Formation, production and viability of oospores of *Phytophthora infestans* from potato and *Solanum demissum* in the Toluca Valley, central Mexico

Wilbert G. FLIER[1]*, Niklaus J. GRÜNWALD[2], William E. FRY[2] and Lodewijk J. TURKENSTEEN[1]

[1] *Plant Research International, P.O. Box 16, NL-6700 AA Wageningen, The Netherlands.*
[2] *Plant Pathology Department, Cornell University, Ithaca, NY 14853, USA.*
E-mail: w.g.flier@plant.wag-ur.nl

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Aspects of the ecology of oospores of *Phytophthora infestans* were studied in the highlands of central Mexico. From an investigation of a random sample of strains, it was found that isolates differed in their average capability to form oospores when engaged in compatible pairings. Most crosses produced large numbers of oospores but a few yielded none and some yielded only a few oospores. The results reveal that oospore production and fecundity is dependent on both isolates and the combining ability of a specific combination of parental strains. On average, 14% of the oospores produced were viable as determined by the plasmolysis method. Viability ranged from a low 1% in one cross to a high of 29% in another cross. Oospores were found in 10–20% of naturally infected *Solanum demissum* leaves from two different collections, and leaflets with two lesions per leaflet produced more oospores than did leaflets with 3–5 lesions per leaflet. There was no consistent trend for preferential mating between isolates from the same location or host.

INTRODUCTION

Phytophthora infestans the causal organism of late blight in potatoes, is a heterothallic oomycete with two compatibility groups, referred to as mating types A1 and A2 (Shaw 1996). Oospores are produced when two compatible fungal strains of opposite mating types interact (Galindo & Gallegly 1960). In central Mexico, the presumed centre of origin of *P. infestans*, both mating types are present in approximately equal frequencies (Goodwin *et al*. 1992, Grünwald *et al*., unpubl.), and oospores are commonly found in potato crops (Niederhauser 1956, Gallegly & Galindo 1958). It is generally believed that each compatible cross between strains will yield oospores (Hohl & Iselin 1984), although the ability of paired isolates to form oospores in both artificial media (Judelson, Spielman & Shattock 1995, Judelson 1996, Lee, Mizubuti & Fry 1999) and host tissues (Mosa *et al*. 1991) seems to vary among combinations of parental strains. The viability of oospores produced can vary considerably between crosses (Pittis & Shattock 1994). There are several reports that describe the relative compatibility of Mexican strains of *P. infestans* (Shattock, Tooley & Fry 1985, 1986, Spielman, McMaster & Fry 1989, Spielman *et al*. 1990, Al-Kherb *et al*. 1995, Judelson 1997). In addition, there are strong indications that there is diversity among isolates in their preference to serve as male or female parents (Judelson 1997). However, most reports are based on experiments with a limited number of isolates. To date, only fragmentary data exist on the variation in mating ability, oospore production and viability present in *P. infestans* populations. A better understanding of the relative mating compatibility of isolates and the viability of the oospores produced is important in order to predict the relative importance of oospores in late blight epidemics in areas where A1 and A2 strains were only recently introduced.

The population of *P. infestans* in the Toluca Valley in central Mexico is considered to be the most diverse in the world (Tooley, Fry & Villareal Gonzalez 1985, Fry & Spielman 1991, Goodwin *et al*. 1992) and offers the unique opportunity to improve our knowledge about the effect of a genetically diverse population on mating behaviour and oospore viability. To date, no information is available on presence and implications of isolation barriers on the production and viability of oospores of *P. infestans*. The presence of pre- or post-reproduction isolation could provide some support for the hypothesis of differentiation and eventually sympatric speciation (Kondrashov & Kondrashov 1999) in local populations of *P. infestans* in central Mexico. Dieckmann & Moebeli (1999) recently presented theoretical evidence that assortative mating often leads to reproductive isolation between ecologically diverging sub-populations which in turn can lead to sympatric speciation. It has been stated that the speciation process can be accelerated by either resource use through host adaptation (strong selection for virulence genes in the pathogen to neutralise R-genes in *Solanum demissum*) or random genetic drift (Berlocher 1998). It is likely that *P. infestans* populations in populations of *S. demissum* undergo

* Corresponding author.

severe bottlenecks at the end of each growing season, so it is to be expected that random genetic drift plays an important role in shaping populations of *P. infestans* with a very restricted size. The presence of several wild and cultivated host-species of *P. infestans* in central Mexico provides an excellent opportunity to test the hypothesis of sympatric speciation driven by ecological preferences in natural systems. Ordoñez *et al*. (2000) recently provided evidence for the presence of sympatric speciation in *P. infestans* populations. They reported on an Ecuadorian *Phytophthora* A2 population closely resembling *P. infestans* which appears to be strictly isolated from potato isolates by host-plant specificity.

Our objective was to study the ecology of oospores of *Phytophthora infestans* in the highlands of central Mexico. We evaluated oospore production, viability and mating ability in isolates collected from the native host-plant *Solanum demissum* and commercial potato crops in the Toluca Valley. In particular, we were interested in quantifying variation in mating ability, oospore production and oospore viability (using various viability tests) of *P. infestans* using *in vitro* crosses. Isolates were collected in the Toluca Valley and along the slopes of the Volcano 'Nevado de Toluca' in central Mexico in 1997. Secondly, we assessed whether oospore formation occurs on wild *Solanum* species. A final goal was to test whether pre-reproduction or post-reproduction isolating mechanisms exist among isolates from different local populations of *P. infestans* in Toluca. Therefore, we compared oospore production and viability of isolates from three different populations of *S. demissum*.

