An Improved Method for Infecting Tomato Leaves or Seedlings with Oospores of *Phytophthora infestans* Used to Investigate F1 Progeny

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ABSTRACT

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An improved, soil-free laboratory method was developed to induce late blight infection in detached tomato leaves or tomato seedlings with in vivo-produced oospores of *Phytophthora infestans*. Oospores were produced in detached tomato leaves infected with an inoculum mixture of A1 and A2 isolates. The infected leaves were homogenized in distilled water. The homogenates were exposed to two cycles of drying and wetting to kill sporangia, zoospores, and mycelia and, thereafter, mixed with perlite and water. Tomato leaves or seed were floated on the perlite mixture in petri dishes and incubated for up to 30 days. Results from 13 experiments showed that, in dishes containing oospores, 96 of 820 leaflets (11.7%) developed late blight lesions within 6 to 30 days; whereas, in 3 experiments with seedlings, 12 of 1,400 plants (0.9%) were blighted within 17 to 26 days. No late blight developed in leaflets or seedlings maintained in dishes containing homogenates of the control leaves infected with either A1 or A2 inoculum. In all, 234 single-sporangium F1 isolates recovered from 24 leaf lesions produced by four crosses and 27 isolates recovered from 12 seedlings infected with two crosses were examined for sensitivity to metalaxyl and mating type. Results showed that, although the parent isolates were either S (sensitive) or R (resistant) to metalaxyl, most F1 progeny isolates exhibited various levels of intermediate resistance to metalaxyl. Most isolates belonged to either the A1 or the A2 mating type, depending on the cross and lesion. However, some isolates belonged to the unusual mating type A1A2 or were sterile. Current experiments are intended to elucidate the virulence and aggressiveness of these F1 progenies to tomato. This improved method may facilitate our understanding of the role of in vivo-produced oospores in the epidemiology of late blight in tomato.

Oospores are sexual spores of oomycetes (Stramenopiles). In the oomycete plant pathogen *Phytophthora infestans* (Mont.) de Bary, oospores are produced by mating of A1 and A2 isolates. They may be formed in agar culture as well as in host tissues, including leaves, stems, stolons, tubers, fruit, and seed (2,4,5,7,11,12,18,21–24,29,31). Oospores of *P. infestans* are readily produced in detached leaves of potato and tomato (5), as well as in green mature fruit of tomato (23) upon co-inoculation with A1 and A2 sporangia, but only rarely in potato tubers (21). In potato crops growing under field conditions, oospores are produced commonly in lower bottom leaves touching the soil, but rarely in upper leaves (4). Overhead irrigation of potato crops enhanced the production of oospores in the upper leaves (4).

Oospores may play a central role in the epidemiology of late blight in potato (1,7,9,11,18,22,29,31) and tomato (3,10,22–25) because they are the only structures of *P. infestans* that can survive

for prolonged periods outside the host tissues. They were reported to survive in the field as demonstrated by their ability to initiate the late blight disease in situ or in detached leaves in the laboratory (7,22,28,29). In Sweden (1,28), circumstantial evidence suggested that epidemics were initiated from soilborne oospores. In Central Mexico, soilborne oospores were reported to survive winter fallow and infect potato plants in the field (9). Also, the fact that seedborne oospores can induce late blight infection in tomato seedlings (24) makes the oospores an important tool for initiating epidemics of late blight in tomato. Due to their recombinant nature, oospores may produce new genotypes of the pathogen with altered characteristics, including sensitivity to fungicides, virulence race structure, and aggressiveness, thus changing the epidemiology of the disease (1–3,9–12,18,22,27–29,31).

To ensure in experiments that oospores are the sole source of infection, the vegetative structures of *P. infestans* (sporangia, zoospores, and mycelia) accompanying the oospores were differentially eliminated by either cell-wall-degrading enzymes (18), freezing and thawing (29), or repeated wetting and drying of the inoculum (9). Sporangia of *P. infestans* were shown to be extremely vulnerable to repeated wetting and drying due to deterioration of their inner mitochondrial membranes (6). In-

deed, through our recent study, we showed that repeated drying and wetting, but not repeated freezing and thawing, of infected tomato leaflet-homogenates containing both oospores and vegetative structures of *P. infestans* totally eliminated all pathogen structures except the oospores (25). This technology of repeated wetting and drying was employed with oospore soils in Central Mexico (9) to assess the infectivity of soilborne oospores of *P. infestans* after intercropping. It also is used to infect grape leaves with oospores of the downy mildew pathogen *Plasmopara viticola* (U. Gisi, *personal communication*).

