ZOILA ROSA MAGNOLIA SANTA CRUZ CRUZADO

VIABILIDADE DE OÓSPOROS ORIUNDOS DE CRUZAMENTOS DE ISOLADOS BRASILEIROS DE *Phytophthora infestans*

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

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(Presidente da Banca)

Sem a força de Deus não teria tido a coragem de enfrentar tudo

Em memória do meu tio Julio Ugás Chamochumbi

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BIOGRAFIA

Zoila Rosa Magnolia Santa Cruz Cruzado nasceu em Lima, Peru em 22 de Março de 1966. Filha de Jorge Diógenes Santa Cruz Díaz, jornalista, e de Rosa María Cruzado Pacheco, secretária e ama de casa.

Magnolia obteve o título de Bióloga na "Universidad Nacional Pedro Ruiz Gallo" em Lambayeque, Peru, em 1994. No mesmo ano, ela começou trabalhar como assistente de pesquisa no Laboratório de Virologia no Centro Internacional de la Papa (CIP-Lima, Peru), onde tinha também desenvolvido a tese de bacharelado. Seis anos depois, foi transferida ao Laboratório de Patologia Molecular, no mesmo centro de pesquisa, e ai ela trabalhou até Fevereiro de 2004.

Em Março de 2004, Magnolia iniciou o curso de Mestrado em Fitopatologia na Universidade Federal de Viçosa em Minas Gerais, Brasil, para obter o título *Magister Scientiae*.

CONTEÚDO

RESUMO

SANTA CRUZ CRUZADO, Zoila Rosa Magnolia. M.S. Universidade Federal de Viçosa. Agosto de 2006. **Viabilidade de oósporos oriundos de cruzamentos de isolados brasileiros de *Phytophthora infestans*.** Orientador: Eduardo Seiti Gomide Mizubuti. Co-Orientadores: Luiz Antonio Maffia e Robert Weingart Barreto.

Isolados de *Phytophthora infestans* foram obtidos de folhas de batata e tomate e frutos de tomate coletados das regiões Sul, Sudeste, Nordeste e Centro Oeste do Brasil. Onze isolados do grupo de compatibilidade A1 e quatro do A2 foram pareados em meio de cultura, para avaliar a produção de oósporos. Após quantificação de oósporos resultantes de cada pareamento, duas variáveis, número de oósporos determinado em meio de cultura (NOMC) e número de oósporos extraídos do meio (NOEXT), foram submetidas à análise de dialelos parciais, com apenas F_1, para estimar possíveis diferenças quanto à fecundidade dos cruzamentos realizados. Avaliaram-se a viabilidade e germinação *in vitro* de oósporos extraídos do meio de cultura. Avaliou-se a viabilidade por meio do teste de plasmólise em NaCl e a produção de tubo germinativo foi determinada em ágar-água. Em todos os cruzamentos houve produção de oósporos. O número de oósporos variou de 233 a 3900/ml. As capacidades geral (CGC) e específica (CEC) de combinação variaram entre os isolados e foram significativas ($P < 0,0001$). Para NOMC e NOEXT, o isolado do grupo de compatibilidade A2 com maior valor de CGC foi o Pib-750. Do grupo A1, os valores mais altos de CGC foram para os isolados Pib-754 e Pib-742, com base em NOMC e NOEXT, respectivamente. Baseados nos valores de CEC, a combinação mais adequada para produzir oósporos foi Pib-731 x Pib-755, para ambas as variáveis. Não houve tendência efeito de proximidade geográfica sobre a fecundidade dos cruzamentos. Embora os cruzamentos tenham produzido oósporos, suas taxas de viabilidade e germinação foram baixas, em média, 0,3% e 0,03%, respectivamente. Apesar da baixa viabilidade, a produção de oósporos pode constituir-se em ameaça para as culturas de batata e tomate como fonte de variabilidade genética de *P. infestans*.

ABSTRACT

SANTA CRUZ CRUZADO, Zoila Rosa Magnolia. M.S. Universidade Federal de Viçosa. August, 2006. **Viability of oospores originated from crosses of Brazilian isolates of *Phytophthora infestans*.** Adviser: Eduardo Seiti Gomide Mizubuti. Co-Advisers: Luiz Antonio Maffia and Robert Weingart Barreto.

Phytophthora infestans isolates were obtained from infected potato or tomato leaves and tomato fruits collected from the South, Southeast, Northeast, and Central-West regions in Brazil. Eleven isolates of the A1 mating type and four of the A2 were paired in culture media to assess production of oospores. After quantifying the number of oospores obtained from each pairing, two variables, number of oospores produced in culture medium (NOCM) and number of oospores extracted from the medium (NOEXT), were subjected to a partial diallel analysis with F_1's only, in order to estimate the fecundity of the crosses. Oospore viability was estimated with a plasmolysis in NaCl test and germ tube formation was determined in water agar. All crosses produced oospores. The number of oospores varied among the crosses and ranged from 233 to 3900/ml. The general combination ability (GCA) and specific combination ability (SCA) varied among isolates and were significant ($P < 0.0001$). For both NOCM and NOEXT the A2 mating type isolate with the highest GCA value was Pib-750. Among the A1 mating type isolates, highest GCA values were estimated for Pib-754 and Pib-742, based on the NOCM and NOEXT, respectively. Based on SCA, the best isolate combination for oospores production was between Pib-731 x Pib-755, for both variables. There was no clear trend regarding a possible geographical proximity effect on the fecundity of crosses. Although the crosses produced oospores, viability and germination rates were low, on average 0.3% to 0.03%, respectively. Despite the low viability, production of oospores can be a threat to potato and tomato crops as a source of genetic diversity for this pathogen.

1. INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fourth most important food crop and the vegetable most widely cultivated in the world, reaching an annual production of 300 million tons (CIP, 2002) from 19 million ha spread in all continents (FAO, 2002). Tomato (*Solanum lycopersicum* L. = *Lycopersicon esculentum* Mill.) is the second most important vegetable crop in the world with approximately 100 million tons of fresh fruit produced from 3.7 million ha (FAO, 2002). The two species are hosts of *Phytophthora infestans* (Mont.) de Bary, an oomycete that causes late blight, the most devastating disease of both crops worldwide (Groves & Ristaino, 2000; Mizubuti, 2001). Since the Irish famine in 1845, late blight has conveyed considerable efforts aimed at understanding disease development and control. Nevertheless, nowadays late blight still results in yearly losses of around 5 billions of dollars (Judelson & Blanco, 2005). Because of the destructive epidemics that may occur under suitable environmental conditions (mild temperature and high humidity) intensive chemical sprays programs are often used in potato and tomato crops. The ultimate goal of many research programs is to reduce fungicide usage and relieve the downside aspects of the intensive use of chemicals.

