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Chang, Tun-tschu, Ph.D.

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GENETICS OF *PHYTOPHTHORA*: EVIDENCE FOR HYBRIDIZATION

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN BOTANICAL SCIENCES

(Plant Pathology)

AUGUST 1990

By

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ABSTRACT

Single-zoospore cultures from two isolates (A^1 and A^2) of _Phytophthora_ _infestans_ after metalaxyl treatment consisted of A^1 and A^2. Metalaxyl also caused mating type change in 3 A^1 of _P_. _parasitica_. The conversion of A^1 to A^2 by metalaxyl is postulated as a possible origin of A^2 of _P_. _infestans_ in Europe.

When _P_. _parasitica_ was treated with metalaxyl for 6 weeks, many of its zoospore progeny became resistant to metalaxyl. Exposure to metalaxyl also caused zoospore progeny to change colony morphology, growth rate, ability to produce sporangia and ability of zoospores to form colonies, indicating that metalaxyl is mutagenic.

The optimum conditions for activation of oospores of _P_. _infestans_ were to treat oospores with 0.25% $KMnO_4$ for 15 min. Light was required for oospore germination during germination but not during maturation.

Isozyme patterns of _P_. _infestans_ show that selfed progeny from isolates heterozygous at PEP and GPI-1 loci segregated 1:2:1, and that all selfed progeny from homozygous isolates were identical with their respective parents indicating that _P_. _infestans_ is diploid in vegetative state. All sexual progeny from the cross $903S^rX947Cp^r$ were resistant to either chloramphenicol or streptomycin and were hybrid at PEP locus suggesting that

streptomycin-resistance and chloramphenicol-resistance genes were located in cytoplasm.

Selfed progeny from metalaxyl-resistant (M^r) mutants of P. parasitica segregated 3 resistant : 1 sensitive. Selfed progeny from chloroneb-resistant (Cn^r) mutants also segregated 3 resistant : 1 sensitive, indicating that metalaxyl and chloroneb resistance in these mutants are each conferred by a single dominant gene in heterozygous condition. Progeny from the pairing between homozygous M^r and wild type consisted of selfs and hybrids. Progeny from the pairing between homozygous Cn^r resistant to chloramphenicol and resistant to streptomycin consisted of hybrids resistant to either chloramphenicol or streptomycin, suggesting that chloramphenicol-resistance and streptomycin-resistance genes are present in cytoplasm. Progeny from the pairing between homozygous M^r, Cp^r A^1 and homozygous Cn^r, S^r A^2 consisted of 4 selfs from A^1, 6 selfs from A^2, 46 hybrids from the union of A^1 oogonium with A^2 antheridium, and 92 hybrids resulting from the union of A^2 oogonium with A^1 antheridium.

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INTRODUCTION

Although A^1 mating type of *Phytophthora infestans* is present throughout the world, A^2 mating type of this species was known to be present only in Mexico (56) until 1984 when Hohl and Iselin (21) reported the discovery of A^2 isolates of *P. infestans* in Switzerland. Since then A^2 isolates of *P. infestans* have been found in Scotland (31), England and Wales (59), Japan (33), and in potato tubers imported into United Kingdom from Egypt (53). Since the appearance of A^2 mating type of *P. infestans* followed the commercial usage of metalaxyl in Europe for control of late blight of potato and tomato, and certain fungicides have been shown to cause mating type change in *Phytophthora* (5, 27, 29), it was considered possible that metalaxyl application may be a factors leading to the recent appearance of A^2 mating type of *P. infestans* outside Mexico.

Results from previous *Phytophthora* studies showed no evidence for hybridization between different species(8, 9, 17). Moreover, genetic exchange in pairings between different mating types of the same species remains uncertain due to lack of appropriate genetic markers (4, 45, 49). Since cross fertilization in intraspecific mating is a normal phenomenon, it was proposed that hybridization does occur in addition to selfing in the direct mating between different mating types of the same species of *Phytophthora*. Inheritances of isozyme patterns, antibiotic resistance and

fungicide resistance were investigated and the characters were subsequently used to evaluate the hypothesis.

Since previous reports have shown low and inconsistent germination of *P. infestans* oospores (18, 36, 42, 48, 56), factors affecting oospore germination of *P. infestans* were also studied.

LITERATURE REVIEW

Some species of the genus Phytophthora are homothallic but others are heterothallic and require the presence of opposite mating types known as A^1 and A^2 for induction of gametangia and oospores. Stability of the sexual behavior differs within species of the genus. Certain isolates of heterothallic species produced oospores in old single cultures (6, 14, 27, 37, 57, 65) and some isolates of P. palmivora and P. parasitica changed from heterothallic to homothallic after long-term storage (11, 27, 63). Moreover, homothallic P. drechsleri gave rise to both self-fertile and self-sterile single-zoospore cultures (32).

Heterothallic species of Phytophthora are unique in that formation of oospores occurs in pairings between two morphologically and physiologically distinct species, such as between primitive soil-borne P. cinnamomi and advanced air-borne P. infestans (26, 43). This fact suggests that chemical stimulation may be involved in oospore formation during sexual reproduction, a hypothesis originally proposed by Ashby in 1929 (6). However, Ashby did not rule out the possibility of the occurrence of cross fertilization but subordinated it to selfing.

