	IBIRAIARAS	RS	03/05.
Pib-140	TOM	A1	Ib	CAJURÍ	MG	98/00.	Pib-892	TOM	A1	Ib	TIJUCAS	SC	03/05.
Pib-139	TOM	A1	Ib	CAJURÍ	MG	98/00.	Pib-893	TOM	A1	Ib	SÃO FRANCISCO DE PAULA	RS	03/05.
Pib-13	TOM	A1	Ib	CARANDAÍ	MG	98/00.	Pib-894	TOM	A	Ib	SÃO JOSÉ DOS AUSENTES	RS	03/05.
Pib-145	TOM	A1	Ib	COIMBRA	MG	98/00.	Pib-904	TOM	A1	Ib	CAÇADOR	SC	03/05.
Pib-146	TOM	A1	Ib	COIMBRA	MG	98/00.	Pib-908	TOM	A1	Ib	SANTA MARIA	RS	03/05.
Pib-147	TOM	A1	Ib	COIMBRA	MG	98/00.	Pib-926	TOM	A1	Ib	TIJUCAS	SC	03/05.
Pib-166	TOM	A1	Ib	CAJURÍ	MG	98/00.	Pib-951	TOM	A1	Ib	TIJUCAS	SC	03/05.
Pib-17	POT	A2	IIa	CARANDAÍ	MG	98/00.	Pib-888	POT	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-18	POT	A2	IIa	CARANDAÍ	MG	98/00.	Pib-889	POT	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-184-5	POT	A2	IIa	VENDA NOVA DO IMIGRANTE	ES	98/00.	Pib-890	POT	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-192	TOM	A1	Ib	PARAGUAÇU	MG	98/00.	Pib-895	TOM	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-191	POT	A2	IIa	PARAGUAÇU	MG	98/00.	Pib-896	POT	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-195	POT	A2	IIa	PARAGUAÇU	MG	98/00.	Pib-897	POT	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-195	POT	A2	IIa	PARAGUAÇU	MG	98/00.	Pib-898	POT	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-2	POT	A2	IIa	URUBICI	SC	98/00.	Pib-899	POT	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-20	TOM	A1	Ib	CARANDAÍ	MG	98/00.	Pib-900	TOM	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-200	TOM	A1	Ib	IGARAPÉ	MG	98/00.	Pib-901	TOM	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-204	TOM	A1	Ib	IGARAPÉ	MG	98/00.	Pib-902	TOM	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-205	TOM	A1	Ib	IGARAPÉ	MG	98/00.	Pib-903	TOM	A2	Ib	MARIA DA FÉ	MG	08/10.

Cod.	Host	M.T.	mtDNA	City	State	Year	Cod.	Host	M.T.	mtDNA	City	State	Year
Pib-21	POT	A2	IIa	CARANDAÍ	MG	98/00.	Pib-905	POT	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-220	TOM	A1	Ib	SÃO JOÃO BATISTA	SC	98/00.	Pib-906	TOM	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-223	TOM	A1	Ib	PIRAÚBA	MG	98/00.	Pib-907	TOM	A2	Ib	MARIA DA FÉ	MG	08/10.
Pib-224	TOM	A1	Ib	PIRAÚBA	MG	98/00.	Pib-909	TOM	A1	Ib	DOMINGOS MARTINS	ES	08/10.
Pib-233	TOM	A1	Ib	ALFENAS	MG	98/00.	Pib-910	TOM	A1	Ib	DOMINGOS MARTINS	ES	08/10.
Pib-235	TOM	A1	Ib	ALFENAS	MG	98/00.	Pib-911	TOM	A1	Ib	VENDA NOVA DO IMIGRANTE	ES	08/10.
Pib-234	POT	A2	IIa	ALFENAS	MG	98/00.	Pib-912	POT	A2	Ib	ITAJUBÁ	MG	08/10.
Pib-54	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-913	TOM	A2	Ib	ITAJUBÁ	MG	08/10.
Pib-236	TOM	A1	Ib	ALFENAS	MG	98/00.	Pib-914	TOM	A2	Ib	ITAJUBÁ	MG	08/10.
Pib-155	TOM	A1	Ib	CAJURÍ	MG	98/00.	Pib-915	TOM	A2	Ib	PERDIZES	MG	08/10.
Pib-240	TOM	A1	Ib	VARGINHA	MG	98/00.	Pib-916	TOM	A2	Ib	PERDIZES	MG	08/10.
Pib-241	TOM	A1	Ib	VARGINHA	MG	98/00.	Pib-917	TOM	A2	Ib	PERDIZES	MG	08/10.
Pib-247	TOM	A1	Ib	VIÇOSA	MG	98/00.	Pib-918	TOM	A2	Ib	PERDIZES	MG	08/10.
Pib-247	TOM	A1	Ib	VIÇOSA	MG	98/00.	Pib-919	TOM	A2	Ib	PERDIZES	MG	08/10.
Pib-254	TOM	A1	Ib	VENDA NOVA DO IMIGRANTE	ES	98/00.	Pib-920	TOM	A2	Ib	PERDIZES	MG	08/10.
Pib-329	TOM	A1	Ib	VENDA NOVA DO IMIGRANTE	ES	98/00.	Pib-921	TOM	A2	Ib	POUSO ALEGRE	MG	08/10.
Pib-255	TOM	A1	Ib	BOTUCATU	SP	98/00.	Pib-922	TOM	A2	Ib	POUSO ALEGRE	MG	08/10.
Pib-255	TOM	A1	Ib	BOTUCATU	SP	98/00.	Pib-923	TOM	A2	Ib	POUSO ALEGRE	MG	08/10.
Pib-257	TOM	A1	Ib	BOTUCATU	SP	98/00.	Pib-924	TOM	A2	Ib	POUSO ALEGRE	MG	08/10.
Pib-259	TOM	A1	Ib	LAVRAS	MG	98/00.	Pib-927	TOM	A2	IIa	GUARAPUAVA	PR	08/10.
Pib-259	TOM	A1	Ib	LAVRAS	MG	98/00.	Pib-928	TOM	A2	IIa	????	RS	08/10.
Pib-3	POT	A2	IIa	URUBICI	SC	98/00.	Pib-929	POT	A2	IIa	VARGEM GRANDE DO SUL	SP	08/10.
Pib-30	TOM	A1	Ib	CARANDAÍ	MG	98/00.	Pib-930	TOM	A1	Ib	URUBICI	SC	08/10.
Pib-30	POT	A2	IIa	CARANDAÍ	MG	98/00.	Pib-931	POT	A1	Ib	SÃO GOTARDO	MG	08/10.
Pib-32	POT	A2	IIa	CARANDAÍ	MG	98/00.	Pib-932	POT	A1	Ib	SÃO GOTARDO	MG	08/10.
Pib-33	POT	A2	IIa	CARANDAÍ	MG	98/00.	Pib-933	POT	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-4	POT	A2	IIa	URUBICI	SC	98/00.	Pib-934	POT	A1	Ib	SÃO GOTARDO	MG	08/10.
Pib-46	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-935	TOM	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-48	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-936	TOM	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-49	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-937	TOM	A1	Ib	SÃO GOTARDO	MG	08/10.
Pib-5	POT	A2	IIa	SÃO JOAQUIM	SC	98/00.	Pib-938	POT	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-51	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-939	TOM	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-54	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-940	TOM	A2	Ib	SÃO GOTARDO	MG	08/10.

Cod.	Host	M.T.	mtDNA	City	State	Year	Cod.	Host	M.T.	mtDNA	City	State	Year
Pib-52	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-941	TOM	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-53	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-942	TOM	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-6	POT	A2	IIa	SÃO JOAQUIM	SC	98/00.	Pib-943	POT	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-61	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-944	TOM	A1	Ib	SÃO GOTARDO	MG	08/10.
Pib-62	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-945	TOM	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-63	POT	A2	IIa	BARBACENA	MG	98/00.	Pib-946	POT	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-50	TOM	A1	Ib	BARBACENA	MG	98/00.	Pib-947	TOM	A1	Ib	SÃO GOTARDO	MG	08/10.
Pib-66	POT	A2	IIa	BARBACENA	MG	98/00.	Pib-948	POT	A1	Ib	SÃO GOTARDO	MG	08/10.
Pib-68	POT	A2	IIa	BARBACENA	MG	98/00.	Pib-949	POT	A1	Ib	SÃO GOTARDO	MG	08/10.
Pib-67	POT	A2	IIa	BARBACENA	MG	98/00.	Pib-950	POT	A2	Ib	SÃO GOTARDO	MG	08/10.
Pib-12	POT	A2	IIa	CARANDAÍ	MG	98/00.	Pib-952	POT	A2	Ib	VIÇOSA	MG	08/10.
Pib-208	POT	A2	IIa	SANTO AMARO	SC	98/00.	Pib-953	POT	A2	Ib	VIÇOSA	MG	08/10.
Pib-88	TOM	A1	Ib	CAJURÍ	MG	98/00.	Pib-954	TOM	A2	Ib	VIÇOSA	MG	08/10.
PiB-209	POT	A2	IIa	SANTO AMARO	SC	98/00.	Pib-955	POT	A2	IIa	VARGEM GRANDE DO SUL	SP	08/10.
PiB-221	POT	A2	IIa	SÃO JOAQUIM	SC	98/00.	Pib-956	POT	A2	IIa	VARGEM GRANDE DO SUL	SP	08/10.
Pib-90	TOM	A1	Ib	CAJURÍ	MG	98/00.	Pib-957	TOM	A1	Ib	VARGEM GRANDE DO SUL	SP	08/10.
Pib-96	TOM	A1	Ib	VIÇOSA	MG	98/00.	Pib-958	TOM	A1	Ib	VARGEM GRANDE DO SUL	SP	08/10.
Pib-97	TOM	A1	Ib	VIÇOSA	MG	98/00.	Pib-959	TOM	A1	Ib	CAXIAS DO SUL	RS	08/10.
Pib-97	TOM	A1	Ib	VIÇOSA	MG	98/00.	Pib-960	TOM	A1	Ib	VIÇOSA	MG	08/10.

POT = potato; TOM = tomato; M.T. = Mating type.

Table S2. Allelic frequency at ten loci of the three different sample seasons of *Phytophthora infestans* isolates. Homogeneity test of allelic distribution and the probability for each locus. Hard-Weinberg (H&W) test of equilibrium for each locus.