Rational usage of fungicides to control late blight can be achieved with epidemiological studies conducted under a holistic approach which involves studies of the ecological and genetic factors that affect epidemics (Milgroom & Peever, 2003; Mizubuti & Fry, 2006). Knowledge about the biology and ecology of *P. infestans* is crucial, because of the influence of these aspects on the dynamics of pathogen populations. Monitoring the evolution of pathogen populations is important to support breeding programs oriented towards host resistance and proper fungicide management. Changes of characteristics in a population are due to evolutionary mechanisms. In *P. infestans* populations, selection (Gavino *et al.*, 2000; Montarry *et al.*, 2006), mutation or migration (Fry & Goodwin, 1997; Goodwin *et al.*, 1998) and sexual reproduction (Gavino *et al.*, 2000) have been documented. The role of sexual reproduction has raised interest in the past years due to its potential impact on both evolution of populations of *P. infestans* and late blight management (Drenth *et al.*, 1995; Elliot, 1996; Cohen *et al.*,

1997; Mayton *et al.*, 2000; Flier *et al.*, 2001; Hammi *et al.*, 2001; Levin *et al.*, 2001; Knapova *et al.*, 2002; Fernández-Pavia *et al.*, 2004). Sexual reproduction in most organisms is a widespread phenomenon that promotes genetic variability and thus, the rapid advance of evolution (Barton & Charlesworth, 1998).

Phytophthora infestans is a heterothallic species that reproduces either sexual or asexually. Individuals of the mating types A1 and A2 must be paired for sexual reproduction to take place (Erwin & Ribeiro, 1996; Goodwin & Drenth, 1997). Isolates of A1 and A2 mating types can produce antheridia and oogonia, that can act as male or female to produce oospores, the spores of sexual origin (Judelson, 1997). The oospore is thick walled (1-3μm) with an external five-layer side and an internal thicker and electron transparent layer. Oospores mature at about 30 days. When mature, viable oospores germinate and one or more germinative tubes emerge from any part of the spore. Germ tubes can turn into mycelium or produce a terminal sporangium which releases zoospores that swim and infect host tissues (Erwin & Ribeiro, 1996).

Production of viable oospores has important evolutionary and epidemiological implications. Studies on oospore biology started at nearly 50 years ago when they were first reported from the Toluca Valley, Central Mexico, where oospores can be found in infected plants and in infested soil (Gallegly & Galindo, 1958). Oospores are considered a threat to crops because they can overwinter and be a source of inoculum and sexual recombination can originate more aggressive lineages (Fry & Goodwin, 1997; Flier *et al.*, 2002). Due to its thick outer wall, oospores are better adapted to survive long periods in the soil (Flier *et al.*, 2002). In the Netherlands, oospores incorporated in sandy or clay soils survived for 48 or 34 months, respectively (Turkensteen *et al.*, 2000), while in Mexico viable oospores were observed for up to two years after potato harvesting (Fernández-Pavia *et al.*, 2004). In India, viability of oospores buried in soil in two different regions with temperatures of -5°C to 44°C decreased with time, but germination was detected in both regions for more than 5 months and lesions were found at the base of the stems of potato plants cultivated in infested soils (Singh *et al.*, 2004). Oospore survival capacity also affects evolution of *P. infestans* populations by reducing the "bottleneck effects"(Goodwin, 1997), i.e. by lessening the effects of random genetic

drift caused by extreme population reduction during the crop-free season (Flier *et al.*, 2002). In this way, they prevent drastic reduction of genetic variability.

Sexual reproduction can contribute to the formation of a more genetically diverse population in which variation in disease intensity capacity can result. Recombination might produce individuals with adaptability and virulence factors that are different from those of either parent (Mayton *et al.*, 2000). In the United States, it has been found that more recent populations have greater diversity and are the result of sexual recombination, as suggested by a large set of markers (Gavino *et al.*, 2000). Israeli populations of *P. infestans* have been changing through the last two decades and one of those changes was the increase of aggressiveness to tomato, probably due to recombination events (Cohen, 2002).

In Brazil, tomato and potato are mostly grown in the central and southern regions. The first studies aimed at determining the occurrence of the two mating types were in the 1950's and only isolates of mating type A1 were found in potato and tomato (Ciccarone *et al.*, 1959). In 1988, isolates of the A2 mating type were reported for the first time in Brazil (Brommonschenkel, 1988). Since then, isolates of the A1 and A2 mating types causing late blight epidemics in tomato and potato fields, respectively, have been reported (Reis *et al.*, 2003). Although isolates of both mating types can be found throughout Brazil, the population of *P. infestans* appears to remain clonal and host specific (Reis *et al.*, 2003; Suassuna *et al.*, 2004). BR-1 is a clonal lineage comprised of isolates of the A2 mating type and better adapted to potato whereas US-1 is characterized by isolates of the A1 mating type more adapted to tomato (Reis *et al.*, 2003; Suassuna *et al.*, 2004). Even in regions where both crops are largely cultivated in close proximity and in spite of the presence of the two mating types, no oospores have been observed in natural conditions.

Reis *et al*. (2003) raised issues to explain the absence of sexual reproduction in Brazil: 1. reproductive isolation, because of the host specificity; 2. production of non-viable oospores; 3. low fitness of the progeny - crosses between A1 and A2 would occur in nature, fertile oospores would be produced, but the resulting progeny would be less adapted than the parents; and 4. environmental conditions inadequate for formation and/or germination of oospores. The first hypothesis was tested, and host specificity was

clarified as important (Suassuna *et al.*, 2004). Nevertheless, this can not fully explain the apparent absence of sexual reproduction since A1 isolates can infect potato and A2 isolates can infect tomato. Studies to test the other three hypotheses are necessary. Therefore, the objectives of this study were: 1) to assess the production of oospores *in vitro*; 2) to test the viability of these oospores; and 3) to test their germination under controlled conditions.