Change of mating type in Phytophthora

It has been observed frequently that abundant oospores were produced by single isolates of some heterothallic

species of *Phytophthora* after long-term storage, but the isolates usually lost their ability to produce oospores after several transfers (11, 51, 64). Ko (27) reported that oospore formation by aged isolates of *P. parasitica* was due to mating type change of certain cells during the storage period. Noon and Hickman (35) reported that individual A^2 but not A^1 mating type of *P. capsici* produced oospores when grown on a medium containing chloroneb and suggested that this fungicide caused a permanent change of the test organism from self-sterile to self-fertile. When A^2 isolates of *P. parasitica* were grown on the same medium with chloroneb, two of the isolates tested also produced oospores in sectors. However, this was found to be due to the conversion induced by chloroneb of certain propagules from A^2 to A^1 (5, 27). A^1 isolates of *P. parasitica* and *P. cinnamomi* can also be converted to A^2 mating type after chloroneb treatment (5). Another fungicide, ethazol, was found to have similar ability to convert A^1 and A^2 isolates of *P. parasitica* and *P. cinnamomi* to A^2 and A^1, respectively (5, 29).

It has been proposed that the center of origin of *P. infestans* is in Mexico because all the 16 races of *P. infestans* were present in Mexico, although not more than 9 races were found elsewhere (34). Moreover, the A^1 and A^2 mating types had a ratio approximating 1:1 among 95 Mexican isolates tested (19). It has been widely observed that A^1 mating type of *P. infestans* was worldwide in distribution,

but that the A^2 mating type was confined to Mexico (56). However, in 1981 A^2 isolates of P. infestans were found in Switzerland (21). Since then A^2 isolates have also been found in Scotland (31), in England and Wales (59) and in Japan (33). A^2 isolates were also found in potato tubers imported into United Kingdom from Egypt (53). Since all the A^2 isolates were detected almost simultaneously from different countries in Europe, these isolates may have evolved from local A^1 isolates via genetic change triggered by factors such as fungicides. It is also possible that A^2 isolates may have existed in Europe at a low and undetected frequency until recently. Moreover, the possibility that some or all A^2 isolates found in Europe may have been imported directly or indirectly from Mexico with host plants also has not been ruled out.

Mutagenic effect of fungicides

The effects of fungicides on fungi are usually only temporary. The test organisms become normal again when transferred to the fungicide-free medium. However, permanent changes induced by fungicides have also been found occasionally. Dunrieu-Trautmann and Tavlitzki (16) reported that chloramphenicol and ethidium-bromide induced permanent change of Ustilago cynodontis from hyphal cells to yeast-like colonies. Shaw and Elliott (54) also obtained some stable morphological variants of P. cactorum when a wild type was treated with streptomycin. The mutagenic effect of

metalaxyl on species of _Phytophthora_ has not been investigated.

Nature of mating in Phytophthora

Although oospore formation by heterothallic species of _Phytophthora_ when paired on the opposite side of the polycarbonate membranes indicates the regulation of sexual reproduction by sex hormones, it did not rule out the possibility of hybridization in direct matings (27). Evidence for exchange in the pairings between different species of _Phytophthora_ has not been provided in any study. Boccas (8) reported that protein patterns of the offsprings from the pairings of _P_. _capsici_ X _P_. _palmivora_, _P_. _parasitica_ X _P_. _capsici_ and _P_. _megakarya_ X _P_. _parasitica_ were all single parental types with the exception of one culture which was of unknown origin. Boccas and Zentmyer (9) showed that all 29 single-zoospore cultures obtained from the cross between _P_. _cinnamomi_ and _P_. _parasitica_ displayed protein band patterns _P_. _parasitica_. Based on morphological characters and protein patterns, Erselius and Shaw (17) reported that the progeny from the mating between _P_. _megakarya_ and _P_. _palmivora_ resulted from selfing. Lengths of sporangial stalks of progenies from the pairing between different morphological forms of _P_. _palmivora_ also were similar to parental types (23).

Whether genetic exchange occurs in the direct parings between different mating types of the same species of

Phytophthora has been the subject of controversy. The pairings between race 3 and race 1, 2 of *P. infestans* resulted in two race 0 offspring in one test (36) and 10 non-pathogenic offspring in another (30). The offspring from the pairing between race 3 and race 0 of *P. infestans* consisted of one non-pathogenic, six race 0 and one race 4 (30). Since vegetative state of *Phytophthora* has been shown to be diploid (40), above data could have resulted from hybridization, selfing, or both.

Mating type has been frequently used as a genetic marker in intraspecific matings of *Phytophthora*. The results have been inconsistent and inconclusive. For example, Khaki and Shaw (24) reported that the variation of the ratios of A^1 : A^2 among progenies from the crosses between different isolates of *P. drechsleri* ranged from 0 : 48 (with 9 neuter) to 30 : 0 (with 3 neuter). Shattock et al. (49) also showed that the distribution of A^1 : A^2 among progenies from the pairing of different isolates of *P. infestans* varied from 4 : 12 (with 3 self-fertile) to 63 : 33 (with 38 self-fertile). The ever variable results of mating type ratio of the progenies from the intraspecific crosses is mainly due to the instability of the mating type trait. Ann and Ko (4) reported that the distribution of A^1 : A^2 among progenies from selfing of A^1 isolates of *P. parasitica* varied from 87 : 3 (with 5 self-fertile) to 73 : 35 (with 5 self-fertile), and the ratios of A^1 : A^2 among progenies from selfing of A^2 isolates ranged from 10 : 112

to 67 : 67 (with 1 self-fertile). It is apparent that mating type is not a suitable marker for genetic studies in *Phytophthora*. These results also do not support the hypothesis that A^1 is homozygous recessive aa, and A^2 is heterozygous Aa (40), because homozygous recessive A^1 should not segregate in selfing.