Floating detached leaves of potato or tomato on water suspensions of oospores (22) or floating leaves of potato on soil suspensions harboring oospores (7,29) have been reported to produce late blight lesions in the leaves. However, neither method produced infection when we employed them in petri dishes (*unpublished data*).

The purpose of this study was to develop an improved petri dish baiting technique to obtain oospore infection in detached tomato leaves, as well as in germinating tomato seed. The method involved mixing of in vivo-produced oospores with perlite and water in petri dishes and floating healthy detached leaflets of tomato or tomato seed on the surface of this mixture. Genetic analysis of the F1 progeny isolates obtained by this method may facilitate our understanding of the biology and epidemiology of late blight in tomato in nature.

MATERIALS AND METHODS

Oospore production in tomato leaves.

Oospores were produced in detached tomato (*Lycopersicon esculentum* L) leaves of the susceptible inbred line ZH as described before (5). Briefly, 20 leaves were placed on moist filter paper in a plastic dish (20 by 20 by 3 cm, Nunk, Roskilde, Denmark) and inoculated with mixed (1:1) sporangia of various A1S + A2R isolates (S = sensitive to metalaxyl and R = resistant to metalaxyl; Table 1). Control leaves were inoculated with sporangia of only the corresponding A1S or A2R isolates. Oospores were formed in the A1S + A2R inoculated leaves but not in the control leaves inoculated with either the A1S or A2R isolates. At 10 days after inoculation, leaves infected with A1S + A2R, as well as control leaves infected with each of the corresponding A1S or the A2R parental

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isolates, were collected separately and each homogenized in 50 ml of distilled water.

Differential eradication of vegetative structures from leaf homogenates. Our previous data (6) showed that repeated wetting and drying was detrimental to sporangia of *Phytophthora infestans*. Another observation revealed (see Results) that repeated freezing and thawing (29) was only partially effective in killing the sporangia, zoospores, or mycelial fragments. Therefore, the following method was used to differentially eliminate sporangia, zoospores, and mycelia present in the leaf homogenates, while keeping oospores viable. Aliquots (5 ml) of the above leaf homogenates were pipetted into 9-cm petri dishes (2 cm in height) and the dishes were placed (without lids) in a strongly ventilated biological hood at 25 to 30°C for 24 h to dry up the liquid homogenates. Then, 10 ml of water was added to each dish, and the homogenates were resuspended in the water with the aid of a sterile painting brush and dried again for 24 h. The dry homogenates were resuspended in 20 ml of water and used for baiting. The infectivity of the vegetative structures was tested by placing 10 20- μ l droplets/leaf on 20 detached tomato leaves.

The perlite tomato-baiting method. In preliminary experiments, 20 g of air-dried natural or autoclaved sandy loam soils each was mixed with 10 ml of the homogenates containing oospores, and tomato leaves were floated on top of the soil to allow for oospore infection. Most leaves turned yellow in about a week and no late blight lesions developed. Therefore, the following perlite baiting method was adopted. Perlite (2 g of no. 4; Agrikal, Kibbutz Habonim, Israel) was added to each petri dish containing 20 ml of the homogenate suspension. After thorough mixing with the aid of a glass rod, two leaves of tomato (ZH) or 25 seed of the dwarf tomato cv. Roter Gnom (Syngenta Crop Protection, Basel) were placed on the surface of the mixture in each dish. Dishes were covered with lids and incubated at 20°C (12 h of light/day, 100 μ E m⁻² s⁻¹) for 4 weeks to allow infection by the oospores.

Leaves retained their green appearance (except those that became infected by *P. infestans*) for the whole experimental period. Seed germinated in about 4 days and developed cotyledonary leaves in about 10 days. At this stage, lids were removed from the dishes containing the seedlings and the seedlings were placed in sealed transparent plastic boxes (20 cm in height) to allow for the growth of the seedlings.

Recovery of progeny isolates. To produce single-sporangium isolates (SSIs), sporangia were moved from a sporulating leaf lesion into cold distilled water and their concentration was adjusted to 50 sporangia/ml. Droplets (10 μ l) were placed individually on glass slides and examined under the microscope. Droplets which contained a single sporangium were taken up and each placed on a potato tuber disc. In all, 100 tuber discs of potato (cv. Alpha, 1 cm in diameter, 3 mm thick) were used per sporangial sample. Inoculated tuber discs were maintained on dry filter paper in closed petri dishes at 20°C in the dark. About 5 to 30 discs showed sporulation of *P. infestans* at 7 days post inoculation (dpi). Similar infectivity rates were reported previously (17). Each such sporulating disc was considered as producing an SSI. These SSIs were used for the assays described below.