MATERIALS AND METHODS

Source of isolates

All isolates except for the two reference strains (collected in 1996) were collected in the Toluca Valley in 1997. The selected strains represent three distinct geographical and host origins: (1) commercial potato production fields in the valley of Toluca; (2) small farmers' fields with local varieties of *Solanum tuberosum* ('papa criolla') on the slopes of the volcano; and (3) populations of wild *S. demissum* occurring in pine forests on the Nevado de Toluca near the community of Loma Alta.

In the case of the valley and criolla survey, 20 potato fields were visited from which 212 isolates ($\geqslant$ 10 from each field) were randomly sampled. For *S. demissum*, 66 isolates were obtained from three patches near Loma Alta. Reference strains Pic96001 and Pic96002 (Table 1), which had been isolated from local commercial potato crops, were kindly provided by Telesforo Zavala (INIFAP-Mexican National Potato Program). The isolates have been added to the PICTIPAPA-CEEM culture collection in Toluca, Mexico and are also maintained at Plant Research International, Wageningen, The Netherlands. Isolates are available for research purposes on request.

Isolation and culture

Phytophthora infestans strains were isolated from infected leaflets showing single lesions. Pieces of infected tissue adjacent to the sporulating region of the lesion were placed under potato tuber slices and incubated at 17–19 °C for 5 d until sporulation appeared. An inoculation needle was used to transfer mycelium from the tuber slice to Rye A agar (Caten & Jinks 1968) supplemented with ampicillin (200 mg l^{-1}), Benlate (50% WP, 100 mg l^{-1}), PCNB (75% WP, 67 mg l^{-1}), polymixin B (50 mg l^{-1}) and rifampicin (20 mg l^{-1}) (Forbes 1997). Petri plates (9 cm diam) containing the Rye A selective medium were incubated at ambient temperature for 1–2 wk. Subsequently, small pieces of selective medium containing actively growing *P. infestans* hyphae were transferred to Rye A agar plates. Isolates were maintained on Rye A agar at ambient temperature (20 ± 1 °) with transfers every 3–4 months.

Mating type was determined by pairing Mexican isolates individually with tester strains Pic96001 (A1) and Pic96002 (A2) on Rye A agar according to standard procedures (Table 1) (Forbes 1997).

Oospore production in Mexican isolates

Variation in mating ability and oospore production for 28 Mexican isolates (Table 2) was assessed by pairing all 14×14, A1–A2 combinations. Crosses with parental isolates originating from different geographical locations were marked as inter-regional combinations while crosses in which both parental strains originated from the same geographical location were marked as intra-regional combinations. Parental isolates were transferred to Rye A agar plates and cultured for 10 d at ambient temperature (20 ± 1 °). Agar discs (5 mm diam) taken from margins of fast growing colonies of isolates of opposite mating types (one disc per isolate) were placed 30 mm apart in a Petri plate (6 cm diam) containing 5 ml Rye A agar with 0.05 g l^{-1} β-sitosterol to stimulate oospore production. Two plates were prepared for each parental combination. Plates were incubated for 15 d at ambient temperature in the dark. Presence of mate- and self-repulsion, mating region and oospore formation was determined for each plate. We define mate-repulsion as growth inhibition resulting in an uncolonised zone between parental colonies (Shaw 1987, 1991). When these hyphal interactions are observed between neighbouring colonies of the same isolate, the phenomenon was referred to as self-repulsion. The presence of a mating region is marked by extensive stimulation of submerged hyphal growth at the interaction zone between the two parental strains, resulting in a distinct band visible by eye (Shaw 1987).

Oospore production was estimated based on examination of 10 microscopic fields (0.2 mm^2 each) within the interaction zone of the two parental cultures (Smoot *et al*. 1958). The zone in which oospores were formed was first located, and 10 microscopic fields were selected by random movement of the mechanical stage along this zone. The number of oospores in a cross-section from the agar surface to the bottom of the plate was quantified using an oospore production index (OPI). The OPI represents average oospore production in the mating region and is expressed in four classes: 0, 0 oospores mm^{-2}, 1, 1–50 oospores mm^{-2}, 2, 51–250 oospores mm^{-2} 3, 251–500 oospores mm^{-2}, and 4, $>$ 500 oospores mm^{-2}.

In a second experiment, oospore production and viability were studied in more detail to refine the results obtained in the

Table 1. Characteristics of the 32 Mexican *Phytophthora infestans* isolates used in the oospore production experiments.