Overall allele frequencies by locus							
Locus				Homogeneity test		H&W Equilibrium	
Pi02	98/00	08/10	08/10	G^{2a}	*P – Value*	G2	*P – Value*
152		31.25	3.13				
156	2.33		3.91				
158	2.33						
160	32.56	6.25	50.00				
162	61.63	56.25	39.84				
166			3.13				
168	1.16	6.25		141.14	0.000	250.60	0.000
Pi04							
164	2.91		1.56				
166	48.26	43.75	47.66				
170	47.67	50.00	49.22				
173	1.16	6.25	1.56	29.63	0.925	145.89	0.000
Pi89							
177	0.58						
179	70.93	68.75	31.25				
181	26.74	18.75	67.97				
191	1.74	12.50					
201			0.78	123.70	0.000	52.39	0.000
Pi70							
192	84.88	46.88	85.94				
195	15.12	5.13	14.06	41.16	0.000	17.24	0.000
Pi63							
270	19.19	34.38	21.09				
273	16.86	37.50	10.94				
277	1.16	28.13	1.56				
279	62.79		66.41	88.25	0.000	69.77	0.000
P56							
251	29.07	25.00	15.63				
253	70.93	75.00	84.38	44.20	0.000	179.61	0.000
G11							
154	12.79	65.63	16.41				
157	16.28		14.84				
160	17.44	25.00	29.69				
162	19.19		17.97				
164	1.16		1.56				
166	0.58	6.25	7.81				
168	10.47		3.13				
172	13.95		1.56				
192	0.58						
194	3.49		1.56				
200	2.33						

202	1.16		5.47				
208	0.58	3.13		236.39	0.000	523.77	0.000
D13	98/00	03/05	08/10	G2	P – Value	G2	P – Value
106	1.16		0.78				
108	44.19	9.38	64.84				
110	0.58						
112	3.49		5.47				
124	1.74						
136	34.30	75.00	17.97				
140			0.78				
142	9.88	3.13	3.91				
149	1.16	6.25					
152	2.33	6.25	3.13				
164			1.56				
171	0.58						
176	0.58		1.56	200.17	0.001	272.41	0.000
Pi16							
166	4.65		3.13				
170			0.78				
172	1.16		1.56				
174	5.81	12.50	21.88				
176	9.30	3.13	5.47				
178	75.58	78.13	62.50				
182	2.33		0.78				
184	1.16	6.25	3.91	105.30	0.289	167.75	0.000
Pi33							
203	98.26	93.75	96.88				
205	0.58	6.25	3.13				
212	1.16			24.85	0.636	31.12	0.000

[a]G^2 = maximum likelihood statistical significance test.

2. Assessment of the aggressiveness of *Phytophthora infestans* isolates sampled in 2008-2010.

2.1. Abstract

Changes in the genetic structure of the population of *Phytophthora infestans*, the causing agent of potato and tomato late blight, were recently reported. The aggressiveness of Brazilian isolates was assessed to test the hypothesis that there was also a change in pathogenic features leading to the lack of host specificity. Five epidemiological variables were assessed: incubation period (IP), latent period (LP), lesion area (LA), infection frequency (IF) and sporulation capacity (SC). Differences in aggressiveness were measured on detached leaflet and stem fragments of susceptible potato and tomato cultivars. The isolates were separated in two groups based on their host of origin: potato (PG) and tomato isolates (TG). Isolates from both groups were inoculated in their host of origin and in cross inoculation. No differences regarding all variables were detected between PG and TG, and there was no evidence of interaction between groups of isolates and host when variables were assessed in leaflets. However, differences in LA and SC were detected between PG and TG inoculated in potato and tomato stems. Highest values of LA (2.87 cm^2) were recorded for PG in both hosts. Highest values of SC were recorded for PG when inoculated in tomato stem (1599.14 sporangia/cm^2). There is evidence of changes in the aggressiveness of isolates of the current population of *P*. *infestans* when inoculated in stems.

2.2. Introduction

Phytophthora infestans (Mont.) De Bary is the causal agent of late blight, one of the most destructive foliar diseases of potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) crops worldwide (Fry & Goodwin 1997). Late blight epidemics are favored by moderate temperatures and high humidity (Harrison & Lowe 1989) and under favorable conditions more than 15 fungicide sprays per season can be applied on potato and tomato crops to control late blight in Brazil (Miranda et al. 2010; Reis et al. 2005; Lima et al. 2009). In addition to the environmental and health problems the frequent applications of fungicides also lead to an increase of the production costs (Mizubuti & Fry 2006). Estimates of costs of potato late blight control and the yield losses in developing countries amount to more than U$ 2.5 billion per year (CIP 2009).

The Brazilian population of *P. infestans* was structured by two dominant clonal lineages, BR-1 (A2 mating type) associated with potato and US-1 (A1 mating type) associated with tomato (Reis et al. 2003). However, recently it was demonstrated that the Brazilian population of *P. infestans* changed given that genetically distinct isolates were found in potato fields (Oliveira et al. unpublished). Growers have noticed more severe late blight epidemics and an increase in the occurrence of stem blight. These facts can probably be related to the genetic changes in the population of *P. infestans* in Brazil.

The detection of both mating types in the same potato field can alter the development of late blight epidemics and make disease control more cumbersome and costly. In the presence of sexual reproduction, one would expect an increase in the number of genotypes, including more aggressive ones, which in turn can alter the host specificity of the Brazilian population of *P. infestans*. In some European countries where both mating types occur sexual recombination increased the genetic diversity of the population (Lehtinen et al. 2009) and favored the appearance of more fitted isolates with increased aggressiveness and more capable of overcoming resistance genes (Flier et al. 2003; Young et al. 2009). A shift in population structure can also be noticed regarding host adaptation if sexual recombination takes place. Panmixis reduces the chances of development of host-adapted populations (Hamelin et al. 2005).

Two evolutionary mechanisms are more likely to be involved in the emergence of new genotypes of *P. infestans* with higher fitness: migration and mutation. Multiple introduction events may enhance the genetic diversity by allowing for the entrance of

genotypes carrying new combination of alleles, which may rapidly displace the old population (Montarry et al. 2010). The increase of diversity and changes in the aggressiveness of the population were linked to new variants of the pathogen (Goodwin 1997; Linde et al. 2010). Mutation is an important source of variation for asexual populations because it creates new alleles. However, changes in the population exclusively due to mutation do not become promptly established due to the low rates at which mutations occur. Nevertheless, microorganisms usually have large population size and this can speed up the change of the population and compensate for the low mutation rate (McDonald & Linde 2002). The large population size of the pathogen, very common in the agroecosystems, could increase the probability of emergence of strains more aggressive and/or more fitted (Stukenbrock & McDonald 2008).

The possibility of new introductions of the pathogen in the producing areas pose a threat not only because of the new genotypes themselves, but also for the possibility for sexual recombination when complementary mating types are present in the same field. Constant monitoring of the population should be seen as an important disease management activity. Knowledge of the pathogen population structure and of the epidemiology of late blight is important to the development of new management strategies and for deploying new decision support technologies. Additionally, understanding the evolution of aggressiveness in *P. infestans* also constitutes an interesting field of study. Practical issues such as the implications for durability of cultivar resistance and the efficacy of site-specific fungicide depend on the knowledge of how aggressiveness changes over time, what are the genetic determinants and which factors affect this process. The aim of this study was to assess the aggressiveness of isolates of *P. infestans* collected from lesions formed on potato and tomato leaflets and stems. Two hypotheses were tested: 1. Changes in *P. infestans* population are leading to the lack of host specificity; and 2. Isolates from leaflets and stems differ in aggressiveness.

2.3. Material and Methods

Phytophthora infestans *isolates*

Diseased leaves, fruits, or stems of potato and tomato, with late blight symptoms were collected in the major Brazilian production regions (Fig. 1). Isolates were collected from August 2008 to April 2010, and geographic coordinates were obtained with a portable GPS device. A total of 64 isolates were characterized based on standard procedures that include the study of the morphology (Erwin & Ribeiro 1996), mating type determination (Spielman et al. 1989), and mtDNA haplotype (Griffith & Shawn 1997). Additionally, the isolates were also characterized using ten SSR markers (Knapova & Gisi 2002; Lees et al. 2006; Oliveira et al., unpublished). The isolates were separated into two groups according to the host of origin: potato group (PG) and tomato group (TG). Four isolates of each group were selected based on mating type, dissimilarity among 10 SSR markers, and geographic distance (Table 1). These isolates were used in cross-inoculation experiments as described below.

Aggressiveness of P. infestans

Aggressiveness was evaluated based on the incubation period (IP), latent period (LP), lesion area (LA), sporulation capacity (SC) and infection frequency (IF). The experiment was carried on fully-expanded detached leaflets and stem segments. A sample of three leaflets or three stem segments of each host placed in a gerbox lined with a moistened synthetic sponge was considered an experimental unit. The plant materials were collected from 45-day-old greenhouse-grown plants of potato, cv. Agata, and tomato, cv. Santa Clara. Each PG or TG isolate was inoculated on potato and tomato. Each isolate was inoculated in the plant materials of three experimental units.

For each isolate, a $2x10^4$ sporangia/mL was used to assess the IP; LP; LA; and SC; while a $2x10^3$ sporangia/mL suspension was used for the IFexperiment. To prepare the inoculum suspension, sporangia were first produced in pea-agar medium amended with β-sitosterol, followed by incubation at 18°C in the dark for 10 days. The sporangia from colonies grown in Petri dishes were harvested with 5mL of sterilized distilled water, and kept at 4°C for approximately 45 min, to induce zoospore release. The suspension was sprayed on leaflets of each host of origin, incubating for 6 days at 18°C

with 12h of darkness. When lesions developed and sporulated, the sporangia were harvested from the inoculated leaflets and inoculated on new healthy leaflets. This procedure was repeated one more time (totaling three transfers to leaflets) prior to the inoculation of the experiment to assure inoculum viability. After the third inoculation sporangia were removed from the leaves by cutting the lesion area and washing it with sterilized distilled water. Each suspension was calibrated to the appropriate concentration and kept at 4°C for 45 min to induce zoospore release.

Incubation period, latent period, lesion area and sporulation capacity

A 20 µL-drop of the sporangia suspension was placed in the center of each leaflet or stem segment. The experimental units were transferred to an incubator at 18°C in the dark. To assess the IP, leaflets and stems were observed under a stereoscope at 8-h-intervals from 24-120 h after inoculation (HAI). After 120 HAI leaflets and stems were examined at 24 h intervals until 216 HAI. When typical blight symptoms became visible in at least two of three leaflets or stem fragments of each replication, the IP was considered completed. At the same time, the lesions were observed for the presence of sporulation, and the LP was considered completed when sporangia were visible in at least two of the three leaflets or stem fragments of each replication.

Six days after inoculation, all lesions were photographed with a digital camera and the lesion area was analyzed. The LA was determined using the Imagetool software (UTHSCSA, University of Texas Health Science Center, San Antonio). To assess the SP, lesions were cut from the tissue and transferred to tubes with 2 mL of a preservation solution (1 mL distilled water and 1 mL of 0.04 m $CuSO_4$/0.2 m sodium acetate/ acetic acid pH 5.4) (Suassuna et al. 2004). The tubes were vortexed for approximately 10 s to release the sporangia from the tissue. The number of sporangia present in the suspension was estimated within 36 h using a haemocytometer, and this procedure was done twice, for each sporangia suspension. The SC per unit lesion area was calculated by multiplying the average sporangial concentration (spores mL^{-1}) by volume of preservation solution and dividing it by total lesion area (cm^2) (Kadish et al. 1990).

Infection frequency

The sporangia suspension was sprayed onto leaflets or stem segments. A coverglass with 242 mm^2 were placed inside each gerbox before the suspension was sprayed. After the inoculation, the experimental units were transferred to incubators at 18°C and 16h of light photoperiod, and the number of sporangia in the coverglass was counted under a microscope. The number of individualized lesions formed at 108 HAI in each leaflet or stem fragment was counted and divided by the total of sporangia deposited per area (cm^2) of plant tissue. The total area of the leaflets or stems was determined using the Imagetool software.