2. MATERIALS AND METHODS

2.1. Isolation of *P. infestans*

Samples of leaves and/or fruits of tomato and leaves of potato with typical symptoms of late blight were collected from different regions in Brazil (Table 1). Samples were placed in paper bags and kept in a cooler until being processed. Humid chambers were prepared by using plastic trays covered with plastic bags or in plastic boxes (11 cm width x 11 cm length x 3.5 cm high-gerbox). A piece (10 x 10 cm) of 1 cm-thick foam soaked with distilled water was placed at the bottom of the trays or gerbox. Diseased leaves were placed in humid chambers with the abaxial surface facing up and tomato fruits were thoroughly washed and disinfested with NaClO 2% before placed in the chambers. All samples were kept in growth chamber at 18°C with 12h-photoperiod for 3 to 7 days or until a sporulating lesion was detected. Isolation of *P. infestans* from tomato fruits was accomplished by directly removing pathogen structures from sporulating lesions to Rye B agar (Caten & Jinks, 1968), Rye flour agar (Ribeiro *et al.*, 2000) or V8 agar (Miller, 1955) in Petri dishes. Sporulation on leaves was not as abundant as on fruits, thus diseased leaves were transferred to 50 to 100 ml distilled water in 250 ml Erlenmeyer flasks that were shaked to dislodge sporangia. Inoculum suspension was kept for 1 to 2 h at 4°C to promote formation of zoospores. Asymptomatic tomato (cv Santa Clara) or potato (cv Bintje) leaflets detached from greenhouse-grown plants were placed in humid chambers (two or three leaflets/gerbox) and were inoculated with 700 µl of the inoculum suspension from sporangia removed from their respective originally infected host. Incubation was at 18°C with a 12h light

regime for 3 to 7 days. Sporangia produced on the inoculated leaflets were then transferred to culture media in Petri dishes and incubated as previously mentioned.

Four isolates from tomato and five from potato were provided by Dr. Ailton Reis (CNPH – EMBRAPA) and Dr. César Bauer (CPACT - EMBRAPA). One isolate from tomato (Pib-732) and one of potato (Pib-731) from the collection of the Laboratório de Epidemiologia and previously characterized as A1 and A2, respectively, were also included as testers.

2.2. Production of oospores *in vitro*

To determine the mating type of isolates, each isolate was crossed with the tester isolates, Pib-732 and Pib-731 (Table 1) on clarified V8 10% agar (V8c) (Shattock *et al.*, 1990). To facilitate the extraction of oospores, a lower agar concentration (1%) medium was used. Strips of medium (3 x 0.5 cm) with mycelium of each isolate placed parallel to each other on the surface of the medium in each plate were used to initiate the crosses. Each plate contained one strip from the tester (A1 or A2) and one from the isolate to be identified, placed 3-4 cm apart. Plates were kept at 18°C, in the dark, for 4 to 6 weeks. Production of oospores was assessed microscopically (100X) and an area of 3 cm (length) x 0.5 cm (width) in the center of the plate, between A1 and A2 colonies, was delimited to count the number of oospores produced per plate. The experiment was set in a completely randomized design with three replications (one plate = one experimental unit).

Eleven A1 and four A2 isolates were paired to produce oospores. Selection of these isolates was based in such way that representative isolates from each of the two most important potato and tomato producing regions in Brazil (South and Southeast), were evaluated (Table 1). Counting of the oospores produced on plates was carried out in the region of the colony with the highest density of spores.

2.3. Extraction and germination of oospores

Oospores from the crosses established in 2.2 were extracted and their germination was assessed. A fragment of culture medium within the 1.5 cm^2 area delimited to count the oospores was removed from each plate through a sterilized scalpel. Each fragment was placed in 7 ml of sterilized milli-Q water (SMW) in a glass bottle. Samples were grinded in a homogenizer (Polytron® Model PT 10/35 Brinkmann Instruments, Co.) at 4000 rpm for 2 min (Pittis & Shattock, 1994). At the interval processing of each sample, the blades and the tube of the homogenizer were cleaned using 70% alcohol and rinsed with SMW to avoid cross-contamination. Oospore suspensions obtained were frozen at -20°C for 24 h and when necessary, defrosted at room temperature. An additional treatment with lysing enzymes (SIGMA L1412-10G Lysing Enzymes from *Trichoderma harzianum* 104K1216) was applied to eliminate mycelium and sporangia that remained after grinding. Preliminary tests were carried out to determine the best conditions for the enzyme treatment. Three temperatures (25, 30, and 35°C), three times of incubation in water bath (12, 24, and 36 h) and three enzyme concentrations (1, 2, and 3 mg/ml) were tested. The combination of 30°C - 24 h - 2mg/ml was considered the most suitable. After treatment with lysing enzymes, samples were poured in 1.5 ml Eppendorf tubes and centrifuged 3 times at 3000 g x 3 min to remove excess of enzyme (Pittis & Shattock, 1994). The supernatant (approximately 2/3 of the volume) was gently poured off and the pellet was resuspended in 300µl of SMW. Final suspensions were stored at 4°C. The numbers of oospores per ml of suspension and per plate, were estimated by placing 10µl of the final suspension on a glass slide and counting under a microscope (100X).

From each suspension, an aliquot of 50-500µl (depending on the concentration) containing at least 100 oospores was pipetted to each of three Petri plates (60 x 12mm) containing water agar (1.5% w/v) amended with ampicillin (100mg/l) (Mayton *et al.*, 2000; Knapova *et al.*, 2002). Plates were arranged in trays and kept in a growth chamber at 20°C (±2°C) at approximately 50 cm underneath blue light lamps (Sylvania azul F20W T12 and F40W T12). Germination was assessed under a microscope (100X) two

weeks later and then weekly until the 6th or 8th week (Pittis & Shattock, 1994; Mayton *et al.*, 2000).

In preliminary experiments, the relationship between counting of five microscopical fields and the total number of oospores in the 3 x 0.5 cm fragment was established. As the correlation between the number of oospores found in five fields of observation and the total number in the fragment was significant ($r = 0.87$, $P = 0.0001$), an equation was derived to estimate total number of oospores in a 3 x 0.5 cm fragment (TN) based on the counting of five microscopical fields (N5c) in which: TN = 63.88 + 7.31 (N5c).