Isolate	Host	Origin	Mating type	PEP	GPI	Metalaxyl resistance
Toluca Valley						
Pic97107	Potato cv. Alpha	La Silva	A1	100/100	100/100	Sensitive
Pic97111	Potato cv. Alpha	La Silva	A2	100/100	86/100	Sensitive
Pic97124	Potato cv. Alpha	La Communidad	A2	100/100	100/122	Sensitive
Pic97144	Potato cv. Atlantic	Los Champignones	A1	92/100	122/122	Intermediate
Pic97153	Potato cv. Alpha	PICTIPAPA exp. fields	A2	100/100	100/100	Intermediate
Pic97163	Potato cv. Atlantic	El Cerrito	A1	92/100	86/100	Sensitive
Pic97172	Potato cv. Atlantic	El Cerrito	A2	78/96*	86/100	Sensitive
Pic97175	Potato cv. Atlantic	El Cerrito	A1	100/100	100/100	Sensitive
Pic97196	Potato cv. Alpha	Las Minas	A1	100/100	86/122	Sensitive
Pic97236	Potato cv. Atlantic	La Tolva	A2	100/100	86/100	Sensitive
Nevado de Toluca						
Pic97301	Criolla (unknown)	Loma Alta	A1	100/100	86/86	Sensitive
Pic97322	Criolla (unknown)	Loma Alta	A1	78/100*	100/122	Sensitive
Pic97323	Criolla (unknown)	Loma Alta	A2	92/100	100/100	Sensitive
Pic97333	Criolla (unknown)	Raices	A2	78/100*	86/100	Sensitive
Pic97334	Criolla (unknown)	Raices	A1	92/100	100/111	Sensitive
Pic97348	Criolla cv. Rosita	Loma Alta	A1	100/100	100/100	Sensitive
Pic97349	Criolla cv. Rosita	Loma Alta	A2	100/100	92/100	Sensitive
Pic97391	Criolla cv. Marsiana	Loma Alta	A2	100/100	100/100	Resistant
Pic97441	Criolla (unknown)	Buena Vista	A1	92/100	100/100	Sensitive
Wild Solanum sp.						
Pic97701	*S. demissum*	Loma Alta, patch 1	A2	92/100	100/100	Sensitive
Pic97709	*S. demissum*	Loma Alta, patch 1	A1	92/92	100/100	Sensitive
Pic97711	*S. demissum*	Loma Alta, patch 1	A2	92/100	100/100	Sensitive
Pic97728	*S. demissum*	Loma Alta, patch 2	A2	100/100	100/100	Sensitive
Pic97731	*S. demissum*	Loma Alta, patch 2	A2	100/100	100/100	Sensitive
Pic97735	*S. demissum*	Loma Alta, patch 2	A1	92/100	86/100	Sensitive
Pic97743	*S. demissum*	Loma Alta, patch 3	A1	92/100	100/100	Sensitive
Pic97746	*S. demissum*	Loma Alta, patch 3	A2	100/100	100/100	Sensitive
Pic97750	*S. demissum*	Loma Alta, patch 3	A2	100/100	100/100	Sensitive
Pic97754	*S. demissum*	Loma Alta, patch 3	A2	100/100	100/100	Sensitive
Pic97757	*S. demissum*	Loma Alta, patch 3	A1	100/100	86/100	Sensitive
Reference strains						
Pic96001	Potato cv. Alpha	INIFAP	A1	100/100	100/122	Sensitive
Pic96002	Potato cv. Alpha	INIFAP	A2	100/100	100/100	Sensitive

* Tentatively assigned relative migration of a novel allele (PEP 78).

main experiment and to compare three commonly used oospore viability tests. A subset of eight isolates was randomly selected from the A1 and the A2 isolates used for the first experiment. Four agar discs (5 mm diam), two of each parental isolate, were placed 40 mm apart on 10 ml Rye A agar amended with 0.05 mg l^{-1} β-sitosterol in 9 cm diam Petri dish. The agar discs from the same isolate were placed in the opposing corners so that the four discs formed a square. Three replicates were used.

Oospore production (OPI and oospore counts), viability and germination were determined. Mating regions of 21 d old cultures were excised using a scalpel, transferred to sterile 50 ml centrifuge tubes containing 9 ml sterile double distilled water. The agar was blended for 60 s at 20000 rev. min^{-1} using an IKA T20 homogeniser with the S20 probe. The mean number of oospores was determined in three 50 µl aliquots, and the total number of oospores per mating event was calculated adjusting for the total volume of the homogenised sample.

A NovoZym 234 treatment was used to digest sporangia and mycelial fragments and to promote oospore activation and germination (Shaw 1996). To monitor oospore viability prior to and after NovoZym 234 treatment, the plasmolysis and the tetrazolium test (Jiang & Erwin 1990) were used. For the plasmolysis method, extracted oospores were suspended in 2 M NaCl solution for 3 h. Plasmolysis was assessed by micoscopical observation of 150 oospores in three replicates. Viability is expressed as the percentage of oospores that were plasmolysed. For the tetrazolium test, oospore suspensions were dispersed in 0.1 M phosphate buffer containing 0.1% tetrazolium bromide (MTT). The suspensions were incubated for 2 d at 35 ° and stained oospores were subsequently examined microscopically for colour reactions according to Erwin & Ribeiro (1996). Rose oospores were considered to be dormant, while blue-pink oospores were assumed to be activated (ready to germinate). Unstained or black oospores were considered to be non-viable.