Infected seedlings were seen at 17 to 26 days after seeding in the perlite with oospore homogenate suspension. Infected seedlings were removed and each was sandwiched between two slices of potato tuber (Alpha, 3 mm thick). Sandwiches were placed on dry filter paper in petri dishes in the dark. After 1 week, when sporangia developed on the tuber slices, they were removed and used to produce SSIs in the manner described above or used as bulk isolates. These SSIs and bulk isolates were used similarly for the assays described below.

Sensitivity to metalaxyl. A 25% WP formulation of metalaxyl (Ridomil; Syngenta Crop Protection) was used. Sensitivity to metalaxyl of the parental isolates and each SSI was tested according to Kadish and Cohen (17) with modifications. In all, 10 tomato (ZH) leaf discs (10 mm diame-

ter) were placed with lower surface up on a 7-cm circle of filter paper (Whatman no. 1) saturated with 1 ml of metalaxyl suspension in a 9-cm petri dish. Concentrations (a.i.) of metalaxyl used were: 0, 0.01, 0.1, 1, 10, and 100 μ g/ml. Sporangial concentration of a test isolate was adjusted to 2,000 sporangia/ml and a 10- μ l droplet (containing about 20 sporangia) was placed onto each leaf disc. Plates were incubated at 20°C (12 h of light/day). Two independent tests were performed with each test isolate. At 7 dpi, the infected leaf discs were removed and placed in 10 ml of 50% ethanol solution and vortexed for 15 s, and the number of sporangia produced at each concentration of metalaxyl was counted with the aid of a haemocytometer. The efficacy of metalaxyl in inhibiting the sporulation of the pathogen was calculated as 100 (1 - X/Y), where X = mean number of sporangia/disc produced in leaf discs treated with a certain concentration of metalaxyl, and Y = the mean number of sporangia produced in water control leaf discs. Linear regression analysis was conducted between the log concentration of metalaxyl (μ g/ml) and probit control efficacy of sporulation (%) using SPSS 11.5 for Windows. The concentration of metalaxyl required to inhibit sporulation by 50% (ED₅₀) was calculated based on the linear regression equations. The mean ED₅₀ value from the two replicate experiments, and the standard error of that mean, were calculated for each isolate.

Isolates were classified into four groups according to their sensitivity or resistance to metalaxyl: sensitive = isolates showing a mean ED₅₀ of 0.01 to 0.1 μ g/ml, moderately sensitive = isolates showing a mean ED₅₀ of 0.11 to 1 μ g/ml, intermediately resistant = isolates showing a mean ED₅₀ of 1.1 to 10 μ g/ml, and resistant = isolates showing a mean ED₅₀ of >10 μ g/ml.

Mating type determination. Mating type was determined in detached tomato leaves as described before (5). Briefly, two tomato leaves were placed on moist filter paper in each of three petri dishes. Leaves in dish 1 were inoculated with sporangia of the isolate to be tested, leaves in dish 2 were inoculated with a sporangial mixture made of the tested isolate + A1 tester isolate, and leaves in dish 3 were inoculated with sporangial mixture of the tested isolate + A2 tester isolate. Droplets of a sporangial suspension (10 μ l, 2,000 sporangia/ml) were placed onto each leaflet. The tester isolates used were TO4 (A1) and 367 (A2) (Table 1). Plates were incubated at 20°C with 12 h of light/day. At 10 dpi, leaves were placed in boiling ethanol for 10 min, mounted in 70% glycerol, and examined microscopically for the presence of oospores. The mating types observed were classified as A0 = sterile, no oospores present in any of the three dishes; A1 = oospores present in dish 3 only (mating with A2 only); A2 = oospores present in

Table 1. Origin and characteristics of isolates of *Phytophthora infestans* used in this study

Isolate	Country	Origin	Mating type	Sensitivity to metalaxyl ^a
TO4	Switzerland	Potato	A1	S
AD	Israel	Potato	A1	S
SDUZ	Israel	Potato	A1	S
870	Israel	Potato	A1	S
MIVT	Israel	Potato	A1	S
TH	Israel	Potato	A1	S
96	Switzerland	Potato	A1	S
EH-1	Israel	Potato	A1	S
367	Israel	Potato	A2	R
YAS	Israel	Potato	A2	R
US-8	United States	Potato	A2	R

^a S = sensitive (concentration of metalaxyl required to inhibit sporulation by 50% [ED₅₀] = 0.01 to 0.1 μ g/ml) and R = resistant (ED₅₀ >10 μ g/ml).

dish 2 only (mating with A1 only); and A1A2 = oospores present in both dish 2 and dish 3 but not in dish 1 (mating with both A1 and A2, but self-sterile).