2.4. Test of viability of oospores

Viability of oospores was assessed with a plasmolysis test as described by Jiang & Erwin (1990). From the suspension obtained in 2.3, an aliquot of 50-100 µl (depending on the concentration) containing at least 100 oospores was placed in the center of an excavated plate and, 4 to 5 min later, 2 volumes of 4M NaCl were added. After 30-60 minutes, the number of plasmolyzed oospores were determined by examination under a microscope (100X) (Jiang & Erwin, 1990; Mayton *et al.*, 2000). A plate containing a suspension of oospores killed in boiling water during 10 min was added to the test as negative control.

2.5. Statistical analysis

Correlation analysis was carried out to compare production of oospores per plate and number obtained after extraction. This analysis was performed with The SAS System (version 8.0). To test whether crosses varied according to the different combination of parent isolates, a partial diallel analysis was conducted. Isolates were divided in two groups according to the mating types and the Griffing's model for partial diallel cross analysis was fitted to the data (Cruz & Regazzi, 1997; Sales *et al.*, 2003). Because of the inherent nature of the mating system, only the F_1's were analyzed for the number of oospores produced in culture and the number of oospores extracted.

Assessments of the magnitude of the number of oospores either in culture or extracted were estimated based on the general combining ability (GCA) and the specific combining ability (SCA) using the "method for partial dialleles with only F_1's progeny" (Cruz & Regazzi, 1997). The GCA estimates the ability of the parents to form a base progeny. Parents (individuals) with high values of GCA can be chosen as they are, overall, genetically superior. The SCA is useful to select a specific pair of parents, which is considered the best combination among the ones assessed. Selection of a combination (a pair of individuals) with high SCA can be used to obtain superior hybrids (Cruz & Regazzi, 1997; Sales *et al.*, 2003). A proper version of the Griffing's model was fit to describe experimental observations: $Yij = \mu + g_i + g'_j + s_{ij} + \varepsilon_{ij}$; where Yij is the average number of oospores produced in culture medium or extracted, μ is the overall mean, g_i is the GCA effect for A1 isolates, g'_j is the GCA effect for A2 isolates, s_{ij} is the SCA effect between A1 and A2 isolates, and ε_{ij} is the experimental error. The diallel model was fitted using the software GENES (Cruz, 2001).

3. RESULTS

3.1. Isolation of *P. infestans*

Fourteen isolates from tomato and two from potato from different geographical areas were obtained from the collected samples (Table 1).

Table 1. Characteristics of isolates of *Phytophthora infestans* used in this study. Isolates in bold were selected for the tests

Code	Host	Origin City (State)	Geographical Region	Year	Mating Type
Pib732*	**Tomato**	**Ibiúna (SP)**	**Southeast**	**unknown**	**A1 (tester)**
Pib731*	**Potato**	**unknown (PR)**	**South**	**unknown**	**A2 (tester)**
Pib-733	Potato	Itaverava (MG)	Southeast	2005	nd
Pib-734	Tomato	Itaverava (MG)	Southeast	2005	A1
Pib-735	**Potato**	**Conselheiro Lafaiete (MG)**	**Southeast**	**2005**	**A1**
Pib-736	Tomato	Conselheiro Lafaiete (MG)	Southeast	2005	A1
Pib-737	Tomato	Ressaquinha (MG)	Southeast	2005	A1
Pib-738	**Tomato**	**Carandaí (MG)**	**Southeast**	**2005**	**A1**
Pib-739	Tomato	Ressaquinha (MG)	Southeast	2005	A1
Pib-740	Tomato	Carandaí (MG)	Southeast	2005	A1
Pib-741	Tomato	Coimbra (MG)	Southeast	2005	A1
Pib-749**	**Potato**	**Pouso Alegre (MG)**	**Southeast**	**2005**	**A2**
Pib-742	**Tomato**	**Ibiúna (SP)**	**Southeast**	**2005**	**A1**
Pib-750**	**Potato**	**Capão Bonito (SP)**	**Southeast**	**1997**	**A2**
Pib-743	**Tomato**	**Marechal Floriano (ES)**	**Southeast**	**2005**	**A1**
Pib-744	Tomato	Castelo (ES)	Southeast	2005	A1
Pib-745	**Tomato**	**Sumidouro (RJ)**	**Southeast**	**2005**	**A1**
Pib-746	**Tomato**	**Mucugê (BA)**	**Northeast**	**2005**	**A1**
Pib-747	Tomato	Mucugê (BA)	Northeast	2005	A1
Pib-748	Tomato	Mucugê (BA)	Northeast	2005	A1
Pib-754**	**Tomato**	**Tijucas (SC)**	**South**	**2005**	**A1**
Pib-755**	**Tomato**	**Florianópolis (SC)**	**South**	**2005**	**A1**
Pib-756**	**Tomato**	**Brasília (DF)**	**Central-West**	**2003**	**A1**
Pib-757**	**Tomato**	**Goianápolis (GO)**	**Central-West**	**2004**	**A1**
Pib-751**	Potato	Santa Maria (RGS)	South	2004	A1
Pib-752**	Potato	Ibiraiaras (RGS)	South	2005	A1
Pib-753**	**Potato**	**Castro (PR)**	**South**	**2004**	**A2**

nd = not determined; *from collection of the Laboratório de Epidemiologia do Departamento de Fitopatologia da UFV; ** gently provided by Dr. Ailton Reis and Dr. César Bauer (CNPH and CPACT – EMBRAPA, respectively)

3.2. Production and extraction of oospores

The Pearson correlation coefficient between the number of oospores counted in culture medium and extracted was 0.56 ($P < 0.0001$). All crosses between A1 and A2 isolates produced oospores. Most of the oospores were typical thick-walled and amber colored in culture media (Figure 1); oospores from some crosses looked darker. After extracted (Figure 2), some oospores looked abnormal (uncolored or transparent and/or distorted), but at low percentages (< 25%).

For oospores produced in culture medium, the GCA varied among isolates and was significant in both groups, as well as the SCA was significant (Table 2). Highest number of oospores in culture media was produced in cross Pib-749 x Pib-754 (mean = 2091 ± std. dev. = 343) and the lowest in the cross Pib-750 x Pib-757 (217 ± 19.1) (Table 3).

For number of oospores after extraction, the GCA varied among isolates and was highly significant in both groups (Table 4). The SCA was also significant (Table 4). The average number of oospores extracted varied from 233 oospores/ml (± 152.8) in cross Pib-753 x Pib-735 to 3900 oospores/ml (± 2381.2) in cross Pib-749 x Pib-743 (Table 5).