Statistical analysis

For IP, LP, LA, SP and IF, the experiment was set in a completely randomized design and a factorial linear model was used for the two-way ANOVA:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$$

where μ = overall mean, α_i = fixed effect of the host, β_j = fixed effect of the group of isolates (PG and TG), $(\alpha\beta)_{ij}$ = interaction term of host and group of isolates and e_{ijk} = random experimental error. All analyses were carried out with the R software version 2.11.1 (R Development Core Team 2008).

2.4. Results

Incubation and latent period

The data for detached leaflets and stem segments were analyzed independently. For IP in both leaflets and stems, the host x group interaction was not significant. The host effect was significant (Table 3), therefore IP was significantly different in tomato and potato plants when assessed in both leaflets ($P = 0.004$) and stem segments ($P < 0.001$) (Table 4). The average values of IP in potato leaflets (46.3 h) and stems (55.7 h) were shorter than in tomato leaflets (58.1 h) and stems (97.6 h). There was no significant effect of the group of the isolate (PG and TG) (Table 4).

There were no significant effects of host, group of isolate or the host x group of isolate interaction when LP was assessed in leaflets. In stems the LP varied according to the host and in potato (73.7 h) it was shorter than in tomato (104.3 h) (*P* = 0.0005) (Fig. 2).

Lesion area

The LA differed (*P* = 0.0001) according to the host when assessed on leaflets. In potato the average lesion area was larger (4.1 cm^2) then in tomato (1.3 cm^2), but no significant effect of group of isolate (Table 3) or of the interaction between the host x group of isolate was detected. In stems the LA in potato (0.64 cm^2) was greater (*P* = 0.004) than in tomato (0.40 cm^2) (Table 4). A significant difference (*P* = 0.001) was found for the effect of group of isolate and the average LA caused by PG (2.9 cm^2) was higher than the LA caused by the TG (0.4 cm^2) (Fig. 3). There was no interaction between group of isolate and host.

Sporulation capacity

The host x group of isolate interaction was not significant when analyzing SC . The SC values in leaflets varied according to the inoculated host. The SC was higher (*P* = 0.034) in tomato (1771.3 sporangia/cm^2) than in potato leaflets (451.7 sporangia/cm^2). There was no significant difference between the groups of isolates.

A different result was achieved when analyzing SC in stems. The host x group of isolate was significant (*P* = 0.002) and the isolates originated from potato sporulated more in tomato stem segments (1655.8 sporangia/cm^2) than in potato (918.7 sporangia/cm^2) (Table 4). The opposite occurred for the tomato isolates: sporulation was lower when assessed in tomato stems (375.13 sporangia/cm^2) than in potato stems (1559.1 sporangia/cm^2) (Fig 4.). There was no significant difference due to the inoculated host or group of isolate.

Infection frequency

There was no significant interaction between host and group of isolates. The average IF was significantly different between the leaflets of the two inoculated hosts ($P = 0.031$) (Tables 3 and 4). The number of lesions formed per sporangium in potato leaflet (0.04 lesions per sporangia) was lower than in tomato, where a single sporangium could originate 0.05 lesions (Table 3 and Fig. 2). There was no effect of group of isolate. The IF in stems was not affected by the host of origin, group of isolates or their interaction.

Relationship between aggressiveness components

There were significant correlations between epidemiological components (Table 5). The incubation period on leaflet (IP-L) was positively correlated ($r = 0.35$, $P = 0.037$) with the LP on leaflet (LP-L) and with the sporulation capacity ($r = 0.46$, $P = 0.007$) on leaflets (SC-L), and negatively correlated ($r = -0.33$, $P = 0.036$) with the lesion area in leaflets (LA-L). The IP on stem (IP-S) was negatively correlated with the LA-L ($r = -0.33$, $P = 0.035$) and positively correlated with the LP on stem (LP-L).

When the data were analyzed by pooling the host tissue factor (i.e. considering both stem and leaflets) negative correlations between LA and SC were noticed. There was a negative correlation between the SC-L and the LA-L ($r = -0.49$, $P = 0.002$) and LA-S ($r = -0.43$, $P = 0.012$), and a positive correlation between SC-L and SC on stem ($r = 0.40$, $P = 0.03$). The SC-S was negatively correlated with the LA-L ($r = -0.38$, $P = 0.033$) and with the LA-S ($r = -0.34$, $P = 0.0477$).

The LA-L and LA-S were strongly correlated ($r = 0.63$, $P = 0.0003$). The other aggressiveness components were not significantly correlated to each other. There was no correlation between the IF on leaflet or stem with other components.

2.5. Discussion

The general results reported here provide additional evidence for the occurrence of changes in the genetic composition of the population of *P. infestans* in Brazil. The lack of differential aggressiveness when groups of isolates from potato and from tomato

were inoculated in leaflets was not expected, given the results of a previous study conducted to assess aggressiveness that clearly demonstrated host specificity associated with differences in aggressiveness to potato and tomato leaflets (Suassuna et al. 20004). However, the detection of differential aggressiveness of groups of isolates when inoculated in stems supports the above mentioned changes since, as previously pointed out, growers have noticed an increase in the occurrence and severity of stem blight, predominantly in potato crops. Contrary to the previous study (Suassuna et al., 2004) the design of the current work allowed for testing the hypothesis of differential aggressiveness in stems. A body of evidence corroborates the hypothesis of change in the population of *P. infestans* in Brazil (Oliveira et al., unpublished data) and the current study demonstrated that important shifts also occurred in variables that affect the development of late blight epidemics. Differences in the epidemiological variables in potato and tomato leaflets were due to host difference and were not linked with the origin of the isolates (PG or TG) or with the interaction term between host and the group of isolate.

Regardless of the possibility of no strict clonality of the current population of *P. infestans* in Brazil, only isolates of the A1 mating type were found in tomato fields and seem to be adapted to this host. This association may be due to the quantitative aspect of the host-pathogen relationship, under the influence of the environmental conditions. The climate condition like temperature, leaf wetness period and relative humidity could favor the association of A1 isolates with tomato late blight (Maziero et al. 2009). Tomato fields are predominantly located in areas of lower altitude than potato fields and this may have consequences for the development of late blight in both hosts. The mean temperatures in tomato and potato producing areas are close to 20° C and 17° C, respectively. Previous work with Brazilian isolates from different clonal lineages showed that isolates obtained from tomato are favored by higher temperature (15-27° C) than potato-originated isolates (10-22° C) (Maziero et al. 2009). The experiments were conducted at 18°C a nearly optimum temperature for both, tomato and potato originated isolates. This could have prevented the detection of differences in aggressiveness, mainly by affecting the development of tomato isolates (favored by higher temperatures). Additional studies focusing in the competitive fitness under field conditions are necessary to elucidate the association of A1 isolates with tomato late blight.

The presence of isolates of both mating types in potato fields and the lack of differences in aggressiveness to leaflets could be due to pathogen dispersal and the

intensification of the production with area expansion and continuous cropping. A more likely explanation is the constant inoculum flow among production regions worldwide, linked with emergence of new variants with altered aggressiveness profile. The lack of host specificity may favor the inoculum flow between host species. If true, this implies in the development of a one single population, increasing the necessity of a more integrative management of the disease in both crops (Lebreton et al. 1999). Another implication of the lack of specificity is the increased potential of sexual recombination. Since the tomato population is comprised of A1 isolates and isolates of both mating types can be found in potato fields, the increase in inoculum flow can allow for more frequent occurrence of both mating types in the same field. This will be particularly important for regions where A1 mating type isolates are still absent in potato fields and for places where both mating types occur at unequal distribution.

The development of lesions caused by *P. infestans* in the stems is not well understood. The changes in the Brazilian populations could be linked with the emergence of isolates with differential ability to colonize stem tissues. Differences in aggressiveness in potato and tomato stems were found and there was a correlation between the group of isolate and the epidemiological variables, LA and SC. Few studies were conducted aimed at understanding the development of stem blight because this kind of infection is considered to occur at relatively low frequency (Keil et al. 2010). However, those studies that relate high incidence of stem blight are always linked with sexual reproduction and soil-borne oospores (Widmark et al. 2007).

In this work we noticed changes in the aggressiveness of *P. infestans*, which are probably linked with those changes related in our previous work of characterization of the Brazilian population of the South and Southeast regions (Oliveira et al., unpublished data). This could be due to the emergence of new variants, probably by introduction of new genotypes in Brazilian fields, or maybe by the presence of a putative sexual recombination between A1 and A2 isolates. This changes in the Brazilian population support the idea that horizontal resistance, in potato and tomato crops, is the most feasible strategy of control late blight control, since the speed of changes is high and host resistance controlled by minors genes is consider more durable to long term disease management.

The lack of differences in aggressiveness to leaflets between different groups of isolates, regions and mating type, does not invalidate the claim of changes in the population of *P. infestans*. The lack of host specificity could be linked to the appearance of new variants of the pathogen. In the same way, the change of the population reflects

on differences in aggressiveness in stems. The emergence of isolates with altered capacity of colonization, characterized by the lack of host specificity and isolates from PG group more aggressive to stems tissues can alter the survival abilities and the contribution to the next generation. *P. infestans* is a hemibiotrophyc pathogen that has poor saprophytic ability under tropical conditions, and subject to population bottlenecks (Lima et al. 2009). Under this scenario, the possibility to exploit both host by different subpopulations (PG and TG), that are subject to different selection events, such as resistance genes, host characteristics, climate conditions and fungicides applications, may enhance the long term evolution of the pathogen. According to Lebreton et al. (1999), the pathogenicity to different hosts could be profitable to *P. infestans*, once that reduces the effect of unfavorable periods in the pathogen life cycle. The results obtained in the present study suggest that the genetic changes detected in previous work (Oliveira et al., unpublished data), are leading to differences in the host-pathogen interaction, and are favorable to the constantly re-emergence of potato late blight, in leaflets and stem.

In conclusion, the presence of isolates more aggressive in stems, supports the hypothesis of the presence of new variants in the Brazilian population of *P. infestans*. The lack of host specificity contributes to break the reproductive barrier that was assumed to exist in Brazil. These findings enhance the necessity that cultivars with elevated levels of quantitative resistance, controlled by minors genes, are used in the late blight management programs, and highlight the necessity of assessment of the fungicides sensitivity of the Brazilian population, despite of the changes occurring in Brazil.

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Figure 1. Map showing the collection fields on different regions of Brazil. Brazilian states names are in bold.

Table 1. *Phytophthora infestans* isolates used for aggressiveness tests in potato and tomato detached leaflet and stem.