RESULTS

Differential elimination of vegetative structures of *P. infestans*. Detached tomato leaves infected with A1 or A2 isolates were homogenized in distilled water and the homogenates, containing mycelia, sporangia, and zoospores, were subjected to either two cycles of freezing (in liquid nitrogen) and thawing, one cycle of drying and wetting, two cycles of drying and wetting, or left untreated. Droplets of each homogenate were inoculated onto healthy, detached tomato leaflets in order to examine the infectivity of the pathogen. Percent successful infection, as taken at 7 dpi, was 10, 5, 0, and 100%, respectively. The results showed that all treatments significantly reduced the number of successful infections relative to untreated inoculum. No infections developed only in the treatment of two cycles of drying and wetting. Similar results were obtained when healthy detached leaflets of tomato were floated on the inoculum homogenates or on their mixtures with perlite. These results suggested that the vegetative structures of *P. infestans* fail to survive two cycles of drying and wetting.

Baiting substrate and oospore infectivity. Detached tomato leaves inoculated with a mixture of A1S + A2R sporangia produced numerous oospores by 10 dpi. No oospores were produced in leaves inoculated with sporangia of either A1S or A2R isolates. Leaf homogenates containing oospores and control homogenates with a single isolate were subjected to two cycles of drying and wetting and, thereafter, suspended in water and mixed with

either sandy-loam soil or perlite or left as is, and placed into petri dishes. Detached leaflets of tomato were floated on each mixture and allowed to produce late blight infections in growth chambers at 20°C. Late blight lesions with sporulation of *P. infestans* were observed only in detached leaves (normally one lesion per leaflet) floating on the perlite mixtures containing oospores. Leaflets floating on water suspensions or soil mixtures deteriorated too quickly to allow for disease development. In perlite mixtures, lesions appeared, in some experiments, as early as 6 dpi. In most experiments, they appeared at 8 to 15 dpi whereas, in some, as late as 30 dpi or not at all. The number of tomato leaflets showing infection varied among experiments (see below).

Parental isolates and infectivity of oospores. Results obtained from 13 independent leaf baiting experiments are summarized in Table 2. They were conducted with tomato leaflets floating on perlite mixed with homogenates of blighted tomato leaves containing either oospores (and vegetative structures) or vegetative structures only of *P. infestans*. Each datum represents the number of leaflets out of 20 leaflets/assay (2 leaflet/dish, 10 dishes/assay) on which a late blight lesion had developed. Altogether, 8 A1S and 3 A2R parental isolates (Table 1) and 10 A1 × A2 crosses were used. The results (Table 2) show that late blight lesions developed in only the baiting dishes which contained oospores. Out of 840 leaflets, 97 leaflets (11.5%) developed symptoms of late blight. No blight lesions appeared in the control leaflets (total of 1,120 leaflets). This confirms that the two cycles of drying and wetting employed to the pathogen effectively killed its vegetative structures while keeping oospores infective.

Oospores differed in their infectivity (or perhaps in sensitivity to repeated drying and wetting) depending on their parental isolates (Table 2). For example, oospores produced by mating of YAS × T04 were not infective in each of 5 independent experiments, whereas those produced by 367 × AD (10 experiments) and 367 × SDUZ (11 experiments) produced infection in 18 and 16.5% of all leaflets used, respectively.

Similar results were obtained when germinating seed of tomato served as bait. In three independent experiments, 25 seed were allowed to germinate in each of 10 petri dishes containing perlite mixed with oospores of 367 × AD or 367 × SDUZ. As controls, perlite mixed with homogenates of leaflets inoculated with only 367, AD, or SDUZ were used. No late blight developed in the plants (200 to 230 plants/isolate/experiment) growing in the control dishes. In dishes containing oospores, infected seedlings were first seen at 17 days after seeding as necrosis on the hypocotyls or the cotyledons. In experiments 1, 2, and 3, the number of infected seedlings in the perlite-oospores mixtures was 0, 1, and 11, respectively (about 0.9% of all seedlings). In experiment 2, the one infected plant was recovered from 367 × AD and, in experiment 3, three plants (1.4% of all plants) were recovered from 367 × AD and eight plants (3.5% of all plants) from 367 × SDUZ.