For both number of oospores produced and number of oospores extracted, the A2 isolate with highest GCA was Pib-750 (Tables 3 and 5). Among A1 isolates, the highest value of GCA for oospores produced in culture media was estimated for isolate Pib-754 (Table 3), whereas for number of extracted oospores, the highest GCA was estimated for isolate Pib-742 (Table 5). The cross with highest SCA was Pib-731 x Pib-755, for both variables (Tables 3 and 5).

Based on SCA, the least potentially useful crosses for oospore production after extraction were Pib-731 x Pib-745, Pib-750 x Pib-755, and Pib-753 x Pib-743.

When considering the average estimates of oospores produced, crosses involving parents from South x Northeast (973 oospores) and Southeast x Southeast (972), for number of oospores produced, and Southeast x Southeast (1800) for number of oospores extracted, had the highest values. The lowest value was for crosses made with parents from South x Central West regions (Table 6).

Figure 1. Oospores originated from crosses of Brazilian isolates of *Phytophthora infestans* formed in culture medium.

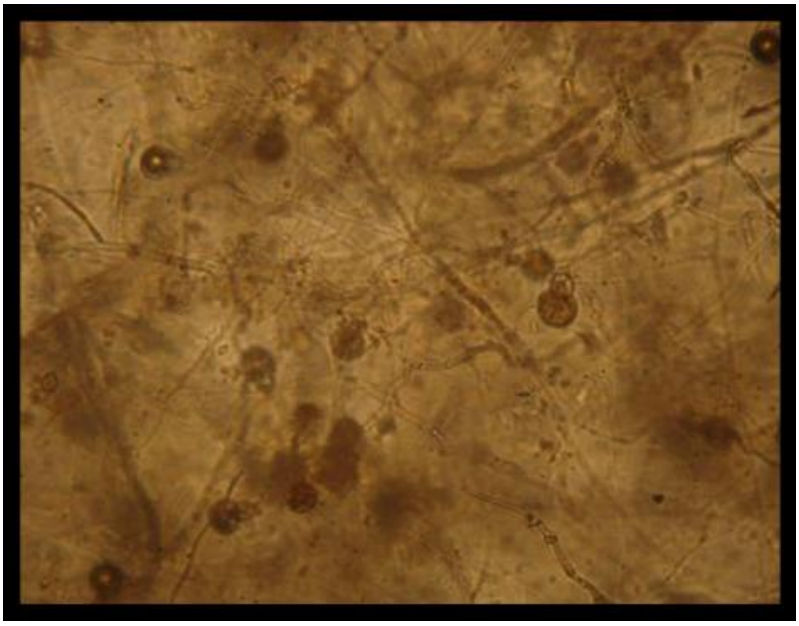

Figure 2. Oospores originated from crosses of Brazilian isolates of *Phytophthora infestans* after extraction from culture medium

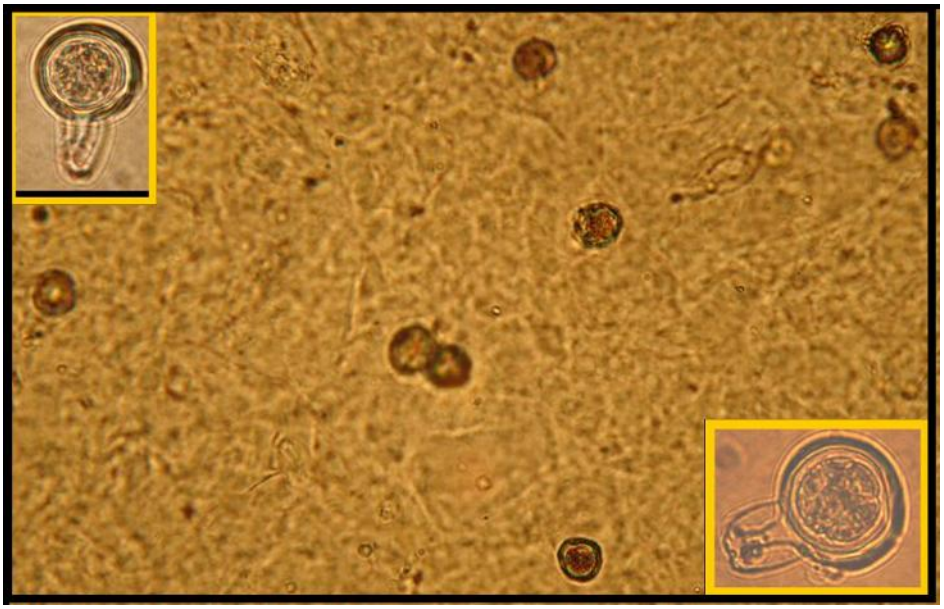

Table 2. Analysis of variance for the number of oospores produced in culture medium after crossing isolates of *Phytophthora infestans* of the two mating types groups (A2 group and A1 group). Estimates of the general (GCA) and specific combining ability (SCA) based on Griffing's partial diallel model for F_1's progenies only.

Source of variation	**DF**	**MS**	**F**	***Probability***
Crosses	43	846444.16	10.87	<0.0001
GCA - Group A2	3	1093432.59	14.05	<0.0001
GCA - Group A1	10	2496983.80	32.08	<0.0001
SCA Group A2 x A1	30	271565.43	3.49	<0.0001
Error	88	77815.0		

Table 3. Average number of oospores counted per plate for each cross of A1 and A2 isolates of *Phytophthora infestans*. Numbers in parenthesis indicate the general combining ability (GCA) of the isolate, and numbers in brackets indicate the specific combining ability (SCA) of a particular cross.