Germination of oospores was assessed using a plating technique described previously. A NovoZym 234 (Novo Biolabs) treatment was applied to lyse any mycelial fragments and sporangia in the suspension. A NovoZym 234 solution (50 mg NovoZym 234 per ml deionised water) was added to

each of the crude oospore suspensions and incubated at 20 ° for 24 h. After digestion, oospores were washed in three successive steps using 25 ml deionised water and re-suspended in 10 ml sterile deionised water. Oospore suspensions were then spread on sloppy water agar (5 g l^{-1}) and incubated at 20 ° under cool blue fluorescent light. Oospore germination was assessed after 14 d and expressed as percentage germination (Shaw 1996).

Oospore production in wild Solanum *species*

Three populations of *Solanum demissum* were extensively monitored throughout the summer of 1997 for the presence of *Phytophthora infestans*. We defined a population here as a distinct group of *S. demissum* plants growing within a relatively small area (10–100 m^2) in a small valley on the slope of the Volcano Nevado de Toluca near Loma Alta. Population 1 consisted of approx. 500 plants of *S. demissum* growing in an open pine forest in the vicinity of an abandoned 'Criolla' potato field. At the time of collection, 2–5% of the leaf area was infected by *P. infestans*, with most plants showing at least one sporulating lesion on lower leaves. Population 2 comprised approx. 250 plants and was located in the same valley separated from population 1 by at least 100 m of dense pine forest. Population 3 was located near a 'Criolla' field under shrubs and bushes, at least 700 m distant from population 1 and 2 and consisted of approx. 200 *S. demissum* and 100 *S. xedinense* plants. Leaflets displaying multiple lesions were collected from two populations (1 and 2) of *S. demissum*. No leaflets with multiple lesions were found in population three during the 1997 field season.

Infected leaflets were examined for presence of oospores. Leaflets with multiple lesions were incubated for 6 d at ambient temperature on water agar (10 g l^{-1}) in Petri dishes (9 cm diam). Leaflets were then clarified in boiling ethanol (96% v/v) for 5 min, bleached in 1% NaHClO for at least 6 h and mounted on microscope slides with glycerol. The entire clarified leaflets were examined for presence of oospores using a bright field microscope at a magnification of 10×10 and 10×40. The number of oospores per leaflet was counted.

Variation in fecundity of Phytophthora infestans *isolates from* Solanum demissum

Isolates of *Phytophthora infestans* collected in 1997 from three populations (as described above) of *S. demissum* were crossed to investigate the presence of reproductive isolation barriers by comparing intra- and inter-population variation in mating ability, oospore production and oospore viability. Isolates were collected on 11 Sept. 1997. Crosses that involved isolates collected from different populations of *S. demissum* were marked as inter-population matings, while crosses involving isolates from the same location were marked as intra-population matings.

Five A1 isolates were mated with nine A2 isolates, including mating type tester strains Pic96001 and Pic96002. All crosses were performed according to the method described above using two replicates. Oospore density was determined by counting the number of oospores in ten randomly selected microscopic fields. Viability of oospores was assessed using the plasmolysis method.

Statistical analysis

All statistical analyses were performed using the Genstat 5 version 3.4.1 statistical package (Payne *et al*. 1993). The presence of isolate specific effects and cross specific effects, analogous to the concept of general combining ability (GCA) and specific combining ability (SCA) in plants and livestock (Pooni, Jinks & Singh 1984, Falconer & Mackay 1996, Conner *et al*. 1998) was investigated, using OPI as an estimation of oospore production. The OPI was $\log_e$-transformed since variance increased with means. Analysis of variance (ANOVA) was conducted to determine the relative importance of GCA and SCA on oospore production and viability. The experimental design and data analysis were adopted from Falconer & Mackay (1996). In the ANOVA the main effect of isolate is considered to be an estimate for GCA while the interaction effect of specific parental combinations resembles SCA. The significance of both factors GCA and SCA was tested using Fisher's *F* test. Associations between the presence of a mating region, mate-repulsion and oospore production in *in vitro* crosses and independence of the frequency of oospore production in wild *S. demissum* leaflets were evaluated using contingency tables. A log-linear regression model was fitted to the counts. The test of independence was based on the χ^2 approximation (Payne *et al*. 1993). Spearman rank correlation coefficients were calculated for the various oospore production and viability parameters.

RESULTS

Oospore production in Mexican isolates

In vitro pairings of A1 and A2 *Phytophthora infestans* isolates from commercial potato fields, Criolla fields and populations of *Solanum demissum*, led to production of oospores in all parental combinations tested, with the exception of pairings in which isolate Pic97301 (A1) was involved. Oospores were only produced in 4 out of 13 pairings when isolate Pic97301 was included as one of the parents (Table 2). In some mating combinations, only a few oospores were produced. No self-fertile isolates were observed.

In most parental combinations, a characteristic mating region was present at the interaction zone between two isolates. The presence of oospores, absence of sporangiophores and high density of both surface and sub-surface hyphae characterise this region. The presence of a mating region was found to be strongly associated with production of oospores ($P < 0.001$). Mate repulsion was observed in a few crosses, but appeared to have little effect on oospore production.