Sensitivity to metalaxyl of progeny isolates. Mean ED₅₀ values of metalaxyl for 25 SSIs produced from 25 families (24 lesions produced on leaves and 1 produced in a seedling) are given in Figures 1 through 3.

Mean ED₅₀ values varied among crosses, families (lesions), and isolates (Figs. 1–3). Data for each cross are compiled in Figure 4.

Table 2. Infectivity of oospores of *Phytophthora infestans* mixed with perlite to detached tomato leaflets^a

Inoculum in perlite	Number of leaflets infected out of 20 leaflets inoculated in 13 baiting experiments												
	1	2	3	4	5	6	7	8	9	10	11	12	13
TO4 × 367	0	0	0	3	0	2	3	–	–	–	1	2	–
AD × 367	1	–	0	0	0	5	7	–	5	–	1	7	10
SDUZ × 367	1	1	1	2	2	2	5	8	–	4	6	–	6
870 × 367	1	–	–	–	–	–	–	–	–	–	–	–	–
MIV × 367	1	–	–	–	–	–	–	–	–	–	2	–	–
TH × 367	1	–	–	–	–	–	–	–	–	–	–	–	–
96 × 367	...	–	–	–	–	–	–	–	–	–	–	–	1
TO4 × YAS	–	0	0	0	0	0	–	–	–	–	–	–	–
EH1 × US-8	–	–	–	–	–	–	–	–	–	–	5	...	–
TO4	0	0	0	0	0	0	0	–	–	–	0	0	–
AD	0	–	0	0	0	0	0	–	0	–	0	0	0
SDUZ	0	0	0	0	0	0	0	0	–	0	0	–	0
870	0	–	–	–	–	–	–	–	–	–	–	–	–
MIVT	0	–	–	–	–	–	–	–	–	–	0	–	–
TH	0	–	–	–	–	–	–	–	–	–	–	–	...
EH-1	–	–	–	–	–	–	–	–	–	–	0	–	–
96	–	–	–	–	–	–	–	–	–	–	–	–	0
367	0	0	0	0	0	0	0	0	0	0	0	0	0
YAS	–	0	0	0	0	0	–	0	–	–	–	–	–
US-8	–	–	–	–	–	–	–	–	–	–	0	–	–

^a The perlite contained homogenates of blighted tomato leaves carrying either vegetative or vegetative and sexual structures of the indicated isolates of *P. infestans*; – = not done.

Cross 367 × AD produced four types of isolates with the following frequencies of metalaxyl sensitivities: 17% of the isolates were sensitive, 30% moderately sensitive,

44% intermediately resistant, and 11% resistant. Cross 367 × SDUZ produced the same four groups of isolates but at proportions of 27, 47, 25, and 1%, respectively.

Cross 367 × T04 produced two types of isolates, with 20% of them being moderately sensitive and 80% intermediately resistant. Cross 367 × 96 produced only

Fig. 1. Sensitivity to metalaxyl of F1 progeny isolates of nine families derived from three crosses of *Phytophthora infestans* (experiments 5 and 6).

one type of isolate, all of them intermediately resistant to metalaxyl (Fig. 4). The overall proportions of the above four groups among the 250 isolates was 16, 32, 47, and 5%, respectively, which represent a ratio of about 3:6:9:1. The response to

metalaxyl of the isolates (family 267) derived from the infected seedling of experiment 2 is represented in Figure 2A. No SSIs were prepared from the 11 infected plants recovered from experiment 3. Sensitivity to metalaxyl of *P. infestans* recov-

ered from these plants was assayed with bulked sporangia. Seven bulks had an ED_{50} of 0.11 to 1 and four bulks had a value of 1.1 to 10 $\mu\text{g/ml}$. The natural variation in metalaxyl sensitivity within parental isolates was small. For example, ED_{50} in SSIs

Fig. 2. Sensitivity to metalaxyl of F1 progeny isolates of nine families derived from one cross (367 x AD) of *Phytophthora infestans* (experiment 13).

derived from TO4 ranged between 0.06 and 0.1 $\mu\text{g/ml}$.