Isolate	MT	mtDNA	Host	Sample origin
Pib-810	A2	IIa	Potato	Maria da Fé, MG
Pib-811	A2	Ib	Potato	Perdizes, MG
Pib-841	A2	IIa	Potato	Vargem Grande do Sul, SP
Pib-869	A2	IIa	Potato	Guarapuava, PR
Pib-844	A1	Ib	Tomato	Viçosa, MG
Pib-847	A1	Ib	Tomato	Caxias do Sul, RS
Pib-860	A1	Ib	Tomato	Tijucas, SC
Pib-866	A1	Ib	Tomato	Domingos Martins, ES

Table 2. Epidemiological variables related to Brazilian isolates of *Phytophthora infestans* and differences related to the two hosts (potato and tomato) and tissues (leaflet or stem), group of isolate according to the host of origin and the interaction term between host and host of origin of the isolates.

	Host		group of isolate		group of isolate			
					PG		TG	
Aggressiveness component	Potato	Tomato	Potato Group (PG)	Tomato Group (TG)	Potato	Tomato	Potato	Tomato
Incubation period (h) - Leaflet	46.33	58.13	53.10	50.16	48.00	58.67	44.67	57.48
Incubation period – Stem	55.67	97.58	73.82	77.44	54.44	97.07	56.89	98.00
Latent period (h) - Leaflet	75.60	83.73	76.85	82.87	73.56	80.80	78.67	89.60
Latent period (h) – Stem	73.73	104.36	86.18	87.67	71.52	106.33	76.44	102.10
Infection frequency[a] (leaflet)	0.04	0.05	0.05	0.04	0.04	0.06	0.03	0.05
Infection frequency (stem)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Lesion area (cm^2) - Leaflet	4.06	1.34	0.68	2.53	4.14	1.73	4.16	0.90
Lesion area (cm^2) – Stem	0.65	0.40	2.87	0.37	0.89	0.47	0.42	0.30
Sporulation capacity (leaflet)	451.68	1771.28	807.27	1440.80	410.89	1243.28	491.63	2525.56
Sporulation capacity (stem)	1292.92	1472.84	1303.29	1376.59	918.74	1655.79	1599.14	375.13

[a]Number of developed lesions per sporangium deposited.

Table 3. ANOVA table for the experiment conducted with detached leaflet and stem of two hosts of potato or tomato, group of isolate defined according to the host of origin and the interaction between Brazilian isolates of *Phytophthora infestans* and the different hosts leaflet (A e C) and stem (B e D).

	IP – leaflet				LP - Leaflet				LA – Leaflet				SC – Leaflet				IF - Leaflet			
SV	DF	MS	F	Pr(>F)	DF	MS	F	Pr(>F)	DF	MS	F	Pr(>F)	DF	MS	F	Pr(>F)	DF	MS	F	Pr(>F)
Host	1	1518.8	9.66	0.004	1	567.2	1.62	0.212	1	833082	16.9	0.0001	1	9773073	4.79	0.034	1	0.003	4.98	0.031
group of isolate	1	61.08	0.39	0.537	1	356.6	1.02	0.320	1	68365	1.39	0.244	1	452278	0.64	0.640	1	0.001	1.50	0.226
Host X GI	1	12.53	0.08	0.779	1	26.75	0.08	0.784	1	764	0.02	0.901	1	708799	0.34	0.559	1	0.001	0.01	0.912

IP – incubation period; LP – latent period; LA – lesion area; SC – sporulation capacity; IF – infection frequency; GI – Group of Isolate

	IP – Stem				LP - Stem				LA - Stem				SC - Stem				IF - Stem			
SV	DF	MS	F	Pr(>F)	DF	MS	F	Pr(>F)	DF	MS	F	Pr(>F)	DF	MS	F	Pr(>F)	DF	MS	F	Pr(>F)
Host	1	20160	42.3	$7.5xe^{-8}$	1	8037	15.2	0.0005	1	11245	9.35	0.004	1	313397	0.31	0.578	1	5xe-5	0.77	0.386
group of isolate	1	34	0.07	0.791	1	8.4	0.02	0.90	1	14991	12.5	0.001	1	458376	0.46	0.178	1	2xe-6	0.03	0.862
Host X GI	1	6.5	0.02	0.908	1	178.8	0.34	0.564	1	1403.9	1.17	0.286	1	11412727	11.4	0.002	1	2xe-4	3.71	0.601

IP – incubation period; LP – latent period; LA – lesion area; SC – sporulation capacity; IF – infection frequency; GI – Group of Isolate

Table 4. Pearson's correlation matrix, showing above the correlation between the epidemiological components, and below the significance of the tests.

	LA-S	SC-L	SC-S	IF-L	IF-S	IP-L	IP-S	LP-L	LP-S
LA-L	0.625***	-0.485**	-0.378*	-0.024	0.143	-0.332*	-0.331*	-0.299	-0.209
P-value	*P = 0.0003*	*P = 0.0023*	*P = 0.0327*	*P = 0.8788*	*P = 0.4346*	*P = 0.0364*	*P = 0.0348*	*P = 0.0909*	*P = -0.209*
LA-S		-0.425*	-0.342*	-0.037	0.329	-0.090	-0.195	-0.265	-0.254
P-value		*P = 0.0122*	*P = 0.0477*	*P = 0.8075*	*P = 0.0613*	*P = 0.5757*	*P = 0.2092*	*P = 0.1367*	*P = 0.1531*
SC-L			0.404*	0.293	-0.113	0.463**	0.198	0.281	0.332
P-value			*P = 0.0298*	*P = 0.0827*	*P = 0.5813*	*P = 0.0067*	*P = 0.2617*	*P = 0.1253*	*P = 0.0683*
SC-S				0.033	-0.359	0.308	0.058	0.155	0.109
P-value				*P = 0.8549*	*P = 0.0778*	*P = 0.0817*	*P = 0.7521*	*P = 0.4121*	*P = 0.5732*
IF-L					0.030	0.255	-0.048	0.019	0.182
P-value					*P = 0.8622*	*P = 0.0949*	*P = 0.7504*	*P = 0.9124*	*P = 0.2959*
IF-S						0.268	0.116	0.011	0.253
P-value						*P = 0.1257*	*P = 0.5062*	*P = 0.9565*	*P = 0.2130*
IP-L							0.171	0.354*	0.212
P-value							*P = 0.2787*	*P = 0.03709*	*P = 0.4411*
IP-S								0.139	0.704***
P-value								*P = 0.2359*	*P = 0.0004*
LP-L									-0.304
P-value									*P = 0.1024*

LA = lesion Area; SC = sporulation capacity; IF = infection frequency; IP = incubation period; LP = latent period; -L = leaflet; -S = stem

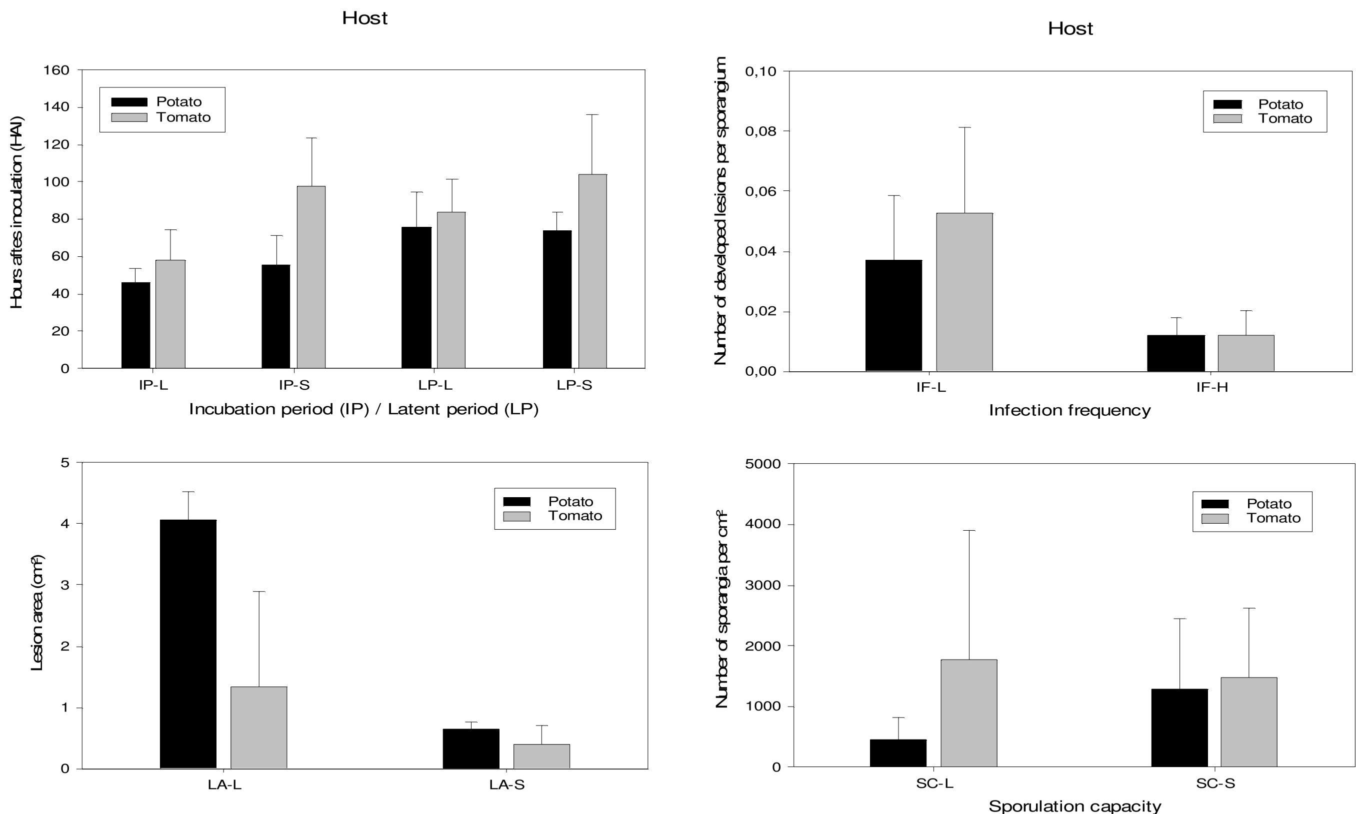

Figure 2. Bar-chart comparing epidemiological components associated to *Phytophthora infestans* on detached leaflet (L) of potato and stem(S) and tomato with the standard deviation bar. Differences based on inoculated host. (IP = incubation period; LP = latent period; IF = infection frequency; LA = lesion area; SC = sporulation capacity).