The average value for OPI was 3.0 and variability in OPI was higher for A1 isolates (range 0.5–3.8) than for A2 isolates (range 2.5–3.4) (Table 2). The greater OPI diversity for A1 isolates is mainly caused by two isolates (Pic97107 and Pic97301), which in general produced very few oospores. Differences in oospore production between combinations of isolates (Table 2) were significantly dependent upon parental isolates (GCA) ($P < 0.001$) and specific combinations of

Table 2. Average oospore production index (OPI) values (0, 0 oospores mm^{-2}; 1, 1–50 oospores mm^{-2}; 2, 51–250 oospores mm^{-2}; 3, 251–500 oospores mm^{-2}; 4, > 500 oospores mm^{-2}) for *in vitro* matings between Mexican A1 and A2 isolates of *Phytophthora infestans*, at the interaction zone between the two isolates.

Isolate	A1 isolates														
A2 isolates	Pic97107	Pic97144	Pic97163	Pic97175	Pic97196	Pic97301	Pic97322	Pic97334	Pic97348	Pic97441	Pic97709	Pic97735	Pic97743	Pic96001	Average
Pic97111	1	3	4	4	2.5	0	3	3	3.5	4	4	3.5	3	4	3.0
Pic97124	1	3	4	3.5	2.5	0	2	3.5	3	3	4	3.5	4	4	2.9
Pic97153	1	4	4	3	4	0	3	4	4	4	4	3	4	3.5	3.3
Pic97172	3	4	3.5	4	3.5	0	1	3	4	3	4	4	4	4	3.2
Pic97236	2	4	3.5	3	1.5	1	4	3.5	3.5	3	–	2.5	4	2.5	2.9
Pic97323	1.5	4	4	3.5	2.5	0	2.5	4	4	3.5	3	3	4	3.5	3.1
Pic97333	1	3	3	3	2	2.5	2.5	2.5	4	3.5	3.5	3.5	4	4	3.0
Pic97349	1	3	2	3.5	3.5	1	4	3	4	2	2	3	4	3.5	2.8
Pic97391	0.5	3.5	4	4	2	0	4	3.5	4	3.5	3.5	2.5	4	2.5	3.0
Pic97701	1.5	4	4	2.5	2.5	0	2	4	3.5	4	4	4	4	3.5	3.1
Pic97711	1.5	3.5	2	4	2	0	1.5	2	3	1	2	4	4	4	2.5
Pic97728	3	3.5	2	3	3.5	0	3.5	1.5	1	4	4	2.5	2.5	4	2.7
Pic97754	0	3.5	4	3.5	2.5	2	2	4	3.5	2.5	4	4	4	4	3.1
Pic96002	3	3.5	4	3.5	2.5	–	3.5	4	4	4	4	4	4	4	3.4
Average	1.5	3.5	3.4	3.4	2.6	0.5	2.8	3.3	3.5	3.2	3.5	3.4	3.8	3.6	3.0

–, not determined due to contamination.

Table 3. Average oospore production index (OPI) values for inter- and intra-population crosses for the three regional populations of *Phytophthora infestans* tested. Statistical significance using the Fisher's protected least significant difference (LSD) means comparison method.

	Type of mating			
Origin	Inter-population crosses (n)	Intra-population crosses (n)	LSD $_{(0.95)}$	$F_{\text{probability}}$
Toluca Valley	3.00 (170)	3.04 (25)	0.37	0.814
Slopes of Volcano	3.01 (175)	2.90 (20)	0.40	0.577
Populations of *S. demissum*	2.96 (183)	3.58 (12)	0.51	0.016

isolates (SCA) ($P < 0.001$) based on ANOVA. The GCA:SCA variance ratio was calculated as 1.765, indicating that the effect of general combining ability on oospore production is almost twofold that of specific combining ability.

Average oospore production was compared for crosses in which both parental isolates originated from the same geographical region (intra-regional combination) and crosses in which parental isolates were collected from different geographical regions (inter-regional combination) (Table 3). Isolates from *S. demissum* tend to be better parents when mated with each other since intra-regional combinations led to a significantly higher average OPI value ($P < 0.05$) compared to inter-regional combinations (Table 3). No preferential differences in OPI were found for isolates originating from the Toluca valley or the slopes of the Nevado de Toluca.

The oospore production data from the viability experiment were in agreement with the results obtained in the first experiment. Among the 10 matings between Mexican isolates of *Phytophthora infestans*, the average number of oospores produced varied between 1.6×10^3 and 1.5×10^5 oospores per Petri dish (Table 4). Three matings involving isolate Pic97107 yielded low numbers of oospores in comparison with other crosses in the same experiment (Table 4). Both GCA ($P < 0.001$) and SCA ($P = 0.019$) effects were found to influence the number of oospores produced in a mating event although the GCA:SCA ratio of 43.4 indicates that the effect of general combining ability on oospore production is more important than specific combining ability, based on our results using 10 crosses. A Spearman rank correlation was calculated to compare direct oospore counting and OPI values. Oospore production data generated by both methods were highly

Table 4. Oospore production and viability measurements in 10 *in vitro* matings of *Phytophthora infestans* isolates of central Mexican origin.