Antibiotic resistance has also been used as genetic markers for determining genetic exchange in intraspecific mating of *Phytophthora* (24, 55, 61). However, the results are inconclusive because the location of resistant genes are in nuclei or cytoplasm has not been determined. Ann and Ko (4) found that the progenies from the crosses between chloramphenicol-resistant and streptomycin-resistant isolates of *P. parasitica* were resistant to either chloramphenicol or streptomycin but not both, indicating that there was no genetic exchange for these markers. However, hybridization is not ruled out if resistance genes are located in the cytoplasm.

Isozyme patterns have been used to show that majority of progenies from direct matings between different isolates of *P. infestans* were from hybridization (45, 49). However, in these studies the assumption that isozyme patterns of the parental isolates were controlled by homozygous genes have not been supported experimentally. Only nine selfed-oospore cultures were analyzed (48).

MATERIALS AND METHODS

Microorganisms

The history of Phytophthora isolates used in this study is given in Table 1. All Phytophthora isolates were from single zoospores and maintained in sterile distilled water at 24 C (10) with the exception of Phytophthora infestans which was maintained at 19 C in darkness.

Isolates of P. infestans were grown on V-8 juice rye agar modified from rye agar described by Caten and Jinks (13). Whole rye grains 50 g were soaked in 1,100 ml distilled water at 24 C for 24-36 hr followed by autoclaving for 30 min. The broth was filtered through four layers of cheesecloth and the filtrate was adjusted to the final volume of 1,000 ml with distilled water. V-8 juice rye agar was prepared by adding 5% V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar to the rye broth. P. parasitica was grown on V-8 agar consisting of 10% V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar.

Isolation of single zoospores

A small piece (3 X 3 X 2 mm) of P. infestans culture was placed at the center of a V-8 rye agar plate. After incubation at 19 C for 6 days in darkness, 10 ml of sterile distilled water was added to each plate to dislodge the sporangia from sporangiophores. Zoospores were obtained by chilling the sporangial suspension at 5 C for 3 hr. About

Table 1. List of isolates of Phytophthora used

Species and culture no.	Mating type	Habit association	Donor
P. parasitica			
P991	A^1	Citrus soil	G. A. Zentmyer
6128	A^1	Eggplant fruit	W. H. Ko
6133	A^1	Guava seedling	W. H. Ko
P731	A^2	Citrus soil	G. A. Zentmyer
6167	A^2	Papaya fruit	M. Aragaki
6195	A^2	Gazania	C. Hodges
6317	A^2	Guava seedling	W. H. Ko
6115	A^2	Roselle stem	W. H. Ko
6134	A^2	Eggplant fruit	W. H. Ko
P. infestans			
533	A^1	Mexico; potato	W. H. Fry
Pinp-1[a]	A^1	Hawaii; potato	W. H. Ko
Pint-1[b]	A^1	Hawaii; tomato	W. H. Ko
550	A^2	Mexico; Solanum sp.	W. H. Fry

[a] Representing 26 tested cultures obtained from the same place.
[b] Representing 8 tested cultures obtained from the same place.

100 zoospores were spread on rye agar. After incubation at 19 C for 3-5 days, colonies originating from single zoospores were transferred onto V-8 rye agar. Five colonies were evenly distributed around the edge of each plate. For P. parasitica, single-zoospore cultures were obtained by the method of Ko (27).

Formation of oospores

V-8 juice rye agar and V-8 juice agar were used for oospore formation of P. infestans and P. parasitica, respectively. Direct mating was done by placing two pieces of agar culture (3 X 3 X 3 mm) of the opposite mating types 5 mm apart on an agar block (15 X 15 X 3 mm) at the center of a small Petri plate (60 mm). These plates were then sealed with two layers of parafilm and incubated in darkness for 10 days at 24 C and 19 C for P. parasitica and P. infestans, respectively, for oospore formation. Cultures of P. parasitica were incubated for a total of 2 to 4 months under fluorescent light (2,000 lux) for oospore maturation (3). Cultures of P. infestans were maintained continuously at 19 C in darkness for an additional 10 days for oospore maturation. The polycarbonate membrane method described by Ko (25) was used to produce selfed oospores. P. infestans culture block (15 X 10 X 3 mm) of a 4-day-old A^1 or A^2 mating type was placed in the center of a Petri dish to serve as oospore producer. The culture block was covered with a sterile polycarbonate membrane (0.2 um, 90-mm

diameter; Nuclepore Co., Pleasanton, CA 94566) and was chemically induced to produce oospores by pairing on the opposite side of the membrane with a 4-day-old culture block of A^2 or A^1 type, respectively, to serve as the hormone producer. After incubation for 10 days at 19 C in darkness in a moist chamber, the membrane and the hormone producer on it were removed and the culture block below with the oospores on the bottom was further incubated for 2 weeks for oospore maturation under the same conditions as described above. For P. parasitica, selfed oospores were obtained using the method described by Ann and Ko (4).