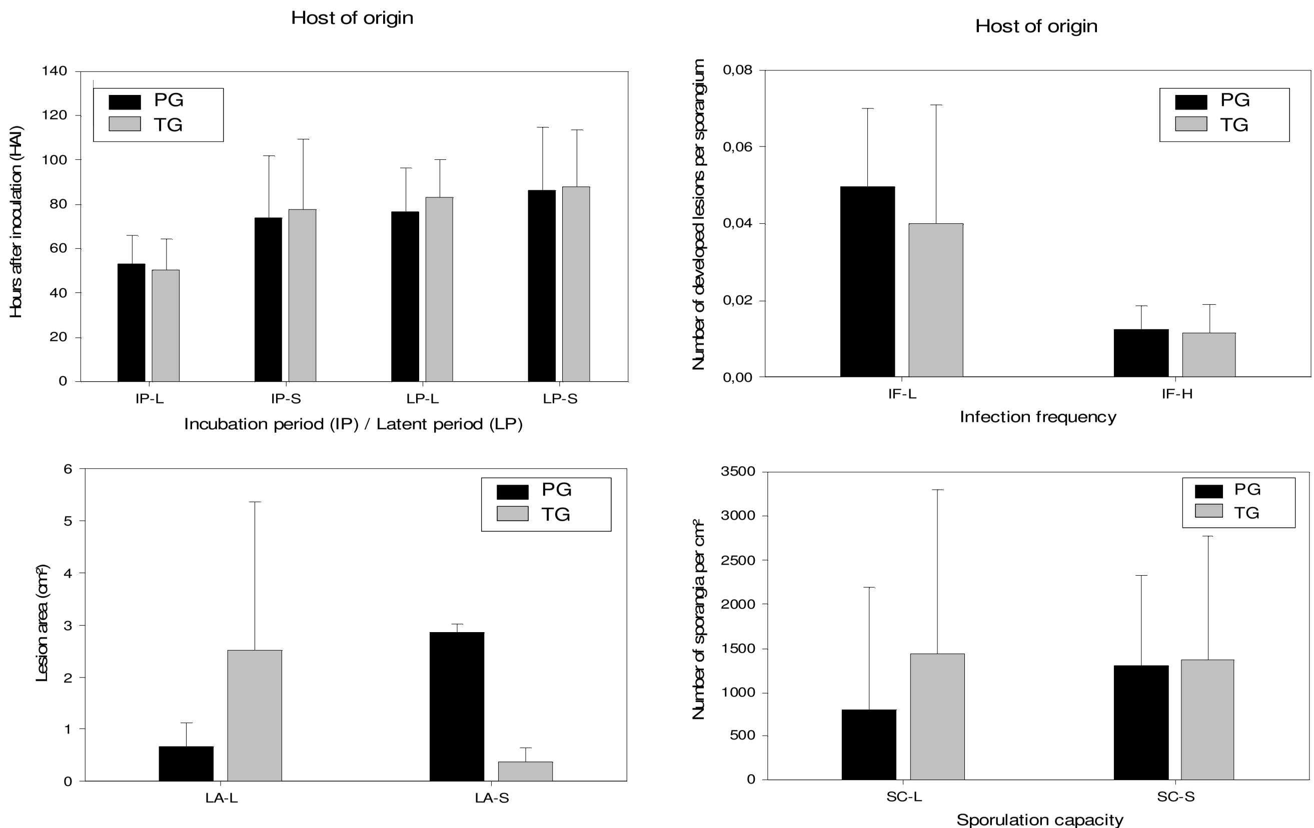

Figure 3. Bar-chart comparing epidemiological components associated to *Phytophthora infestans* on detached leaflet (L) of potato and stem (S) and tomato with the standard deviation bar. Differences based on group of isolate . (PG = potato group; TG = tomato group; IP = incubation period; LP = latent period; IF = infection frequency; LA = lesion area; SC = sporulation capacity).

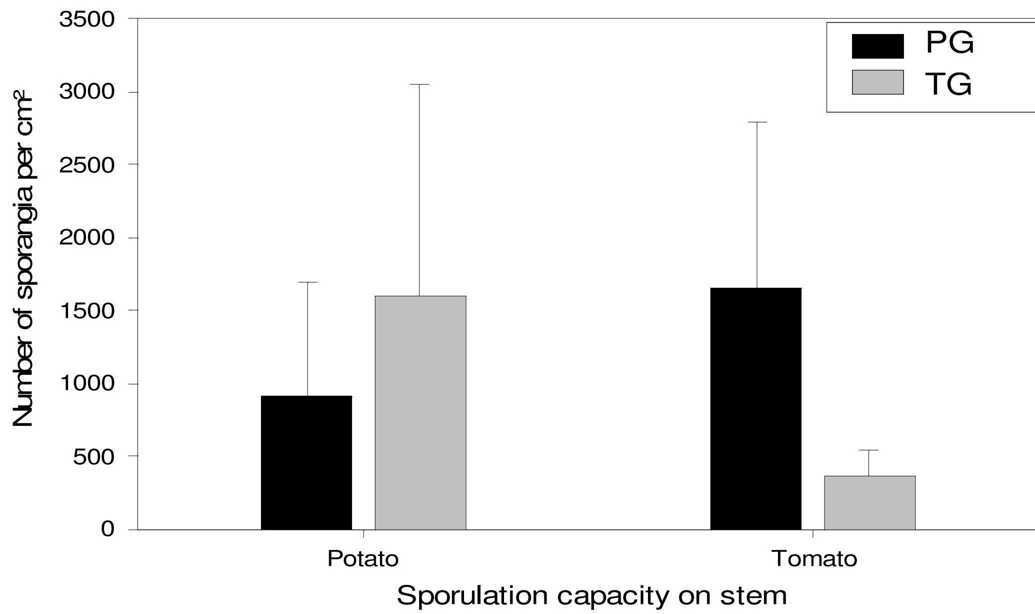

Figure 4. Bar-chart comparing sporulation capacity (SC) of the different isolates of *Phytophthora infestans* on potato and tomato stem fragments with the standard deviation bar. Differences based on the interaction between host and group of isolates. (PG = potato group; TG = tomato group).

3. Sensitivity to protectant and systemic fungicides of isolates of *Phytophthora infestans* collected from potato and tomato fields in Brazil.

3.1. Abstract

The sensitivity to commonly used protectant and systemic fungicides was accessed for Brazilian isolates of *Phytophthora infestans* collected in the South and Southeast regions. Six fungicides, two protectants, mancozeb and chlorothalonil, and four systemic, propamocarb-HCl, fenamidone, dimetomorph and mandipropamid were evaluated. Sixty eight isolates were used in sensitivity tests for propamocarb-HCl and 62 isolates for mancozeb, chlorothalonil, fenamidone, dimethomorph and mandipropamid. No loss in sensitivity was detected to mancozeb and chlorothalonil and there was a positive correlation between EC_{50} values of both fungicides. Among the systemic fungicides the highest EC_{50} values were observed for propamocarb-HCl. There was a positive correlation between propamocarb-HCl EC_{50} values and geographic distance. The frequency of isolates with EC_{50} above 10 µg mL^{-1} was higher in the South region (36% of the isolates) than in the Southeast (7.1%). There was no evidence of resistance to the other systemic fungicides and no significant correlation was found between EC_{50} values of the fungicides and geographic region or host of origin. There was no evidence of cross-sensitivity between the different groups of fungicides.

3.2. Introduction

Late blight epidemics caused by the oomycete *Phytophthora infestans* (Mont.) de Bary are highly destructive to potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) crops when moderate temperature and high humidity prevail in the fields (Harrison & Lowe 1989; Fry 2008). Fungicide application is the main control measure used to reduce crop losses due late blight. In Brazil, more than 15 sprays per season are applied to potato and tomato fields to control the disease (Reis et al. 2005; Lima et al. 2009; Miranda et al. 2010).

Both protectant and systemic fungicides are used in most late blight management programs. The most commonly used protectant fungicides with multi-site activity are copper oxychloride, chlorothalonil, and mancozeb (Kato et al. 1997). Protectant fungicides can be used in mixtures or alternately sprayed with site-specific systemic fungicides such as phenylamides (mefenoxam), QoI's (famoxadone and fenamidone), carbamates (propamocarb-HCl) and the carboxylic acid amides (CAA's) derived products (dimethomorph and mandipropamid) (Reis et al. 2005; Pérez et al. 2009).

Resistance to site-specific fungicide was reported as a problem in Brazil about 10 years ago, but changes in the pathogen population and the introduction and use of new fungicides require frequent monitoring of the sensitivity of *P. infestans*. The first characterization of the sensitivity of Brazilian isolates of *P. infestans* to fungicides regularly used to control late blight detected high frequency of isolates resistant to mefenoxam, but there was no evidence of resistance to cymoxanil, chlorothalonil, and mancozeb (Reis et al. 2005). Mefenoxam is a phenylamide systemic fungicide that interferes with the synthesis of the ribosomal RNA (rRNA) (Gisi & Cohen 1996; Gisi & Sierotzki 2008). After its release in the market phenylamides were highly effective against oomycetes, but because of the intensive usage, resistant isolates have emerged and were selected in many areas, including in Brazil (Reis et al. 2005). Meanwhile, other compounds were developed and made available to growers. The availability of new fungicides contributed to a change in recommendation procedures.

Different site-specific fungicides are now used in both potato and tomato fields in all producing areas in Brazil. One of the most widely used is propamocarb (carbamate group). The proposed mode of action is on cell membrane permeability and fatty acids synthesis, but the exact biochemical mode of action is still not understood

(Möller et al. 2009), but resistant isolates have been detected in *Pythium* spp. (Moorman & Kim 2004). Different studies conducted in Nordic countries and in the Toluca Valley, Mexico, the putative center of origin of *P. infestans*, did not detect insensitivity to propamocab HCl. Comparing populations sampled between 1997-2000 no change in the sensitivity was noticed, but some isolates were able to grow at 1,000 μg mL^{-1} (Lehtinen et al. 2003). In the Toluca Valley the situation was not different, no insensitivity was noticed, but some isolates were able to grown at concentrations higher than 100 μg mL^{-1} of propamocarb-HCl (Grünwald et al. 2006). More recently resistance to this compound was noticed in German isolates. Ten of seventy-six isolates had ED_{50} values higher than 5,000 μg mL^{-1} (Möller et al. 2009).

The quinone outside inhibitor compounds (QoI's) form a group of fungicides used in potato and tomato crops aimed at controlling late blight. This group is composed by the strobilurins (i.e. azoxystrobin and piraclostrobin), famoxadone, and fenamidone. The last two fungicides are registered to control late blight in Brazil and commercial products are sprayed in potato and tomato crops. Considering the QoI's group as a whole, additional selection pressure is exerted due to the application of azoxystrobin to control early blight, a fungal disease caused by *Alternaria grandis* on potato and *A. tomatophila* on tomato (Rodrigues et al. 2010). The QoI is a well studied group of fungicides and resistance to these compounds is known to occur in several plant pathogenic fungi. Point mutations in the target site of the fungicide, i.e. the mitochondrial cytochrome b gene, is the main mode of resistance described (Gisi & Sierotzki 2008). However, knowledge about other modes of resistance, including an alternative oxidase pathway and metabolism of the active ingredient (Fernández-Ortuño et al. 2010), is starting to accumulate. The well known point mutations that confer QoI resistance are the amino acid changes at positions 143 (G143A) and 129 (F129L), in the mtDNA cytochrome b. These changes are associated with qualitative resistance to this group of fungicides (Fernández-Ortuño et al. 2010).

The CAA's group is effective against downy mildews in several crops and late blight. The mode of action of these compounds remains largely unknown. Several studies proposed that the mechanism of fungicidal activity is the alteration on cell wall structure (Cohen & Gisi 2007; Gisi & Sierotzki 2008) by blocking phospholipid biosynthesis through the inhibition of phosphatidylcholine (lecithin) (Griffiths et al. 2003; Rubin et al. 2008; Blum et al. 2010) and the disruption of F-actin (Zhu et al. 2007; Rubin et al. 2008). One interesting characteristic of the CAA group is the inhibition of zoospore encystment of *Phytophthora* spp. and *Plasmopara viticola*,

causing lysis and preventing the regeneration of the protoplasts (Cohen et al. 2007; Gisi & Sierotzki 2008).