A1 isolates (group II)	A2 isolates (group I)			
	Pib-731 (110)	Pib-750 (127)	Pib-753 (-265)	Pib-749 (28)
Pib-743 (266)	1409 [159]	697 [-153]	512 [-301]	1153 [295]
Pib-757 (-561)	1360 [-110]	217 [-127]	424 [265]	465 [-28]
Pib-754 (427)	1257 [-148]	1411 [583]	770 [-450]	2091 [14]
Pib-746 (-561)	1136 [-110]	873 [-127]	809 [265]	729 [-28]
Pib-742 (411)	1101 [-34]	548 [-102]	1323 [-143]	853 [279]
Pib-735 (-561)	1097 [-110]	802 [-127]	261 [265]	1150 [-28]
Pib-755 (55)	1048 [633]	997 [-526]	563 [73]	1279 [-179]
Pib-732 (325)	975 [139]	1379 [-141]	619 [188]	868 [-186]
Pib-738 (360)	951 [-215]	1698 [555]	273 [-351]	1031 [11]
Pib-756 (-561)	817 [-110]	1604 [-127]	305 [265]	960 [-28]
Pib-745(399)	344 [-95]	378 [292]	729 [-76]	1011 [121]

Table 4. Analysis of variance for the number of oospores obtained after extraction from culture medium, from crossing isolates of *Phytophthora infestans* of the two mating types groups (A2 group and A1 group). Estimates of the general (GCA) and specific combining ability (SCA) based on Griffing's partial diallel model for F_1's progenies only.

Source of variation	**DF**	**MS**	**F**	***Probability***
Crosses	43	1932262.11	42005.69	<0.0001
GCA - Group A2	3	2263020.96	49196.10	<0.0001
GCA - Group A1	10	5197061.67	112979.60	<0.0001
SCA Group A2 x A1	30	810919.71	17628.68	<0.0001
Error	88	46.00		

Table 5. Average number of oospores of *Phytophthora infestans* counted after extraction from plates. Numbers in parenthesis indicate the general combining ability (GCA) of the isolate, while those in brackets indicate the specific combining ability (SCA) of a particular cross.

A1 isolates (group II)	A2 isolates (group I)			
	Pib-731 (58)	Pib-750 (221)	Pib-753 (-379)	Pib-749 (100)
Pib-743 (488)	2367 [676]	3267 [245]	300 [-654]	3900 [-267]
Pib-757 (-779)	1800 [-58]	333 [-221]	467 [379]	367 [-100]
Pib-754 (546)	933 [-16]	1667 [-146]	1467 [-579]	2033 [742]
Pib-746 (-779)	1500 [-58]	1000 [-221]	1467 [379]	2000 [-100]
Pib-742 (788)	1400 [-391]	600 [745]	2033 [-188]	2100 [-167]
Pib-735 (-779)	2000 [-58]	1733 [-221]	233 [379]	1100 [-100]
Pib-755 (-37)	1233 [1001]	2533 [-629]	1000 [104]	1500 [-475]
Pib-732 (713)	600 [-49]	2000 [-713]	500 [354]	1867 [408]
Pib-738 (154)	1367 [-291]	1400 [845]	367 [-188]	2167 [-367]
Pib-756 (-779)	700 [-58]	2000 [-221]	367 [379]	667 [-100]
Pib-745 (463)	333 [-699]	467 [537]	1933 [-363]	1000 [525]

Table 6. Number of oospores produced in culture media by crosses of Brazilian isolates of *Phytopthora infestans* and of extracted, averaged by region of origin of the isolates.

Region of A1 Isolates	Region of A2 Isolates			
	South		Southeast	
	Produced (oospores / 1.5 cm^2)	Extracted (oospores / ml)	Produced (oospores / 1.5 cm^2)	Extracted (oospores / ml)
South	910* ± 305	1158 ± 243	--	--
Southeast	961± 494	1323 ± 811	972 ± 305	1800 ± 1015
Northeast	973 ± 231	1483 ± 23	801 ± 102	1500 ± 707
Central-West	727 ± 476	833 ± 659	812 ± 612	842 ± 787

*average ± standard deviation

3.3. Oospore viability

Very low viability was recorded for most assessed oospores (Table 7). There was a low number of plasmolysed oospores (Figure 3). Oospore viability ranged from 0 to 1.3% across all crosses. From 44 crosses, 15 resulted in no plasmolysed oospores. Plasmolysed oospores were detected in all crosses between the A1 isolate Pib-755 and the four A2 isolates. Ten of 11 crosses between the isolate Pib-750 with A1 isolates resulted in viable oospores. All combinations, where both parents were from the South region, resulted in viability of at least 0.3% (Pib-731 x Pib-754, Pib-731 x Pib-755, Pib-753 x Pib-754, and Pib-753 x Pib-755).

Figure 3. Viable (plasmolysed = P) and non viable (no plasmolysed = NP) oospores after treatment with NaCl.

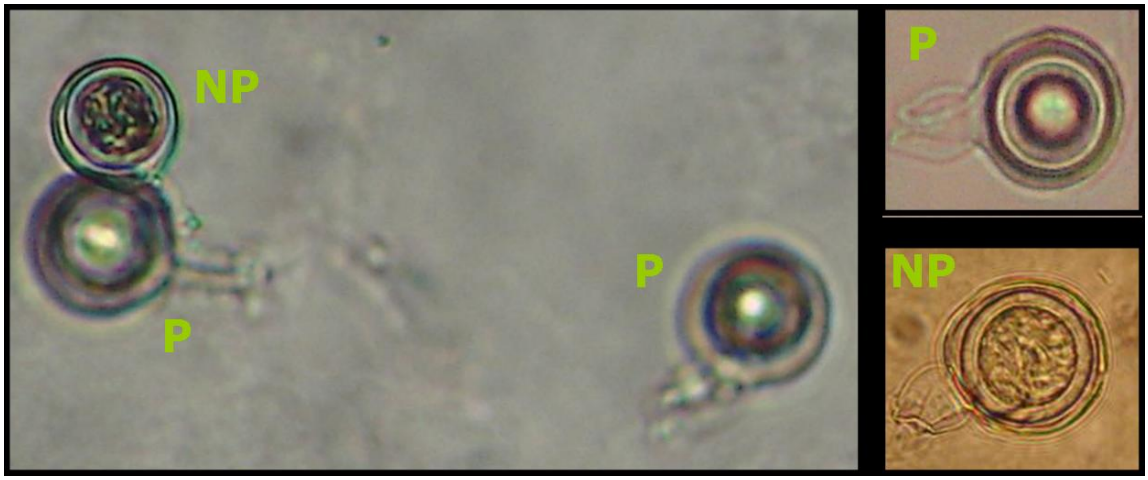

Table 7. Viability (%) of oospores from crosses of Brazilian isolates of *Phytophthora infestans*