Germination of oospores

The method of Ann and Ko (3) was used for germinating oospores of P. parasitica. For P. infestans, oospore suspension was obtained by grinding each culture containing oospores with 50 ml of distilled water in an Omni mixer at 4,500 rpm for 1 min. The suspension was filtered successively through a 53-um and a 20-um sieve. Oospores retained on the 20-um sieve were washed with tap water and resuspended in 10 ml of sterile distilled water. Oospore suspension was mixed with an equal volume of freshly prepared 0.5% $KMnO_4$ solution. After shaking for 15 min on a shaker, oospores were washed free of $KMnO_4$ on a 20-um sieve with tap water. About 100-200 oospores were spread on S+L medium of Ruben et al. (38) amended with 0.01% asparagine and 2% Bacto agar. After autoclaving, the medium was

supplemented with 100 ug of ampicillin, 50 ug of nystatin and 10 ug of pentachloronitrobenzene per milliliter to prevent growth of possible contaminants. After incubation at 19 C under light for 10 to 20 days, germinating oospores were individually transferred to V-8 rye agar.

Analysis of glucose phosphate isomerase (GPI-1) and peptidase (PEP) of P. infestans

Preparation of samples -- Two pieces (5 X 5 X 3 mm) of culture blocks of *P. infestans* were transferred to 25 ml of V-8 rye broth in a 250-ml flask. After incubation for 8-10 days at 19 C in darkness, mycelial mass was removed from the liquid medium and washed with distilled water on a Whatman #1 filter paper. Approximately one part by weight of fresh vacuum-dried mycelia was homogenized with one part of extraction buffer (0.05 M tris-HCl, pH 7.1) with about 0.08 g of fine sea sands using a prechilled motar and pestle. Samples were centrifuged in a Sorvall RC 2-B refrigerated centrifuge with SS-34 rotor at 10,000 rpm for 40 min. One volume of the supernatant was mixed with 1/2 volume of 50% (V:V) glycerol water solution containing 0.002 % bromophenol blue as a tracking dye. Electrophoresis was initiated using these prepared samples. Two replicates were used for each culture and the experiment was repeated once.

Gel preparation -- The buffer systems of gels and electrodes were modified from Tooley et al. (62) for PEP and from Selander et al.(44) for GPI-1. The gel for running PEP

contained 8% acrylamide/bis, 0.355 M tris-borate-Na_2EDTA (0.225 M tris-0.125 M boric acid-0.005 M Na_2EDTA), 0.1% TEMED (N,N,N',N'-tetramethylethylenediamine), and 0.05% APS (ammonium persulfate). The gel for running GPI-1 consisted of 7.5% acrylamide/bis, 0.11 M continuous tris-citrate I at pH 6.7 (0.08 M tris-0.03 M citric acid, pH adjusted with 1 N NaOH), 0.1% TEMED and 0.05% APS. All reagents except APS and TEMED were mixed together followed by degassing under vacuum for at least 15 min. APS and TEMED were added to degassed solution and the solution was poured into a glass slab mold (16 cm wide, 20 cm long and 1 mm thick). A 15-well-comb was placed on the top of the glass slab mold. The gels were polymerized for 45 to 60 min. The comb was removed from the polymerized gel and the wells were rinsed thoroughly with distilled water.

Sample loading -- Ten ul of each sample for GPI-1 or 20 ul of each sample for PEP was carefully transferred into a well with a microsyringe. After allowing the samples to migrate for 5 min, the excess samples were removed from the wells with a syringe.

Electrophoresis -- Electrophoresis was carried out in 0.206 M continuous tris-citrate I electrode buffer at pH 6.3 (0.149 M tris-0.057 M citric acid, pH adjusted with 1 N NaOH) for GPI-1 and 0.284 M tris-borate-Na_2EDTA electrode buffer (0.18 M tris-0.1 M boric acid-0.004 M Na_2EDTA) for PEP. Gels were run at 45 mA per 2 gels for 1 hr followed by 70 mA per 2 gels for 8 hr for GPI-1, and at 140 volts for 1

hr followed by 220 volts for 4.5 hr for PEP. Bio-Rad PROTEAN II vertical slab cell and Bio-Rad model 3000 Xi power supply were used for the electrophoresis process. From enzyme extraction to the electrophoresis process, the samples were maintained at or below 5 C to prevent degradation of the enzymes.

Enzyme staining and genic nomenclature -- Staining procedure used was that of Allendorf et al.(1) and Shaw and Prasad (50). After completion of the electrophoresis process, gels for GPI-1 were immersed in staining solution consisting of 150 ml 0.1 M tris-HCl at pH 8.0, 15 mg $NADP^+$ (Sigma N-5050), 120 mg $MgCl_2$, 3 mg PMS (phenazine methosulfate, Sigma P-9625), 15 mg MTT (Sigma M-2128), 120 mg fructose-6-phosphate (Sigma F-6134), and 30 ul glucose-6-phosphate dehydrogenase (Sigma G-7877 1.5 mg prot./ml) at 35 C until the bands were noticeably clear. For PEP, gels were immersed in staining solution containing 15 mg o-dianisidine (Sigma D-9143), 150 ml 0.1 M phosphate buffer at pH 7.5, 1.5 ml 0.1 M $MnCl_2 \cdot 4H_2O$, 30 mg glycyl-L-leucine (Sigma G-2002), 40 mg L-amino acid oxidase (Sigma A-9253, from *Crotalus adamanteus* type I), and 2 mg peroxidase (Sigma P9250 from horseradish type II) at 35 C for at least 1 hr until the bands were noticeably clear. The staining reactions were terminated by discarding the staining solution followed by washing 3 times with tap water, immersing the gel slab in 1% acetic acid for several min and rinsing with tap water.