Parental isolates		Oospore production		MTT					
				% activated		% dormant			
A2 parent	A1 parent	Oospores mm^{-2}	Oospores per plate^{-1}	NovoZym –	NovoZym +	NovoZym –	NovoZym +	Plasmolysis % viability	Germination % germination
Pic97111	Pic97107	7.8	2164	0.0	0.0	0.0	0.0	0.0	0.0
Pic97111	Pic97348	650.5	101565	17.6	28.0	17.3	3.0	16.5	3.1
Pic97111	Pic97743	317.3	49950	5.4	14.6	5.9	0.5	4.7	0.0
Pic97333	Pic97107	22.3	1665	0.0	0.0	0.0	0.0	0.0	0.0
Pic97333	Pic97348	247.8	42180	10.5	19.8	9.2	1.7	10.0	1.7
Pic97333	Pic97743	120.3	9435	2.3	8.3	5.6	3.3	1.5	0.0
Pic97754	Pic97107	80.5	17760	0.0	0.0	0.0	0.0	0.0	0.0
Pic97754	Pic97348	483.0	29970	22.5	30.8	16.3	9.5	20.5	1.8
Pic97754	Pic97743	240.3	78255	9.3	19.0	9.6	1.7	12.3	7.4
Pic96002	Pic96001	550.2	148185	49.7	53.7	25.6	13.9	49.5	11.0
$LSD_{(0.95)}$		125.1	21634	5.3	9.0	8.9	3.5	11.5	1.9

Table 5. Spearman rank correlation coefficients of different measurements on oospore production and viability in 10 matings between *Phytophthora infestans* isolates originating from central Mexico.

	Oospore production		Oospore viability						
			MTT prior to NovoZym treatment			MTT after NovoZym treatment			
Components	Oospores mm^{-2}	Oospores per plate	% dormant	% activated	% viable	% dormant	% activated	% viable	Plasmolysis % viability in 2 M NaCl
Oospores per plate	0.82								
% dormant MTT NovoZym −	0.86	0.78							
% activated MTT NovoZym −	0.83	0.80	0.93						
% viable MTT NovoZym −	0.83	0.78	0.97	0.98					
% dormant MTT NovoZym +	0.65	0.52	0.87	0.82	0.87				
% activated MTT NovoZym +	0.86	0.83	0.93	0.95	0.96	0.76			
% viable MTT NovoZym +	0.85	0.81	0.94	0.97	0.97	0.82	0.99		
% viable Plasmolysis	0.87	0.83	0.91	0.94	0.94	0.79	0.93	0.94	
% germination	0.65	0.81	0.78	0.79	0.78	0.64	0.64	0.85	0.81

All correlation coefficients were shown to be significantly different from zero ($P < 0.01$).

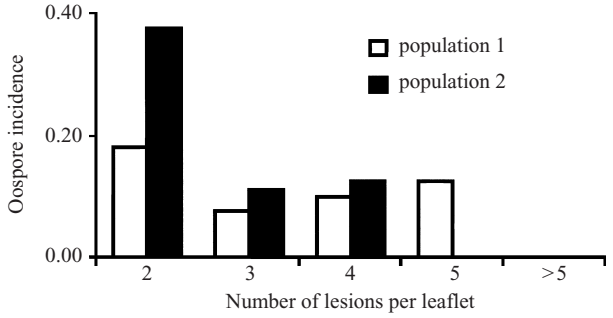

Fig. 1. Incidence of oospores in infected leaflets of different *Solanum demissum* populations, showing 2,3,4,5 or more than 5 lesions per infected leaflet.

correlated ($r = 0.82$; $P < 0.01$), (Table 5). Therefore, OPI values are a useful tool for assessing oospore production.

Variation in oospore viability and germination was evaluated in ten in-*vitro* matings using tetrazolium bromide staining, the plasmolysis method and oospore germination as different measurements for oospore vitality. Oospore viability prior to the NovoZym treatment ranged from 0.0 to 75.3% based on the tetrazolium test, and from 0.0 to 49.5% for the plasmolysis method (Table 4). Digestion of sporangia and mycelial fragments with NovoZym 234 reduced oospore viability for each of the seven matings that yielded viable oospores. After NovoZym treatment, viability varied between 0.0 and 67.6%, based on the estimates obtained with MTT staining (Table 4), as only MTT was used both before and after the NovoZym treatment.

The percentage of viable oospores, based on the MTT test criterion, was consistently higher in every cross than estimates obtained by the plasmolysis method. However, both methods gave highly correlated estimates for oospore viability ($r = 0.94$; $P < 0.01$) (Table 5). The few oospores obtained from the crosses Pic97107 × Pic97111, Pic107 × Pic97333 and Pic97107 × Pic97754 were found to be non-viable, both with MTT and the plasmolysis test. No germination was observed after NovoZym treatment of the oospores produced by these crosses. These results indicate that no viable oospores were produced in these three crosses. The NovoZym treatment induced oospores to shift from a dormant into an activated stage in all seven crosses that yielded viable oospores but did not significantly affect oospore viability since no drop in viability, based on MTT staining, was observed after the NovoZym treatment (Table 4).

The percentage germination in the seven oospore progenies that yielded viable oospores (based on MTT and the plasmolysis criterion) varied between 0.0 and 11.0%. A positive relation was found between oospore viability (as measured by MTT, plasmolysis and germination) and the number of oospores produced in a mating (Table 5); rank correlation coefficients varied between 0.65 and 0.87, and all associations were found to be significant ($P < 0.01$).