Mating type of progeny isolates. The mating type of 87 and 165 SSIs obtained in experiments 5 plus 6 and 13 are presented in Tables 3 and 4, respectively. Although, in most families (lesions), all isolates belonged to one mating type, A1 or A2, in others, two or even three (e.g., family 268) mating types occurred. The proportion of the different mating types among the families was strongly dependent on the cross. The frequency of isolates with the different mating types varied greatly among the crosses (Fig. 5, compiled from Tables 3 and 4). Thus, the

proportion of A1 isolates in progenies derived from the crosses $367 \times \text{AD}$, $367 \times \text{SDUZ}$, $367 \times \text{T04}$, and 367×96 was 75.6, 31.0, 2.5, and 0%, respectively, and the proportion of A2 was 13.7, 53.5, 95.0, and 100%, respectively. The unusual mating type A1A2 was observed in 10 isolates (4%) belonging to three families (268, 280, and 281). Isolates of this mating type produced oospores with either A1 or A2 partner, but were self-sterile when cultured alone on tomato leaves. Of all isolates, nine (3.6%) were sterile (A0) and failed to produce oospores with either A1 or A2. In family 267, derived from an infected seedling, 15 isolates

were A1 and 1 was A2. The other 11 infected seedlings all carried the A1 mating type.

No association was apparent between the sensitivity of an isolate to metalaxyl and its mating type (Table 5). For example, all isolates of families S4 and 283 were A2, yet each isolate exhibited a different level of sensitivity to metalaxyl. Similarly, all isolated of families 269 and 273 were A1, with each isolate exhibiting a different level of sensitivity to metalaxyl.

DISCUSSION

Infection with in vivo-produced oospores of *P. infestans* usually is achieved

Fig. 3. Sensitivity to metalaxyl of F1 progeny isolates of nine families derived from two crosses ($367 \times \text{SDUZ}$ and 367×96) of *Phytophthora infestans* (experiment 13).

by floating healthy leaves of potato on water-saturated soils containing oospores (7,29). In this study, we report on an improved method to induce infection of late blight with oospores of *P. infestans* produced in tomato leaves. With this method, perlite is used instead of soil and detached tomato leaves or tomato seedlings are used as bait instead of potato leaves. The tomato leaves carrying oospores are crushed, subjected to two cycles of drying and wetting, and then mixed with perlite on which healthy tomato leaves or tomato seed are placed. The major advantage of this method is that leaves floating on perlite remain green for about a month, unlike leaves floating on soil, which have to be replaced frequently because of deterioration (29). The prolonged survival of the tomato leaves on the perlite facilitates oosporic infection. Perlite probably is more suitable than soil because it is inert, sterile, and highly aerated. This method also enables quantification of the oospores, produced in planta by different crosses of *P. infestans*, to cause infection in tomato leaves. The method was successfully adapted for *P. viticola* in grape leaves for studying the inheritance of fungicide resistance (U. Gisi, Syngenta Crop Protection; personal communication).

The oospores we used here were produced in planta. Therefore, a major concern was the differential elimination of the vegetative structures (sporangia, zoospores, and mycelia) of the parental isolates present in the infected leaves while keeping the oospores viable. Our previous data (6) show that sporangia of *P. infestans* do not survive repeated drying and wetting due to extensive deterioration of their mitochondrial cristae. Our recent experiments made with homogenates of infected tomato leaves carrying oospores (25) confirmed that two cycles of drying and wetting, but not freezing and thawing, are detrimental to the vegetative propagules of the pathogen but not to the oospores. Similar results were obtained by Fernandez-Favia et al. with oosporic soils (9). Therefore, this procedure was employed in the present study to assure that only oospores were viable.

The perlite–tomato baiting method employed in this study was shown to be effective in producing oosporic infections in tomato leaves and seedlings. Lesions of late blight developed on the bait leaves floating on perlite containing oospores but never on leaves floating on perlite containing just the vegetative structures of the pathogen that had been subjected to two cycles of drying and wetting. Similar results were obtained when germinating tomato seed served as bait. This is the first full report showing the usefulness of perlite in supporting oosporic infections in tomato leaves and seedlings (preliminary results were published earlier; 25).

Although our major objective in this study was to develop an improved method

for oosporic infection, some interesting features of the progeny isolated were observed. The fact that the A1 and A2 parent isolates used for crossings were metalaxyl-

sensitive and -resistant, respectively, enabled us to analyze in vivo both the inheritance of metalaxyl resistance and the inheritance of mating type in the F1

Fig. 4. Overall distribution of progeny isolates of *Phytophthora infestans* derived from four crosses among the four classes of metalaxyl sensitivity.