Based on the system of genic nomenclature described by Allendorf et al. (1), Tooley et al. (62) have designated allelic variants according to relative electrophoretic mobility by using starch gel electrophoresis for these two enzyme systems in P. infestans. The method for designating allelic variants was modified from Tooley et al. (62) in this study. PEP is presumed to be a dimer protein coded by multiple alleles at a locus. The allele used to code a slow-migrating polypeptide in electrophoresis was designated P^1 and the allele used to code a fast-migrating polypeptide was P^2. GPI-1 is also presumed to be a dimer protein coded by multiple alleles at a locus. The allele used to code a slow-migrating polypeptide was designated G^1 and the allele used to code a fast-migrating polypeptide was G^2. The homozygotes showed one band and the heterozygotes showed three bands on the gel after staining (Fig. 1, Fig.2).

Antibiotic resistance of P. infestans

Induction of mutation was achieved by placing four pieces (10 X 10 X 3 mm) of culture blocks on a Petri plate containing V-8 rye agar amended with 50 ug/ml streptomycin sulfate or 200 ug/ml chloramphenicol. Three plates for each isolate were incubated at 24 C and observed weekly. Mutants which appeared as fast-growing sectors, were transferred to V-8 rye agar containing 300 ug/ml streptomycin sulfate or 200 ug/ml chloramphenicol. Antibiotic resistance was determined by placing culture discs (4 mm) on rye V-8 agar

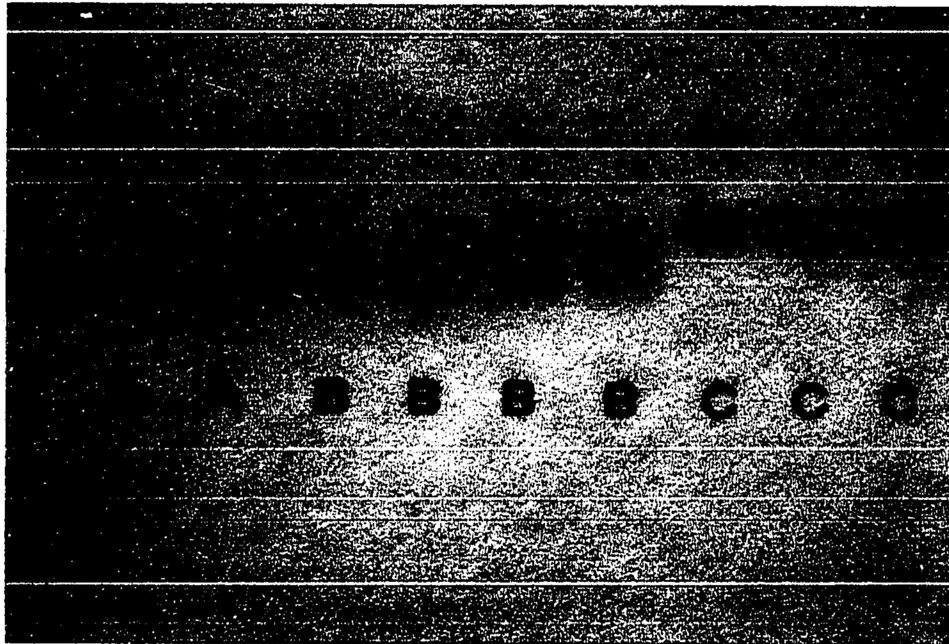

Fig. 1. Zymogram at the PEP locus in vegetative mycelia of *Phytophthora infestans*. Phenotypes with presumed genotypic bases: A = P2P2 fast-migrating homozygous with one banded phenotype, B = P1P2 heterozygous with three banded phenotype, C = P1P1 slow-migrating homozygous with one banded phenotype.