The risks of the establishment of a fungicide resistant population of *P. infestans* depend on pathogen characteristics and on the mode of action of the fungicides. *P. infestans* is classified as a high risk pathogen for resistance to fungicides that act in the RNA polymerase (as the phenylamides) and as medium risk pathogen for fungicides with other modes of action (FRAC 2008). However, with the intensification of application of site-specific fungicides in many crops there has been an increase in the selection pressure for isolates resistant to site-specific fungicides (Brent & Hollomon 2007).

Several factors contribute to an increased concern regarding fungicide resistance in the Brazilian population of *P. infestans*. Commonly, 15 or more fungicide sprays can be made during the crop cycle to control late bight (Reis et al. 2005), however in some extreme cases more than 30 fungicides sprays can be necessary (Maffia & Matsuoka 1980; Nazareno & Jaccound Filho 2003). The large amount of fungicides leads to increased selection pressure to emergence of resistance. The current genetic changes in the population of *P. infestans* with the presence of both mating types (A1 and A2) in some fields in Brazil (Oliveira et al. unpublished), and the potential for sexual recombination to occur could contribute to the appearance of variants in the population that are less sensitive to site-specific fungicides. Under strong selection pressure, the emergence of resistant isolates combined with an effective asexual reproduction process may result in the prevalence of resistant isolates in the population. The aim of this study was to assess the sensitivity of *P. infestans* to fungicides commonly used to control potato and tomato late blight in Brazil.

3.3. Material e Methods

Sampling and isolates

The isolates of *P. infestans* were obtained from diseased plants collected in potato and tomato production regions (Fig. 1) during 2008/2010. All sampled fields were georeferenced with a portable GPS system. Three to 10 leaflets or stems were collected from each sampled field and put in moist chamber at 18°C to induce sporulation. Pathogen isolation was accomplished by harvesting the sporangia from the

lesions and transferring to V8-culture medium (1.0 g CaCo3, 200 mL of V8-juice, 0.05 g of β-sitosterol), amended with ampicillin at 100 mg L^{-1}, rifampicin at 20 mg L^{-1}, pentachloronitrobenzene at 670 mg L^{-1} (PCNB) and benomyl 100 mg L^{-1}. All isolates where characterized for mating type using standard methods (Goodwin et al. 1994).

Sensitivity to chlorothalonil, mancozeb and propamocarb-HCl

The sensitivity of *P. infestans* isolates to the multi-site fungicides chlorothalonil (chloronitriles) and mancozeb (dithiocarbamate), and to the site-specific systemic carbamate fungicide propamocarb-HCl was determined. For chlorothalonil and mancozeb, 62 isolates were used. For propamocarb-HCl, 68 isolates were assayed. Commercial products of the three fungicides were dissolved in dimethyl sulfoxide (DMSO) to the following concentrations of the active ingredient (a.i.): chlorothalonil and mancozeb, 0.0 (control); 0.5; 1; 5; 10; 25 and 100 µg mL^{-1} of a.i. (Reis et al. 2005); and propamocarb-HCl, 0.0 (control); 0.1; 1; 1; 10; 100 and 1000 µg mL^{-1} of a.i (Grünwald et al. 2006). A plug of culture medium with mycelium (6mm diameter) of a 7-day-old actively growing colony of *P. infestans* was cut with a cork borer and transferred to pea-glucose-agar (150 g of frozen peas, 5 g of glucose and 15 g of agar per liter of distilled water) amended with the fungicides at the concentrations cited above, in 4 cm-diameter Petri dishes. Each isolate was incubated at 18°C for 5 days in the dark.

The sensitivity of the isolates was assessed by measuring the diameter of the colony growth in two perpendicular directions, and the mean diameter was used to calculate the area of the circle. Inhibition of mycelium growth was calculated as the ratio of the colony area formed in a Petri dish with culture medium amended with fungicide at a given concentration relative to the growth in the control, without fungicide. The effective concentration required to reduce the mycelia growth by 50% (EC50) was estimated for each of the five replicates by linear regression of the values of growth inhibition by the logarithm of fungicide concentration (Kato et al. 1997; Reis et al. 2005; Günwald et al 2006).

Sensitivity to dimethomorph, mandipropamid and fenamidone

The sensitivity of 62 isolates to dimethomorph, mandipropamid and fenamidone was conducted according to the methods recommended by the Fungicide Resistance Action Committee (FRAC 2008). The isolates were grown on pea-glucose-agar medium plus 0.05 g L^{-1} of β-sitosterol in Petri dishes kept in BOD chamber set at 16°C, in the dark, for 15 days. The sporangia were washed with 5 mL of a nutrient solution [pea broth double concentration (300 g of frozen peas, 10 g of glucose)], at 18±2°C, at room temperature, and the resulting suspension was adjusted in haemocitometer to $2x10^4$ sporangia mL^{-1}.

Commercial products of the three fungicides were prepared to reach the following final concentrations: 0.0; 0.01; 0.03; 0.1; 0.3; 1; 3; 10 μg mL^{-1} of dimethomorph, 0.0; 0.001; 0.01; 0.1; 1; 10; and 100 μg mL^{-1} of the mandipropamid and fenamidone. A 50 μl-drop of the fungicide suspension and a 50 μl-drop of the sporangia suspension were mixed in a well of ELISA plate. Each isolate and fungicide concentration combination was placed in five wells, which were considered as five replicates. The same number of replicates was used for the controls (pea broth + fungicide solution). The initial absorbance was measured in a photometer (Multiskan FC®, Thermo Scientific, USA) at 405 ηm. Plates were put into plastic bags and kept at 18°C in the dark. After 5 days, growth was indirectly measured by absorbance. The values were corrected by subtracting the first reading and/or by comparing with blank wells (Stammler 2006). The mycelia inhibition and EC_{50} were estimated as described above.

Experimental design and statistical analysis

All experiments were set in a completely randomized design, with five replicates for each isolate and fungicide dose. All statistical analyses were conducted using the R software (R Development Core Team 2008). The EC50 values were estimated by linear regression as described above. A distance matrix was generated with the geographic location of the isolates by pairwise comparative analysis and compared with the EC50 using the Mantel's test. This analysis was implemented with the packages "ade4" and

“ecodist” of the R software. A Pearson’s correlation test was conducted to assess the associations between the six different fungicides and to detect cross-resistance.

3.4. Results

Overall sensitivity analysis

Significant variation in the sensitivity to the protectant fungicides, chlorothalonil and mancozeb, and the systemic fungicides, carbamate (propamocarb-HCl), QoI’s (fenamidone) and CAA´s (dimethomorph and mandipropamid) was observed. The mean values of EC50 for the protectant fungicides chlorothalonil and mancozeb were 0.9 ± 1.21 (mean ± standard deviation) and 3.2 ± 4.33 µg mL^{-1}, respectively. Chlorothalonil was the most active protectant fungicide (Table 1). The mean EC50 values for the systemic fungicides propamocarb-HCl, fenamidone, dimethomorph and mandipropamid were 5.1 ± 7.16; 0.3 ± 0.31; 0.3 ± 0.35; and 0.4 ± 0.63 µg mL^{-1}, respectively. Propamocarb-HCl was the least active systemic fungicide tested (Table 1). The analysis of the box plot for the protectant fungicides shows lower variation of EC50 values to 50% of the population (boxes) for chlorothalonil than for mancozeb (Fig. 2). When the systemic fungicides were analyzed, both fungicides of the CAA and QoI’s groups had lower variation in EC_{50} values for 50% of the population compared with the carbamate (propamocarb) (Fig. 2).

Sensitivity to protectant fungicides

The EC_{50} values for chlorothalonil and mancozeb ranged from 0.2 to 8.2 µg mL^{-1} and from 0.3 to 21.5 µg mL^{-1}, respectively (Table 1). For chlorothalonil, the EC_{50} values for 71.0% of the isolates were lower than 1.0 µg mL^{-1}. The EC_{50} values for mancozeb were lower than 1.0 µg mL^{-1} for 41.9% of the isolates, 54.6% between 1-10 µg mL^{-1} and for 6.5% of the isolates the EC_{50} values were greater than 10 µg mL^{-1} (Figs. 3A and B). There was no correlation between sensitivity to chlorothalonil and geographic distance (Table 1). Similarly, there was no correlation between EC_{50} values for mancozeb and geographic region, based on Mantel’s test. Isolates were grouped based on the host of origin and EC_{50} values and the frequency of isolates in each class

was compared by Chi-square test. There was no significant difference between EC_{50} distribution based on host of origin (isolates from potato or tomato), to chlorothalonil (χ^2 = 0.01 P = 0.93) and mancozeb (χ^2 = 0.06, P = 0.82) When the EC_{50} values were analyzed, two different classes of isolates were detected for all regions and host of origin. For both protectant fungicides, the highest frequencies were detected in the class with EC_{50} below 10 µg mL^{-1} of chlorothalonil. Among the potato isolates 69.8% of the isolates had EC_{50} below 1.0 µg mL^{-1}; and for tomato isolates, 77.8% of the isolates had EC_{50} values < 1.0 µg mL^{-1} of a.i. Overall, the highest frequencies of isolates were grouped in the class of EC_{50} values below 10 µg mL^{-1} of a.i. The frequencies of potato and tomato isolates in the class with EC_{50} values less than 10 µg mL^{-1} were 88.0% and 91.8% of the isolates, respectively.

Sensitivity to propamocarb-HCl and fenamidone

The EC_{50} values of propamocarb-HCl ranged from 0.1 to 34.1 µg of a.i. mL^{-1} (Table 1). Among these isolates, 37.1% had EC_{50} values less than 1.0 µg mL^{-1}, 45.2% between 1-10 µg mL^{-1} and 17.7% had EC_{50} higher than 10 µg mL^{-1} (Fig. 3C). There was a positive correlation (r = 0.19 P = 0.007) between sensitivity to propamocarb-HCl and geographic distance (Table 1). This correlation was confirmed by a chi-squared test among the two major sampling regions. The distribution of the EC_{50} values varied with region (χ^2 = 5.1, P = 0.025), and isolates from the South region were less sensitive to propamocarb than isolates from Southeast (Fig. 4). In the South region, 36% of the isolates had EC_{50} values greater than 10 µg mL^{-1}, five times the frequency detected in the Southeast region (7.1%) for the same class. The frequencies of potato and tomato isolates in the class of EC_{50} < 10 µg mL^{-1} were 80.4% and 71.4%, respectively. The distribution of the EC_{50} values did not vary according to the host of origin of the isolates (χ^2 = 0.39, P = 0.533). Three different classes were detected in all regions and host of origin and the highest frequencies were detected for the class of EC_{50} less than 10 µg mL^{-1}.