Table 3. Mating type of progeny isolates originated from three crosses of *Phytophthora infestans* (experiments 5 and 6)

Cross	Family	Number of isolates			
		Total	A1	A2	Not tested
367 × AD	S1	18	17	1	...
	S6	5	5	...	...
	S7	9	...	8	1
	S10	5	5	...	...
	Subtotal	37	27	9	1
367 × SDUZ	S2	2	...	2	...
	S3	5	5	...	...
	S8	3	...	3	...
	Subtotal	10	5	5	...
367 × T04	S4	20	...	19	1
	S5	20	1	19	...
	Subtotal	40	1	38	1
Grand total	...	87	33	52	2

Table 4. Mating type of progeny isolates originated from four crosses of *Phytophthora infestans* (experiment 13)

Cross	Family	Number of isolates				
		Total	A1	A2	A1A2	Sterile
367 × AD	267	16	15	1	...	...
367 × AD	268	18	8	3	6	...
	269	11	11	...	...	...
	270	4	3	1	...	...
	271	3	2	...	...	1
	273	7	7	...	...	...
	275	15	14	...	...	1
	276	8	...	4	...	...
	276A	12	12	...	...	...
	Subtotal	94	72	9	6	2
367 × SDUZ	277	15	...	12	...	3
	278	12	11	...	...	1
	279	8	5	...	...	3
	280	12	1	9	2	...
	281	4	...	2	2	...
	282	10	...	10	...	...
Subtotal	...	61	17	33	4	7
367 × 96	283	10	...	10	...	...
Grand total	...	165	89	52	10	9

progenies. Genetic analyses previously were reported for oospores produced in agar cultures (2,8,11,13–16,18,30).

The analyses were performed with 236 SSIs derived from 24 single leaf lesions of late blight and 16 such isolates derived from a blighted seedling. Lesions were produced by oospores of four A1S + A2R crosses. Most F1 progeny isolates exhibited various levels of intermediate resistance to metalaxyl. No progeny isolate was as resistant as the resistant parent. A similar pattern of variation in F1 was obtained by Knapova et al. (18). Although some of our progeny isolates were A1 sensitive (like the sensitive parent), indicating that they might have evolved from selfing (11,18), others were A2 sensitive (e.g., families S8 and 282), indicating their recombination origin. Indeed, simple sequence repeat (SSR) DNA analyses showed that most SSIs have been derived from mei-

otic progeny (S. Klarfeld, U. Gisi, and Y. Cohen, *unpublished*).

Unlike most other studies (18) in which metalaxyl resistance was quantified by measuring mycelium radial growth in vitro, we used a leaf disc bioassay (17) in which the number of sporangia produced per disc was determined. Isolates were classified into four groups (rather than three; 18) according to their ED₅₀: sensitive, moderately sensitive, intermediately resistant, and resistant. The distribution of the progeny isolates among the four sensitivity groups was strongly dependent on their sensitive parent. In three of four crosses, all, or most, isolates were intermediately resistant (ED₅₀ = 1.1 to 10 µg/ml); whereas, in one cross, most isolates were moderately sensitive (ED₅₀ = 0.11 to 1 µg/ml). The reasons for the difference between the crosses are not clear. Because the same resistant parent (367) was used in all four crosses, the difference

between the crosses is due at least partly to the sensitive parent determining the sensitivity distribution of its offspring.

The literature contains evidence that resistance to metalaxyl in *P. infestans* is controlled by a single, partially dominant nuclear gene, possibly accompanied by minor genes (8,11,15,18,27). The assumption that minor genes might be involved was raised mainly due to the unexpected segregation in F2 or backcross populations. The distortion from Mendelian segregation was explained by the quoted effects of minor loci (8). One report proved a single dominant gene (20).

The data provided here show a large variation in sensitivity levels to metalaxyl, not only among lesions derived from different crosses but also among lesions derived from the same cross as well as among isolates derived from a single lesion of late blight. This might indicate that such minor genes may be contributed by the sensitive parents. Their number and impact probably depend on the nature of the sensitive parent.

Previous studies in *P. infestans* suggested non-Mendelian inheritance, in particular at the mating-type locus (13,14,16,30). Judelson et al. (16) found only two of the four expected combinations of gametes in the progeny of some crosses, suggesting balanced lethals and possibly generated by balanced translocations.

Here, we show that the oospores of *P. infestans* extracted from tomato leaves produced offspring isolates which belong to one, two, or three mating types (excluding nine sterile isolates and five unknowns). The number of mating types and the number of isolates which belonged to each mating type were dependent on the cross. In one cross, only A2 isolates appeared; in the second, an A2:A1 ratio of 38:1 was observed; in the third, a ratio of about 1:2; and, in the fourth cross, a ratio of about 5:1 between A1 and A2 isolates was seen. In the last two crosses, four and six isolates, respectively, belonged to the A1A2 mating type. The reasons for these differences are not clear. Again, because the same A2 parent (367) was used in all four crosses, the differences may be attributed to the A1 parents.