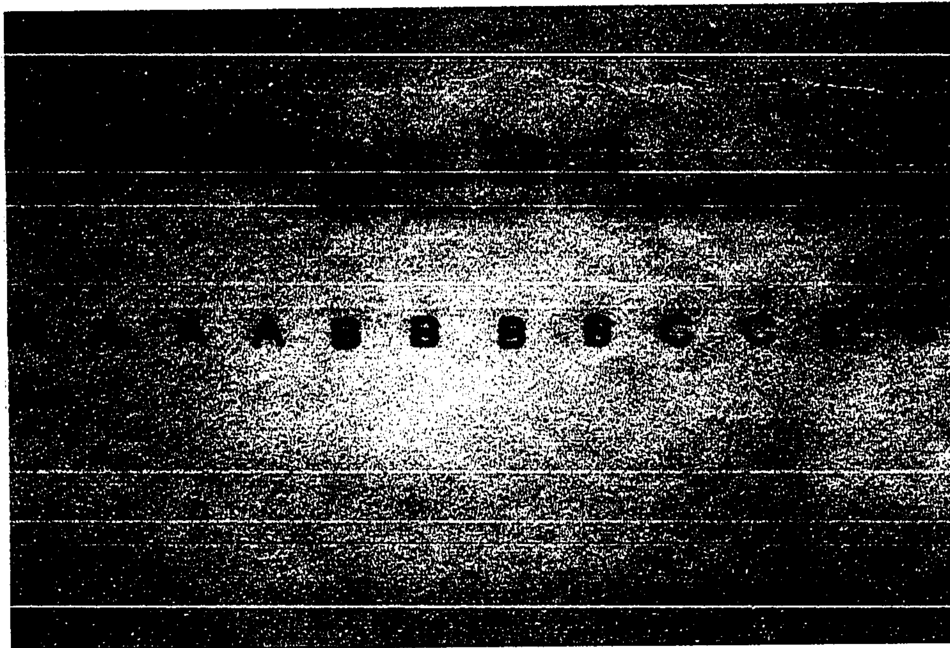

Fig. 2. Zymogram at the GPI-1 locus in vegetative mycelia of Phytophthora infestans. Phenotypes with presumed genotypic bases: A = G1G1 slow-migrating homozygous with one banded phenotype, B = G1G2 heterozygous with three banded phenotype, C = G2G2 fast-migrating homozygous with one banded phenotype.

with and without 300 ug/ml streptomycin sulfate or 250 ug/ml chloramphenicol. Inoculated plates (four culture discs per plate) were incubated at 19 C for 10 days. Those cultures which were sensitive to the antibiotic did not grow or grew sparingly on antibiotic medium, while those which were resistant grew normally and continuously during the incubation period. Two replicates were used for each culture. Symbols that were used concerning antibiotic resistance were Cp^r for chloramphenicol resistance, and S^r for streptomycin resistance. Phenotypes for chemical sensitivity (markers of original traits) including Cp^s and S^s were not mentioned.

Induction of resistance to chloroneb in *P. parasitica*

Isolates $6133Cp^r$ (A^1) and $6134S^r$ (A^2) which were resistant to chloramphenicol and streptomycin, respectively. (4) were used for induction of mutation. Four pieces (10 X 10 X 3 mm) of culture blocks were placed on V-8 agar containing 0 or 100 ug of chloroneb (Terraneb SP, 65% active) per milliliter. Fungicides were added to V-8 agar after autoclaving. For each isolate tested, three plates were used for each concentration. Plates were kept in plastic containers at 24 C and observed weekly for 6-12 weeks. Mutants which appeared as fast-growing sectors, were transferred to V-8 agar containing 100 ug of chloroneb per milliliter.

Metalaxyl treatment

Four pieces (10 X 10 X 3 mm) of culture blocks were placed on V-8 rye agar for *P. infestans* and V-8 agar for *P. parasitica*. The media were supplemented with metalaxyl (Subdue 2E, 25.11% active) at concentrations of 0, 25 or 50 ug/ml after autoclaving. For each isolate tested, three plates were used for each concentration. Plates were kept in plastic containers (32 X 24 X 12 cm) at 24 C and observed weekly for three months. Formation of oospore sectors on the medium was used as an indication of the appearance of the opposite mating type (27). Blocks (5 X 5 X 3 mm) of *P. infestans* and *P. parasitica* oospore sectors or treated cultures were transferred to V-8 rye agar and V-8 agar, respectively, to produce sporangia. Single-zoospore cultures were obtained as described above for determining mating type, metalaxyl resistance and morphological and physiological characters.

Determination of resistance to fungicides and antibiotics in P. parasitica

The method described by Ann and Ko (4) was used to determine chemical resistance of each single-zoospore or single-oospore culture. Metalaxyl resistance was determined by placing culture discs (4 mm) on V-8 agar and V-8 agar amended with 25 ug of metalaxyl per milliliter. Inoculated plates were incubated at 24 C for 7 days. Those cultures which were sensitive to metalaxyl did not grow or grew

sparingly in 7 days on metalaxyl medium, while those which were resistant grew normally and continuously during the incubation period. Chloroneb resistance was similarly determined by comparing growth on V-8 agar and V-8 agar with 100 ug of chloroneb per milliliter. Resistance to chloramphenicol or streptomycin was determined by comparing growth on 5% clarified V-8 agar and 5% clarified V-8 agar supplemented with 250 ug of chloramphenicol or 300 ug of streptomycin sulfate per milliliter. Two plates each with two culture discs were used for each single-spore culture. Symbols used for chemical resistance were Cp^r for resistance to chloramphenicol, S^r to streptomycin, Cn^r to chloroneb and M^r to metalaxyl. Phenotypes for chemical sensitivity (markers of original traits) including Cp^s, S^s, Cn^s and M^s were not mentioned.

To determine the stability of resistance to metalaxyl, resistant mutants were successively subcultured eight times on metalaxyl-free medium at 10-day intervals followed by growth on the medium with and without 25 ug/ml metalaxyl at 24 C for 7 days. Each treatment consisted of two replicates.

Determination of morphological and physiological characters

Four discs (4 mm) of inoculum cut from the advancing margin of a colony were placed at equidistant from each other near the edge of a Petri plate containing V-8 agar for *P. parasitica* and rye V-8 agar for *P. infestans*. After

incubation at 24 C for 4 days for P. parasitica and at 19 C for 8 days for P. infestans, colony morphology and linear growth were recorded. Ability to produce sporangia in P. parasitica was determined by placing a disc of inoculum in the center of a V-8 agar plate. After incubation at 24 C for 4 days, each plate was flooded with 10 ml sterile distilled water. It was then further incubated at the same temperature under light or in darkness to induce sporangial formation. After 4 days. the plates were drained and the sporangia were counted under a microscope. About 200 zoospores were spread on a Petri dish containing 5% V-8 agar. After incubation at 24 C for 2 days, the germination rate was determined and germinated zoospores were marked on the bottom of the dish. The number of colonies developed from germinated zoospores was counted after incubation for two more days. Each treatment consisted of two or three replicates and all experiments were repeated once.