Oospore production in Solanum demissum

Sampling of leaflets with multiple lesions revealed that oospore formation is occurring in *Solanum demissum*. Oospores of *Phytophthora infestans* were observed in clarified leaflets that were examined for presence of oospores using a bright field microscope. Oospores were detected in leaflets showing multiple lesions from both population one and two. Oospore distribution in blighted leaflets appeared to be under-dispersed when whole leaflets were examined, their presence often concentrated in a few hot-spots of oospore production, which we refer to as clusters. Oospores were detected in leaflets with 2–5 late blight lesions per leaflet (Fig. 1), and up to four distinct oospore clusters per leaflet were observed. No oospores were detected in leaflets with 6–12 *P. infestans* lesions. Oospores were found to be present in 5 out of 42 and 5 out of 26 leaflets from population 1 and 2 respectively (Fig. 1). Presence of oospores in leaflets collected from population 1 and 2 did not differ significantly for the two populations assessed ($P = 0.412$). The number of oospores ranged from 10 to ∼ 5000 oospores per cluster.

Variation in fecundity of isolates from Solanum demissum

The number of oospores produced by pairing five A1 and eight A2 *Phytophthora infestans* isolates originating from three

Table 6. Oospore production (mm^{-2}) and viability of oospores, based on the plasmolysis test (%, in parentheses), after *in vitro* pairing of eight A1 and five A2 Mexican isolates of *Phytophthora infestans*.

	A2 isolates								
A1 isolates	Pic97701	Pic97711	Pic97728	Pic97731	Pic97746	Pic97750	Pic97754	Pic96002*	Average
Pic97709	430 (9.0)	488 (1.0)	350 (25.7)	154 (3.7)	546 (12.3)	214 (8.0)	543 (6.3)	–	389 (9.4)
Pic97735	863 (19.7)	743 (3.7)	620 (29.3)	244 (22.0)	257 (22.0)	254 (7.0)	298 (11.3)	327 (13.7)	451 (16.1)
Pic97743	716 (25.3)	312 (4.7)	277 (–)	129 (26.0)	500 (16.7)	1091 (10.0)	260 (22.7)	216 (3.7)	437 (15.6)
Pic97757	491 (11.3)	457 (2.7)	283 (11.0)	352 (11.0)	1018 (18.3)	915 (18.3)	425 (8.7)	492 (8.7)	554 (11.3)
Pic96001*	248 (29.3)	164 (11.0)	320 (19.0)	289 (19.0)	404 (19.7)	367 (20.0)	432 (18.7)	416 (13.0)	330 (17.6)
Average	549 (18.9)	433 (4.6)	370 (21.3)	233 (14.6)	545 (17.8)	568 (12.7)	391 (13.5)	362 (9.8)	432 (14.1)

Least significant difference (LSD $_{(P=0.95)}$) for oospore production = 240; Least significant difference (LSD $_{(P=0.95)}$) for viability = 4.45.
* Reference isolates.

Table 7. Average oospore production (oospores mm^{-2}) and oospore viability (number of parental combinations in brackets) in inter-patch and intra-patch matings between isolates of *Phytophthora infestans* obtained from *Solanum demissum*. Statistical significance was evaluated using Fisher's least significant difference method (LSD). The F-tests apply to inter- versus intra-population oospore production for each origin. Data shown after back-transformation.

	Oospore production				Oospore viability			
Origin	Inter-patch (*n*)	Intra-patch (*n*)	$LSD_{(P=0.95)}$	$F_{probability}$	Inter-patch (*n*)	Intra-patch (*n*)	$LSD_{(P=0.95)}$	$F_{probability}$
S. demissum Pop. 1	429 (26)	459 (2)	254	0.819	14.57 (27)	5.00 (2)	6.52	0.004
S. demissum Pop. 2	431 (26)	432 (2)	254	0.955	13.43 (27)	25.67 (2)	6.36	< 0.001
S. demissum Pop. 3	384 (22)	757 (6)	144	< 0.001	13.75 (27)	15.78 (6)	4.21	0.332

populations of *Solanum demissum*, varied between 154 and 1091 oospores mm^{-2} (Table 6). Both GCA and SCA contributed to the observed variation in oospore production ($P < 0.001$). Preferential mating of isolates originating from the same population in terms of numbers of oospores produced was observed in only one out of three populations (Table 7). Isolates sampled from population 3 showed a two-fold increase in oospore production ($P < 0.001$) when mated with strains originating from that same population (Table 7) compared to matings with isolates from other populations. Average oospore production in population 3 was 384 and 757 oospores mm^{-2} for inter- and intra-population matings respectively.

Considerable differences in oospore viability based on the plasmolysis test were present between both inter- and intra-population crosses. On average, 14.1% of the extracted oospores showed plasmolysis. Viability (based on the plasmolysis test) ranged from 1.0 to 29.3% with crosses Pic97709 × Pic97711 and Pic97735 × Pic97728, respectively (Table 6). Isolates originating from population 1 produced significantly more viable oospores ($P = 0.004$) when crossed with isolates from either population 2 or 3. On average 14.57 versus 5.00% viable oospores for inter-population and intra-population matings were produced, respectively (Table 7). Isolates originating from population 2 produced more viable oospores when involved in intra-population crosses ($P < 0.001$). On average, 3.43 versus 25.67% viable oospores were produced in inter-population and intra-population matings, respectively (Table 6). No differences in oospore viability in isolates originating from population 3 were observed between intra- and inter-population matings (Table 6). No consistent trend indicating the presence of preferential mating or post-reproductive isolation barriers was found.