A1 isolates	**A2 isolates**			
	Pib-731	Pib-749	Pib-750	Pib-753
Pib-732	0*	0.5 ± 0.4	0.3 ± 0.5	0
Pib-735	0	1.3 ± 0.3	0.3 ± 0.6	0
Pib-738	0.2 ± 0.4	0.2 ± 0.4	0.9 ± 0.9	0
Pib-742	0.3 ± 0.5	0	0.1 ± 0.2	0.3 ± 0.5
Pib-743	0.5 ± 0.4	0	0.3 ± 0.6	0.9 ± 0.9
Pib-745	0.3 ± 0.6	0.2 ± 0.4	0	0
Pib-746	0.6 ± 0.7	0	0.3 ± 0.5	0.3 ± 0.3
Pib-754	0.3 ± 0.5	0	1.2 ± 1.3	0.4 ± 0.8
Pib-755	0.3 ± 0.5	1.3 ± 0.4	0.8 ± 0.9	0.4 ± 0.7
Pib-756	0.3 ± 0.5	0	0.5 ± 0.5	0
Pib-757	0.3 ± 0.5	0	0.3 ± 0.5	0

*mean of three replicates ± standard deviation

3.4. Germination

For all crosses, oospore germination was low (Table 8; Figure 4). Germination rates varied from 0% to 0.25% (cross Pib-749 x Pib-742). Oospores from crosses involving isolates of the A1 mating type: Pib-732, Pib-735, Pib-745, Pib-746, and Pib-754, with any A2 isolates did not germinate. Oospores from crosses between the A1 isolate Pib-755 (from the South region) with Pib-749 (Southeast), Pib-750 (Southeast) and Pib-753 (South) germinated.

Only 17% of the oospores classified as viable by the plasmolysis test germinated, while 79% did not and 4% were not assessed because of contamination. Of the apparently non-viable oospores 27% germinated.

Figure 4. Germinated oospores of *P. infestans* in water agar

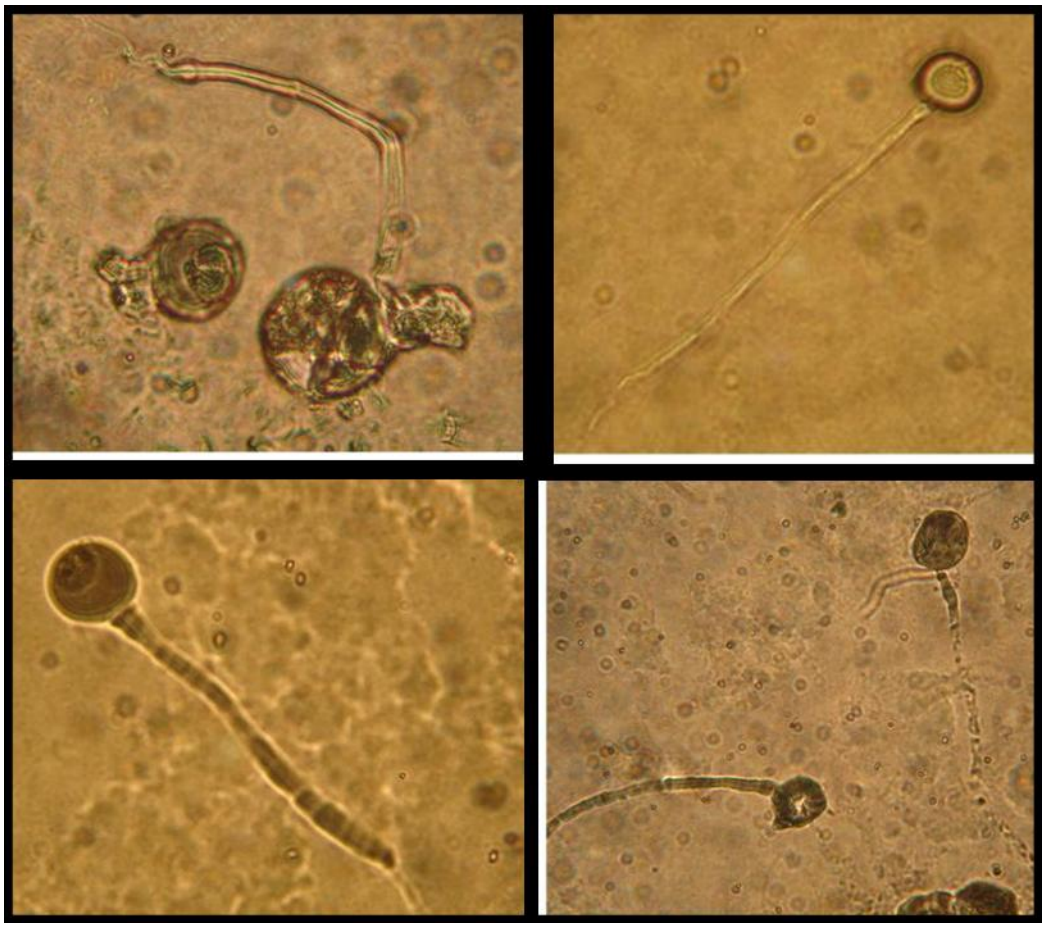

Table 8. Germination (%) of oospores from crosses of Brazilian isolates of *Phytophthora infestans*

	A2 isolates			
	Pib-731	Pib-749	Pib-750	Pib-753
A1 isolates				
Pib-732	0[a] (n [b] = 8)	0 (n = 7)	0 (n = 6)	0 (n = 5)
Pib-735	0 (n = 10)	0 (n = 9)	0 (n = 8)	0 (n = 6)
Pib-738	0.08 ± 0.1 (n = 10)	0 (n = 3)	0 (n = 5)	0 (n = 9)
Pib-742	0 (n = 9)	0.25 ± 0.4 (n = 7)	0 (n = 9)	0 (n = 4)
Pib-743	**Not assessed**	0.11 ± 0.2 (n = 9)	0.17 ± 0.3 (n = 8)	0 (n = 9)
Pib-745	0 (n = 9)	0 (n = 10)	0 (n = 9)	0 (n = 5)
Pib-746	0 (n = 8)	0 (n = 8)	0 (n = 2)	0 (n = 10)
Pib-754	0 (n = 6)	0 (n = 9)	0 (n = 8)	0 (n = 9)
Pib-755	0 (n = 4)	0.11 ± 0.2 (n = 8)	0.11 ± 0.2 (n = 9)	0.08 ± 0.1 (n =11)
Pib-756	0 (n = 6)	0.11 ± 0.2 (n = 9)	0 (n = 10)	0 (n = 8)
Pib-757	0 (n = 9)	0 (n = 6)	0 (n = 10)	0.19 ± 0.2 (n = 10)