Determination of mating types

Mating type of each single-zoospore and single-oospore cultures of P. infestans was determined by pairing a small piece (3 X 3 X 3 mm) of culture with the same size culture of A^1 tester (533) or A^2 tester (550) on a V-8 rye agar block (10 X 10 X 3 mm) in a Petri dish. Ten blocks were placed in a Petri dish at equidistant from each other near the edge of the Petri dish. After incubation at 19 C for 8-10 days in darkness, each block was examined microscopically

for the presence of oospores. Those isolates forming oospores when paired with A^2 tester were designated A^1. Similarly, those forming oospores with A^1 tester were A^2, and those forming oospores with both A^1 and A^2 tester were A^1A^2 which also formed oospores in the absence of either tester. Similar procedure was used to determine the mating type of _P_. _parasitica_ using P991 as A^1 tester and P731 as A^2 tester. V-8 agar blocks were used for the pairings and paired cultures were incubated at 24 C in darkness for 6 days before microscopic observation for the presence of oospores.

Determination of hormone production and reception

To determine hormone production and reception in parent isolates and sexual variants of _P_. _infestans_ induced by metalaxyl treatment, each isolate was paired with A^1 (533) or A^2 (550) tester using the polycarbonate membrane technique developed by Ko (27). Four-day-old cultures on V-8 rye agar block (15 X 10 X 3 mm) were used for both hormone producer and hormone receptor. For _P_. _parasitica_, each isolate was paired with P991 (A^1 tester) or P731 (A^2 tester) on V-8 agar block. One-day-old cultures were used for hormone producer and three-day-old cultures were used for hormone receptor. Oospore production was recorded after incubation in moist chambers for 10 days at 19 C for _P_. _infestans_ and 24 C for _P_. _parasitica_.

RESULTS

Physiological and morphological effects of metalaxyl on *Phytophthora*

Induction of mating type change by metalaxyl -- *Phytophthora infestans*. Isolates of *P. infestans* tested included two Mexican isolates (533 and 550), 34 Hawaiian isolates all of which were A^1, and 87 (35 A^1, 52 A^2) single-oospore cultures from the direct pairing between 533 and 550. Only two single-oospore cultures (902 and 920) formed oospores in sectors after growing on medium containing 20 or 50 ug/ml metalaxyl for 6 weeks. The rest of the isolates did not form oospores after 3 months on the same medium. Single-zoospore cultures obtained from the oospore sector of 902 consisted of 108 A^1, 61 A^2 and 1 A^1A^2 (Table 2). All the 162 single-zoospore cultures from non-treated 902 were A^1. Single-zoospore cultures were obtained from the A^1A^2 type. Among these cultures 41 were A^1 and 3 were A^1A^2. All the 45 single-zoospore cultures obtained from one of the three A^1A^2 type were A^1. Single-zoospore cultures obtained from the oospore sector of 920 consisted of 47 A^1 and 113 A^2 (Table 2). All the 159 single-zoospore cultures from non-treated 920 were A^2.

902 was capable of producing hormone ^{1} and responsive to hormone ^{2}. When its mating type was changed from A^1 to A^2, the hormone produced was changed from ^{1} to ^{2} (Table 3). The A^2 sexual variant 902-1 became responsive to

Table 2. Mating type distribution in single-zoospore cultures from isolates of *Phytophthora infestans* and *P. parasitica*

Species, isolate & mating type		Treatment	No. of single-zoospore cultures		
			A^1	A^2	A^1A^2
P. infestans					
902	A^1	Metalaxyl	108	61	1
902	A^1	None	162	0	0
920	A^2	Metalaxyl	47	113	0
920	A^2	None	0	159	0
P. parasitica					
P991	A^1	Metalaxyl	64	36	0
P991	A^1	None	103	0	0
6128	A^1	Metalaxyl	42	14	0
6128	A^1	None	109	0	0
6133	A^1	Metalaxyl	55	15	0
6133	A^1	None	70	0	0

Table 3. Hormone production and reception in wild types and sexual variants from metalaxyl treatment of *Phytophthora infestans* and *P. parasitica*

Species and isolate	Origin	Mating type	Hormone production[a]		Hormone reception[b]	
			1	2	1	2
P. infestans						
902		A^1	+	-	-	+
902-1	902	A^2	-	+	+	-
902-2	902	A^2	-	+	-	-
920		A^2	-	+	+	-
920-1	920	A^1	+	-	-	+
920-2	920	A^1	+	-	-	-
P. parasitica						
P991		A^1	+	-	-	+
P991-22	P991	A^2	-	+	+	-
P991-25	P991	A^2	-	+	-	-

[a] +, productive; -, non-productive.
[b] +, responsive; -, non-responsive.

hormone ^{1}, while the other 902-2 became non-responsive to both hormones. 920 was capable of producing hormone ^{2} and responsive to hormone ^{1}. When its mating type was changed from A^2 to A^1, the hormone produced was changed from ^{2} to ^{1}. The A^1 sexual variant 920-1 became responsive to hormone ^{2}, while the other 920-2 became non-responsive to both hormones.