Mating type also varied among isolates taken from the same lesion. Of the 25 families examined, 10 produced offspring which all belonged to the A1 mating type; 8 produced isolates which all belonged to the A2 mating type; 4 produced both A1 or A2 isolates, with one of them strongly dominating the population; 2 produced isolates which belonged to either A1, A2, or A1A2; and 1 produced isolates belonging to either the A2 or A1A2 mating type.

It is not possible to determine how many oospores were involved in the formation of each late blight infection in our tomato-bait system. The fact that 18 leaf infections have produced isolates of a single mating

Fig. 5. Overall distribution of progeny isolates of *Phytophthora infestans* derived from four crosses among the four classes of mating type.

Table 5. Relationship between mating type and sensitivity to metalaxyl in progeny isolates of *Phytophthora infestans*

Cross	ED ₅₀ (µg/ml) ^a	Number of isolates				
		Total	A1	A2	A1A2	Sterile
367 × AD	0.01–0.1	18	9	7	— ^b	2
	0.11–1	35	33	2	—	—
	1.1–10	55	49	2	4	—
	>10	13	10	1	2	—
Total	...	121	101	12	6	2
367 × SDUZ	0.01–0.1	18	7	7	3	1
	0.11–1	32	11	19	1	1
	1.1–10	16	3	9	—	4
	>10	1	1	—	—	—
Total	...	67	22	35	4	6
367 × TO4	0.01–0.1	—	—	—	—	—
	0.11–1	14	—	14	—	—
	1.1–10	25	1	24	—	—
	>10	—	—	—	—	—
Total	...	39	1	38	—	—
367 × 96	0.01–0.1	—	—	—	—	—
	0.11–1	—	—	—	—	—
	1.1–10	9	—	9	—	—
	>10	—	—	1	—	—
Total	...	10	—	—	—	—
Grand total	...	237	124	10	10	8

^a ED₅₀ = concentration of metalaxyl required to inhibit sporulation by 50%.

^b — = zero isolates.

type (plus four lesions that produced almost only one mating type) may indicate that one oospore was involved in most infection events.

The appearance of A1A2 isolates is noteworthy. Such genotypes, having conflicting combinations of mating type alleles, are expected to be excluded from the progeny by the model of Judelson et al. (14), but not by the model of Ko (19). A1A2 isolates are sterile when inoculated alone but produced oospores in planta when co-inoculated with either an A1 or A2 partner. A1A2 isolates were reported to emerge after mutagenesis or prolonged culturing on metalaxyl (26). One such isolate was recovered by us from tomato seed harboring oospores (24). In Israel, 7 of 53 isolates obtained recently from potato fields were found to belong to the A1A2 mating type (*unpublished*). No A1A2 isolates were previously reported in the field in Israel (3).

The variation in the phenotypic characteristics of the isolates recovered from oospores in this study stand in accordance with the recent observations of Van der Lee et al. (30). They analyzed the inheritance of molecular markers in two F1 sexual progenies of field isolates. The progenies mapped were generated from oospores obtained by infecting potato leaves with the parental strains and were recovered from sporulating lesions formed on leaves floating on water mixed with soil containing the oospores (*in vivo*). They reveal trisomic progeny and chromosomal rearrangements. A marker linked to the mating-type locus appeared to have translocated to another linkage group. Translocations created the possibility of obtaining one, two, or three copies of a locus or region. These authors concluded, "Remarkably, when progeny of the same parental strains were generated from oospores obtained by co-cultivation on rye medium and germination of these oospores on water agar (*in vitro*), the progeny showed no distorted segregation ratios for the mating-type locus or for markers linked to the mating type." They hypothesized that trisomy and subsequent instability of chromosomal regions or loss of complete chromosomes contribute significantly to the notorious genetic flexibility of *P. infestans*.

Our present study provides an improved protocol for producing oospore infections in tomato leaves and seedlings. It also provides evidence that the F1 offspring isolates produced from oospores generated *in vivo* vary greatly in their phenotypic traits. Current studies (S. Klarfeld, E. Rubin, and Y. Cohen, *unpublished*) show

that these progeny isolates also segregate for virulence and aggressiveness, with some being more virulent to tomato differential cultivars as well as more aggressive on susceptible tomato cultivars compared with their parents.

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