DISCUSSION

All 28 isolates of *Phytophthora infestans* collected in central Mexico in 1997 were capable of mating with one or more isolates of the opposite mating type. Isolates differed in their average capability to form oospores when engaged in compatible matings, and certain specific parental combinations produced more oospores than other parental combinations. Therefore, the variation present in oospore production could be explained by both general combining ability, i.e. isolate-specific effects and specific combining ability, i.e. combination-specific effects analogous to the concept of GCA and SCA in plants and livestock (Falconer & Mackay 1996). Pittis & Shattock (1994) found a similar pattern of oospore production and viability for *P. infestans* isolates originating from the UK.

All parental combinations tested formed oospores with the exception of strains Pic97301 and Pic97107, which formed oospores with 4 out of 14 and 13 out of 14 compatible isolates respectively. Matings with isolate Pic97301 showed a clear mating region in all cases but this did not always result in oospore formation. The numbers of *in vitro* oospores produced in our experiments were in accordance with earlier reports (Smoot *et al*. 1958, Pittis & Shattock 1994) while low oospore viability in specific crosses has been reported by several authors including Shaw (1991), Shattock *et al*. (1986), and Judelson *et al*. (1995). Our results provide an indication that oospore production and viability are related, as matings which yielded low numbers of oospores showed strongly reduced levels of oospore viability. These observations provide some evidence for the presence of sexual incompatibility or lethal factors in *P. infestans* strains leading to abortion and non-viable oospores (Erwin & Ribeiro 1996). This is the first report of the presence of sexually or genetically induced incompatibility

leading to diversity in mating success in crosses between A1 and A2 *P. infestans* strains in its centre of diversity in the Toluca Valley. Our results support the results obtained by Goodwin *et al*. (1992) who reported the presence of incompatible A1 and A2 *P. infestans* genotypes in the Los Mochis region in northern Mexico.

Differences in oospore production were observed between pairings of isolates collected from potato and *Solanum demissum*. Isolates originating from the wild host *S. demissum* tend to produce more oospores in crosses with compatible strains collected from the same host species than in crosses with isolates collected from potato. The observed differences in oospore production and viability among isolates from different *S. demissum* populations were not consistent.

We conclude that no indications were found suggesting the presence of pre- or post-reproduction isolating mechanisms among isolates from different local populations of *P. infestans* based on *in vitro* formation of oospores. The conclusion that no isolating mechanisms leading to accelerated sympatric speciation are present in *P. infestans* populations in the Toluca Valley is supported by recent allozyme analyses showing no significant sub-structuring among isolates from commercial potato fields, Criolla fields or native *Solanum* species (Grünwald *et al*. 2000). However, recent work based on RFLP and AFLP fingerprinting of isolates from the three different areas indicate restricted gene flow among populations from potato and *S. demissum* (Flier & Grünwald, unpubl.). In a recent study, Ordoñez *et al*. (2000) reported on an Ecuadorian *Phytophthora* A2 population closely resembling *P. infestans* which appears to be strictly isolated from potato isolates by host-plant specificity.

We observed the formation of oospores in blighted leaflets of *S. demissum* in nature. Oospores were commonly detected in leaflets of *S. demissum* showing multiple lesions. The absence of oospores in leaflets with large numbers of lesions might be explained by the rapid decay of such leaflets, the time from infection to extended necrosis and decay being insufficient for oospore formation.

Support for the existence of a sexually reproducing population of *P. infestans* in central Mexico is traditionally based on the presence of the two known compatibility groups (Gallegly & Galindo 1958, Smoot *et al*. 1958) and oospores found in potato crops (Gallegly & Galindo 1958). Despite earlier attempts (Gallegly & Galindo 1958, Rivera-Peña 1990) to detect oospores in alternate hosts, the present study provides the first evidence for the occurrence of oospores in wild *Solanum* species from the Toluca valley. Our results show that oospore formation in *S. demissum* is frequent in leaflets with 2–5 lesions. We did not determine how frequent leaflets with multiple lesions are in nature.

We did not find evidence for the presence of mating preference (as a form of assortative mating) in *P. infestans* populations from the Toluca Valley. Although inherent incompatibility between pairings of A1 and A2 strains of *P. infestans* was observed, no reproductive barriers based on geographical sub-structuring or host-plant specificity for *S. demissum* were detected. However, Ordoñez *et al*. (2000), provided evidence supporting the hypothesis that sympatric speciation, the origin of two or more new species from a single local ancestral population without geographical isolation (Kondrashov & Kondrashov, 1999), is possible in populations of *P. infestans*. In order to test the possibility of sympatric speciation within *P. infestans* in central Mexico, more detailed experiments investigating the presence of population sub-structuring due to host-plant specificity, including the role of self-fertilisation, oospore viability, *in planta* oospore formation and more alternative host-plant species, are needed.

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