Overall, for fenamidome, the EC_{50} values ranged from 0.02 to 1.27 µg mL^{-1}. Among the isolates, 95.2% had EC_{50} values below 1.0 µg mL^{-1} and 4.8% of the isolates had EC_{50} greater than 1.0 µg mL^{-1} (Fig. 3E). There was no correlation between sensitivity to fenamidone and geographic distance (Table 1). The distribution of the

EC_{50} values did not vary according to the host of origin of the isolates ($\chi^2 = 0.02$, $P = 0.914$). Two different classes were detected for all regions and host of origin, and for both, the highest frequencies were detected for the class of EC_{50} below 1 µg mL^{-1} of a.i. The frequencies of isolates with EC_{50} values below 1 µg mL^{-1} were 94.3% to potato and 96.1% to tomato originated isolates.

Sensitivity to CAA´s

The EC_{50} values for dimethomorph ranged from 0.03 to 1.46 µg of a.i mL^{-1}. and between 0.05 to 1.40 µg mL^{-1} to mandipropamid (Table 1). Most isolates (90.3%) had EC_{50} values lower than 1.0 µg mL^{-1}, and only 9.7% of the isolates had EC_{50} higher than 1.0 µg mL^{-1} of a.i. For mandipropamid the values and frequency on each class were very similar to those obtained in the dimetomorph experiment. Most isolates (93.6%) had EC_{50} below 1.0 µg mL^{-1} and 6.5% of the isolates had EC_{50} values greater than 1.0 µg mL^{-1} (Figs. 3D and F). There were no correlation between the EC_{50} values of the two CAA's and the geographic distance. Likewise, the distribution of the EC_{50} values did not vary according to the host of origin for either dimethomorph ($\chi^2 = 0.09$, $P = 0.77$) or mandipropamid ($\chi^2 = 0.02$, $P = 0.91$). Two different classes were detected for all regions and for the two host of origin. For both classes the EC_{50} values were below 10 µg mL^{-1} a.i. The frequencies of potato isolates with EC50 less than 1.0 µg mL^{-1} for dimethomorph and mandipropamid were 90.6% and 94%, respectively. All tomato isolates had $EC_{50} < 1.0$ µg mL^{-1} for both fungicides. No insensitivity to these two fungicides was detected.

Cross-sensitivity

The sensitivity of isolates to the protectant fungicides was positively correlated ($r = 0.3$, $P = 0.023$), indicating cross-sensitivity between chlorothalonil and mancozeb. However, no significant correlation was detected when the hypothesis of cross-sensitivity between unrelated fungicides was tested; i.e. the two protectant fungicides against the five systemic products, or between the different systemic compounds (data

not shown). Similar results were obtained when cross-sensitivity was examined for CAA fungicides mandipropamid and dimetomorph (r = 0.23, $P = 0.09$).

3.5. Discussion

The population of *P. infestans* in Brazil is sensitive to the protectant and most of the site-specific fungicides used to control late blight. Between the protectant fungicides, the EC50 values for mancozeb were higher than for chlorothalonil, but there was no evidence of a shift in the distribution of EC50 values when compared with previously published data (Reis et al. 2005). The variation factor for the sensitivity of the isolates to chlorothalonil was 40.8 and to mancozeb the variation was 71.8; similarly to values reported for mancozeb in North America (Sujkowski et al. 1995; Kato et al. 1997), and for both fungicides in Brazil (Reis et al. 2005). The effectiveness of both mancozeb and chlorothalonil was expected, since the multi-site target mode of action of these fungicides with a broad range of activity, makes the changes by mutation in independent genes that code unrelated proteins, a rare event. However, similar to what happens with other polygenic traits, such as quantitative resistance of plants to pathogens, mutations in multiple genes could occur over the time and a gradual decline in efficacy would be perceived (Gullino et al. 2010). To date, there is no evidence of such phenomenon occurring in population of fungal pathogens to either mancozeb or chlorothalonil.

Mancozeb and chlorothalonil are both considered as low risk fungicides for resistance development (Brent & Hollomon 2007). The positive correlation between the EC_{50} values for the two fungicides may indicate that sensitivity to these compounds can be linked. Resistance to multiple compounds, like multi-site fungicides, is in general explained by the increase in the activity of the ABC transporters (Stergiopoulos et al. 2003) or by the over production of thiols which detoxify the fungicides (Leroux et al. 2002). These mechanisms can be considered as general response of fungal cells to chemical intoxication (Latijnhouwers et al. 2003). This could explain why some isolates are less sensitive to two different compounds. Multi-drug resistance mediated by ABC transporter was described for *Aspergillus nidulans* (Andrade et al. 2000) and *Botrytis cinerea* (Hayashi et al. 2001) to systemic fungicides of the azole group.

The development of new fungicides and the reduced efficacy of phenylamide compounds contributed to the widespread usage of propamocarb, QoI's and, lately, the

CAA's derived products (Reis et al. et al. 2005; Günwald et al. 2006; Lehtinen et al. 2008). Associated with this change, there was an increase of the selective pressure by intensive usage of other “new” fungicides, propamocarb; fenamidone; dimethomorph; and mandipropamid. Fortunately, the isolates were sensitive to all systemic fungicides, including the propamocarb-HCl, for which the highest EC_{50} values were found. Nevertheless, because this is the first study that assessed the sensitivity of Brazilian isolates of *P. infestans* to these four systemic fungicides, no previous data are publicly available for comparisons. The values of EC50 to propamocarb-HCl were the highest among the systemic fungicides and the EC50 of 17.7% of the isolates was > 10 µg mL^{-1}. In a preliminary test some isolates grew in medium amended with 100 µg of propamocarb mL^{-1}, so an extra dose (1000 µg mL^{-1}) was included in the tests. Eight isolates of the 68 evaluated were able to grow at 1000 µg mL^{-1}. Relatively high EC_{50} values (> 10 µg mL^{-1} of a.i.) were already reported for isolates from Nordic countries (Lehtinen et al. 2008) and in Mexico (Grunwald et al. 2006). However, these isolates were not considered as resistant (Grunwald et al. 2006; Lehtinen et al. 2008). When the isolates were compared with the baseline in Nordic countries the range of EC_{50} was not different from that of the baseline isolates (Lehtinen et al. 2008). For the Mexican isolates, when the exposed population was compared to the baseline, there was a reduction in the frequency of isolates with EC_{50} above 3 µg mL^{-1} of a.i.. Furthermore, some isolates grew and sporulated at high fungicide concentration, but could not infect potato leaves. Despite the lack of a baseline population, it is not possible to ascertain the presence of resistant isolates, but our results indicates that the excessive use of this molecule could lead to field resistance.

The reasons for the higher frequency of isolates with high EC_{50} values in the South of Brazil cannot be properly explained at this point. However, evidence of sexual recombination events were recently detected in this region (Oliveira et al. unpublished data), and this could have contributed to the emergence of less sensitive isolates. In addition, potato and tomato crops are extensively cultivated in the South region and the favorable environment plus the constant selective pressure by fungicides applications could explain the differential sensitivity to propamocarb-HCl. This observation strengthens the need to implement a policy of frequent monitoring of pathogen population regarding fungicide sensitivity. Effective use of fungicides is an economical and an ecological issue. The current concerns related to the environmental impacts caused by the Brazilian agriculture demand for scientifically-based remediation actions, which in turn depend on information generated by population monitoring.

There was no evidence for insensitivity to fenamidone. The target site of this compound is the ubiquinol oxidation center (Qo site) of the cytochrome bc1 complex, and the inhibition occurs because the QoI binds to the Qo center affecting the respiratory chain. Resistance to QoI in the oomycete *Plasmopara viticola*, causal agent of the grape downy mildew, was detected in 2000 (Genet et al. 2006; Gisi & Sierotzki 2008). But to date, there are no reports of resistance of *P. infestans* to this group of fungicides. However, following the FRAC guidelines for the use of QoI fungicides (mixing and alternating fungicides of different groups) in programs aimed at managing late blight is advisable. The population of *P. infestans* is highly sensitive to CAA's fungicides. The dimethomorph is a CAA fungicide that has been frequently used to control late blight in the past 10 years in Brazil. Otherwise, mandipropamid is a recently developed fungicide (Harp et al. 2006). Cross resistance in other oomycetes among CAA fungicides is common (Gisi et al. 2007; Gisi & Sierotzki 2008). Values of EC_{50} of the isolates collected in Toluca Valley were similar, with no more than 7% of the isolates with EC_{50} values above 1 μg mL^{-1} (Günwald et al. 2006). The EC50 values to mandipropamid in the Brazilian population were similar to those estimated for an exposed European and for the Israeli baseline populations (Cohen et al. 2007). There is no known case of field resistance of *P. infestans* to CAA's, but it has been demonstrated that mutants resistant to the fungicides can be generated under laboratory conditions (Rubin et al. 2008; Stein & Kirk 2004). Apparently, there is a high cost in fitness associated with resistance to CAA compounds and this can explain the absence of field resistance in populations of *P. infestans* (Stein & Kirk 2004). Similarly to the case of QoI, resistance to the CAA's was found in populations of *P. viticola* (Gisi et al. 2007). The resistance was assumed to be due to two recessive nuclear alleles that were fixed in the population of *P. viticola* (Gisi et al. 2007). It was assumed that resistance of *P. infestans* to CAA's could be under similar genetic control. Three reasons were considered for the absence of field resistance: (i) the need for mutation to occur in many sites, since one or more loci are possibly involved in resistance; (ii) the resistance in *P. infestans* could be lethal or associated with a fitness cost (trade-offs), and (iii) if resistance is due to two recessive genes, recombination is needed to produce isolates with recessive genes in homozygosis (Rubin et al. 2008).

Recent studies reported that the Brazilian populations changed and that host specificity is no longer as strong as it used to be. Hence isolates from tomato and potato do not differ regarding aggressiveness when inoculated in leaflet (Oliveira et al., unpublished data). In the work carried out by Reis et al. (2005), no evidence of

association between sensitivity and mating type (A1 or A2) or between sensitivity and host of origin of the isolates were found. In some places, the sensitivity and resistance to phenylamides was linked with the *P. infestans* clonal lineages and/or mating type (Fry & Goodwin 1997). However, the changes in the Brazilian population could lead to emergence of new strains least sensitive to site-specific compounds and no association of fungicide sensitivity with mating type.

The changes in the population with new variants in some potato fields, suggests the involvement of sexual recombination in the South region (Oliveira et al. unpublished). Together with the putative mixed reproduction model, sexual reproduction increases the risks of emergence of resistant isolates of *P. infestans* to fungicides. The expected risk of developing fungicide resistance is determined by the evolutionary potential of the *P. infestans* population. Thus, the constant monitoring of changes in the population structure including the sensitivity to fungicides are key components for effective late blight management (Cooke et al. 2003; Lees et al. 2006; Russell 2005). Monitoring is particularly required in areas of highly favorable environmental conditions and where year-round crops can be harvested. Most potato and tomato producing regions in Brazil fit into this scenario. It is claimed that the contribution of fungicide resistance monitoring in tropical areas is expected to be more relevant to late blight management than in temperate regions.