[a]mean of three replicates ± standard deviation; [b]number of plates assessed

4. DISCUSSION

The absence of a recombining population of *P. infestans* in Brazil can not be attributed to the sterility of oospores. It was possible to reject hypothesis 2 of Reis *et al*. (2003) and conclude that there is a chance for the establishment of a sexual population of *P. infestans* in Brazil. Variation in the number of oospores produced per cross observed in this study was in the range reported in other studies (Pittis & Shattock, 1994; Flier *et al.*, 2001; Oliva *et al.*, 2002). Nevertheless, the maximum number obtained with the Brazilian isolates was lower than those reported elsewhere. When estimating the density of oospores produced per cm^2 of agar, the numbers ranged from 144 to 1394; the range reported by Pittis & Shattock (1994), Flier *et al*. (2001) and Oliva *et al*. (2002) was 4650 to 57730, 0 to 50000, and 305 to 4550, respectively. In all reports, including the present, the number of oospores was highly dependent on several factors. It is known that culture age, hormone production, mating type (Ko, 1978), host species and/or host genotype (Cohen *et al.*, 1997; Turkensteen *et al.*, 2000), and the isolate genotype either singly or in combination (Flier *et al.*, 2001; Oliva *et al.*, 2002) can affect the number of oospores produced. Regarding the results from this study, hormone production and host aspects can not be inferred because they were not

quantified or utilized. Culture age was not likely to affect the number of oospores once isolates and colonies used to make the crosses had similar age. Most likely, isolate genotype was the most influential factor in number of oospores produced, as revealed by the diallel analysis.

The number of oospores produced and consequently the chances of establishment of a sexual population of *P. infestans* will depend on the genotype of the isolates in a region. The estimated GCA and SCA values had a wide distribution. Isolates and crosses with high values of GCA and SCA were not associated with isolates/crosses with the highest number of oospores. Given the nature of the diallel analysis, this is not unusual. The analysis estimates the genetic contribution of parents to produce oospores (Cruz & Regazzi, 1997; Sales *et al.*, 2003). Based on the higher GCA values, it could be inferred that A1 isolates (Pib-754 and Pib-742) contributed the best to form oospores. Same reasoning applies to the A2 isolate Pib-750. Meanwhile, at the cross level, Pib-731 x Pib-755 had the highest SCA value, both for number of oospores in culture media and after extraction, this was the most suitable combination to produce oospores among the 44 crosses made. Mating interactions are known to be regulated by sexual compatibility loci and a system of sexual preference (Judelson, 1997) that influences the synthesis and response of sexual hormones (Judelson & Blanco, 2005) and consequently on the production of oospores. The fact that oospores were not always concentrated in the area of junction of the colonies but closer to one of the isolates is another clue to reach that conclusion. Therefore, because of isolate genotype and the combination capacity of parents some crosses were better oospore producers than others.

Regarding geographical aspects, crosses made with isolates from the same region resulted in higher number of oospores. Nonetheless, few isolates from the South region were assessed; possibly crosses with a greater number of isolates from this region would allow better inferences and to draw safer conclusions about consistency of the results. As reported elsewhere (Flier *et al.*, 2001) no consistent trend for preferential mating between isolates from the same location or host was detected. According to other studies made *in planta* production of oospores on susceptible host leaflets did not depend on the geographical region (Cohen *et al.*, 1997). If further studies related to the production of oospores from the same region confirm the current trend, then regions in which both

potatoes and tomatoes are extensively cultivated must be under strict monitoring for detecting the establishment of a recombining population of the pathogen.

Crosses involving Brazilian isolates of *P. infestans* were fertile, but viability and germination capacity of oospores produced were low. When viability of oospores from Brazilian isolates of *P. infestans* was assessed by the plasmolysis test, the values were lower than in other already reported: 44.8% (Pittis & Shattock, 1994), 50% (Mayton *et al.*, 2000), or 29% (Flier *et al.*, 2001). Percentages of germinated oospores from crosses of Brazilian isolates of *P. infestans* were extremely low and there was no correlation between the viability and the germination values. Germination of oospores was much lower than levels reported by other authors. Lee *et al*. (1999) found germination rates that ranged from less than 1% in some crosses to 3.1 or 8% in others, while viability varied from 3.7 to 12.3%. In Morocco, Hammi *et al*. (2001) reported germination ranging from 4 to 25%. Jiang and Erwin (1990) suggested that low rates of germination could be due to lack of maturity or factors that affect viability. In the results reported here, low rates of viability coincided with low rates of germination or no germination. However, contrary to the observed by Jiang & Erwin (1990), ungerminated oospores from the present study had "normal" appearance with typical ooplast and thick walls. Similar results were reported by Knapova *et al*. (2002). Some hypotheses regarding low viability can be tested in the future: *i*. the enzymatic treatment affected oospore viability. Novozym 234, the standard enzyme for this purpose until a few years ago, is not longer available and it was necessary to adapt a new protocol for oospore extraction. The protocol developed in this study was suitable for obtaining mycelium/sporangial-free oospores preparations, but no assessment of the potential effect on viability was conducted; *ii*. since oospore maturation time affects viability (Jiang & Erwin, 1990; Pittis & Shattock, 1994), it is possible that using older oospores would have resulted in higher values of viability. Unfortunately, no previous study on the maturation time for oospores done in Brazilian conditions is available; *iii*. environmental conditions for oospore germination differ from those reported in other regions; *iv*. the genetic system controlling oospore formation results in unstable or in oospores with low viability. Because the clonal structure of the current population of *P. infestans* in Brazil, isolates within each clonal lineage would have similar genetic background. Thus, regardless of

sampling from different places, genetically similar crosses would result in low viability oospores.

Even at low viability rates, the production of oospores can pose a threat to tomato and potato production in Brazil. The large acreages of overlapping distribution of the two host crops in regions such as the Central-West and in the Triângulo Mineiro in the Southeast facilitate the occurrence of mating events and oospore formation, which was demonstrated here under controlled conditions. Due to availability of host tissue, a large amount of oospores can be formed and despite the low viability, many can germinate and start late blight epidemics from recombinant isolates. Constant and intensive monitoring should be conducted in these areas aiming at detecting incipient recombination events.

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