The results reported here are of great interest to plant pathologists, extension agents, farmers and the industry sector. Changes in the management of late blight should be considered, hence isolates able to grow at high concentration of propamocarb are present in the population and the excessive use of this fungicide could enhance the selection for resistant individuals. Management strategies that avoid resistance selection are important because: (i) the control of late blight is heavily dependent on fungicides application, (ii) *P. infestans* is a high-risk pathogen, with high evolutionary potential for development of fungicide resistance, and (iii) production of both crops are very expensive. Rotation of systemic fungicides from different chemical groups and mode of action and the application associated with protectant fungicides should always be practiced. The industry should consider the development of systemic fungicides already formulated with with a multi-site product or with a distinct site-specific molecule. Monitoring activities must be carried often to assess changes in fungicide sensitivity and avoid the emergence of field resistance.

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3.7. References

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Fig. 1. Map showing the collection fields on different regions of Brazil. Brazilian states names are in bold.

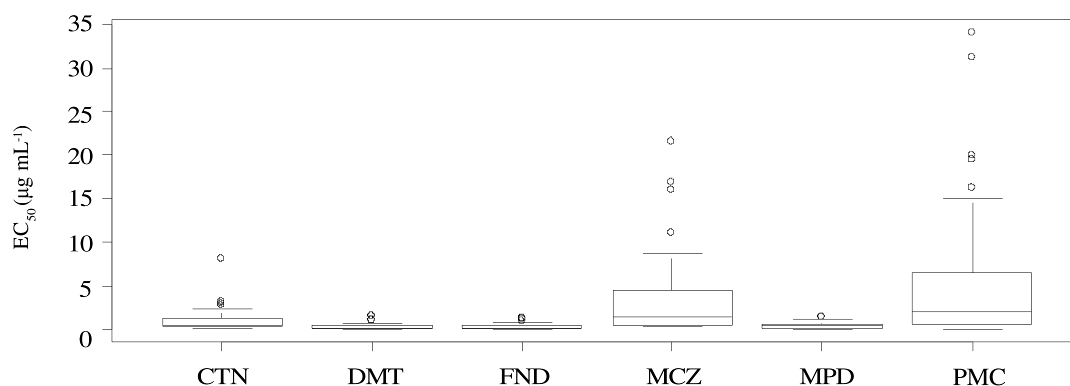

Fig. 2. Box-plots of EC_{50} values of 62 isolates of *Phytophthora infestans* to the protectant fungicides Chlorothalonil (CTN) and Mancozeb (MCZ), carbamate fungicide Propamocarb (PMC), the QoI fungicide Fenamidone (FND), and CAA's fungicides Dimethomorph (DMT) and Mandipropamid (MPD).

Table 1. Maximum, minimum, mean and standard deviation (SD), and the median values of the EC_{50} for isolates of *Phytophthora infestans* and the results of the Mantel's test of correlation between geographic distance and mean EC_{50} values.

	EC_{50} values (µg mL-1)				Mantel Test (EC_{50} Vs Geographic distance)	
	Minimum	Maximum	Mean±SD	EC_{50} Max. (REF)[a]	*Obs*	*P (simulatedb*[a]
Chlorotalonil	0.20	8.16	0.89±1.21	1.47[1]	0.04	0.2468
Dimethomorph	0.03	1.46	0.32±0.35	0.80	0.04	0.2408
Fenamidone	0.02	1.27	0.33±0.31	NR[3]	0.04	0.2597
Mancozeb	0.30	21.54	3.19±4.33	25.7[4]	0.04	0.2478
Mandipropamid	0.05	1.40	0.43±0.63	2.98[5]	0.01	0.3696
Propamocarb	0.10	34.10	5.08±7.16	< 100[6]	0.19[**]	0.0040

[a]EC_{50} values used as references for the maximum values found in the literature. [b]*P* values simulated based on 1000 permutations. [1]Kato et al. 1997; [2]Stein et al. 2003; [3]no reference; [4]Reis et al. 2005; [5]Cohen et al. 2007; [6]In this work the author classified as sensitive isolates with EC_{50} below 100 µg mL^{-1} (Möller et al. 2009).

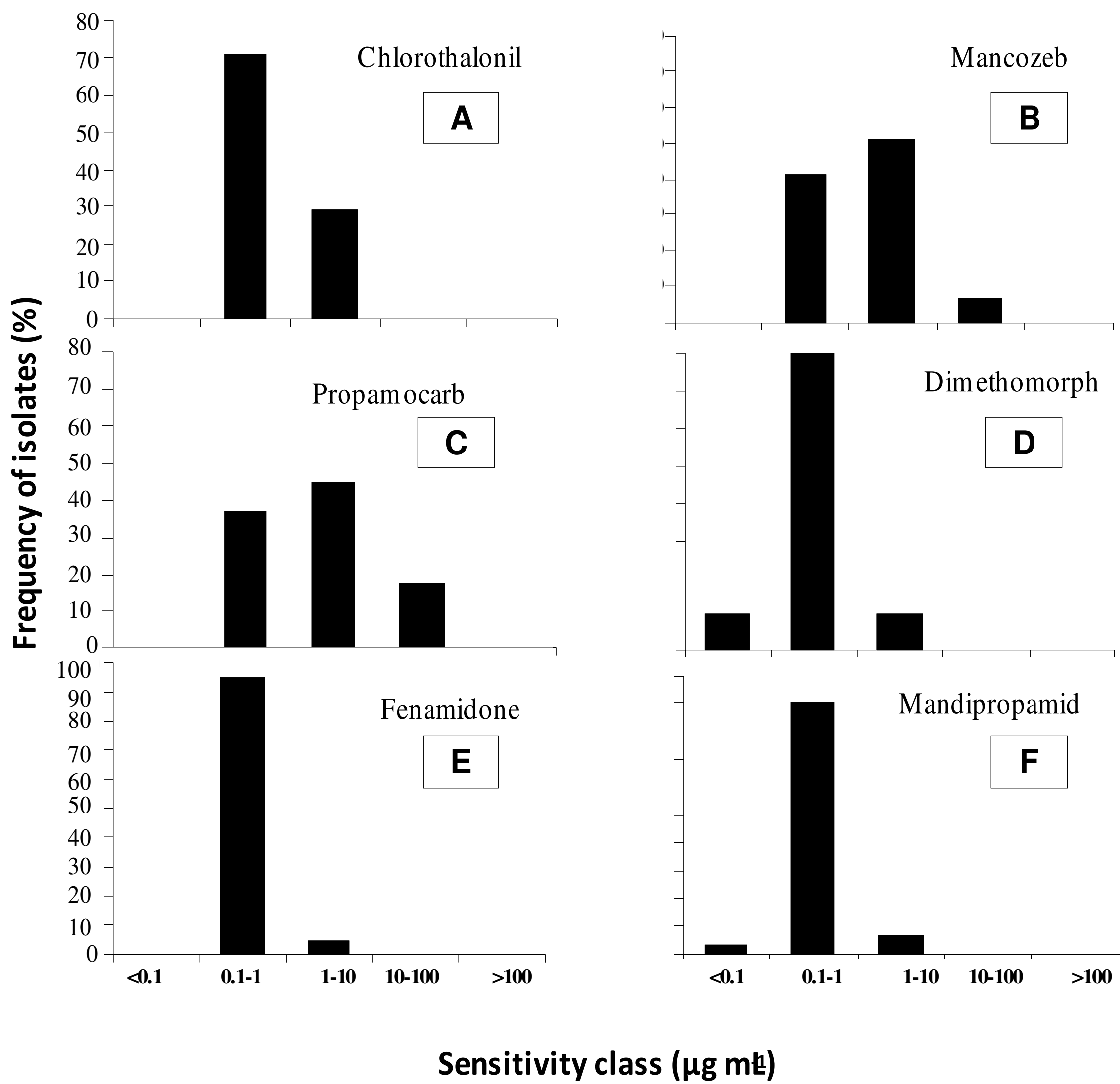

Fig 3. Frequency of isolates of *Phytophthora infestans* in different classes of EC_{50} values estimated for the fungicides: **A**, Chlorothalonil; **B**, Mancozeb; **C**, Propamocarb; **D**, Dimethomorph; **E**, Fenamidone; and **F**, Mandipropamid for 62 isolates.

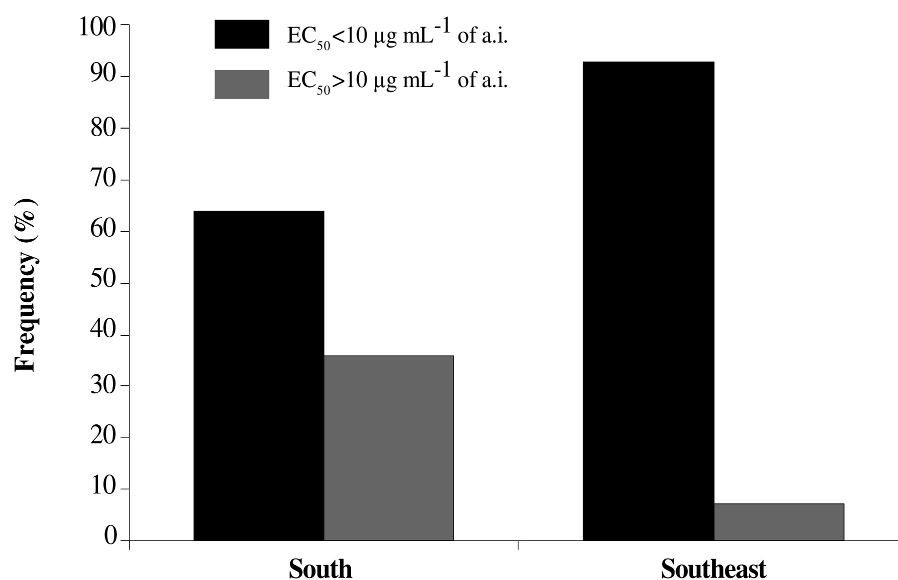

Fig. 4. Frequency of *Phytophthora infestans* isolates in two classes of EC_{50} values estimated for the fungicide propamocarb. Distribution of the isolates according to the sampling region.

CONCLUSÕES GERAIS

- As diferentes sub-populações de *Phytophthora infestans*, definidas com base em hospedeiro, mudaram ao longo dos anos.
- Existem evidências de ocorrência de recombinação sexual na Região Sul do Brasil no período de 2003/2005.
- A alta variabilidade da população brasileira de *Phytophthora infestans* em batateira é mantida por um alto fluxo gênico e raros eventos de recombinação sexual.
- Os componentes epidemiológicos diferiram em função do hospedeiro, exceto para LP-S e IF-S.
- Não houve diferença de agressividade entre isolados dos diferentes grupos quando inoculados em folíolo.
- Os isolados de batateira são mais agressivos que os de tomateiro, quando inoculados em haste.
- Não há evidência de especificidade por hospedeiro.
- Não há evidência de insensibilidade aos fungicidas protetores mancozeb e clorotalonil.
- Há evidência de diminuição da sensibilidade de isolados de *P. infestans* ao fungicida propamocarb na Região Sul do Brasil.
- Não há evidências de insensibilidade aos fungicidas sistêmicos do grupo dos CAA’s, dimetomorfe e mandipropamide, e ao QoI fenamidone.