Phytophthora parasitica. Three A^1 and 6 A^2 of P. parasitica were tested. All the 3 A^1 produced oospores in sectors after growing on medium containing 20 or 50 ug/ml metalaxyl for 4 weeks. None of the A^2 formed oospores after 3 months on the same medium. The single-zoospore cultures obtained from oospore sectors consisted of 64 A^1 and 36 A^2 for P991, 42 A^1 and 14 A^2 for 6128, and 55 A^1 and 15 A^2 for 6133 (Table 2). All the single-zoospore cultures from the non-treated P991, 6128 and 6133 were A^1.

P991 was capable of producing hormone ^{1} and responsive to hormone ^{2}. When its mating type was changed from A^1 to A^2, the hormone produced was changed from ^{1} to ^{2} (Table 3) The A^2 sexual variant P991-22 became responsive to hormone ^{1}, while the other P991-25 became non-responsive to both hormones.

Induction of resistance to metalaxyl -- Single-zoospore cultures were obtained from isolates P991 and P731 of P. parasitica grown for 6 weeks on medium containing metalaxyl. When these were tested for susceptibility to metalaxyl, 21% of the former and 13% of the latter were resistant (Table

Table 4. Response to metalaxyl of single-zoospore cultures obtained from *Phytophthora parasitica* after being grown for 6 weeks on V-8 agar containing 25 ug/ml metalaxyl

		No. of zoospore cultures	
Isolate	Treatment	Resistant	Sensitive
P991	Metalaxyl	21	79
P991	None	0	100
P731	Metalaxyl	13	87
P731	None	0	100

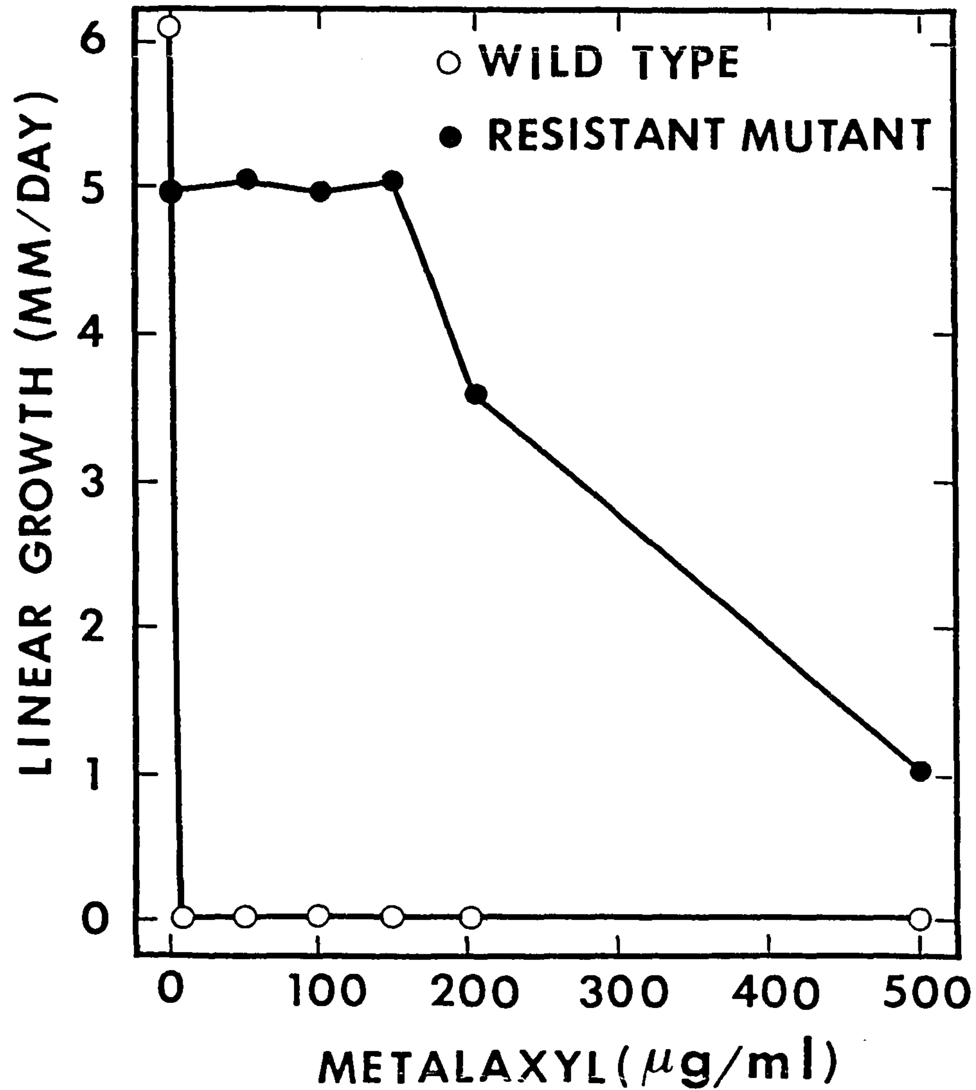

Fig. 3. Growth of wild type (P991) and metalaxyl-resistant mutant of *Phytophthora parasitica* on 10% V-8 agar supplemented with various concentrations of metalaxyl.

Table 5. Relative growth of metalaxyl-resistant mutants of *Phytophthora parasitica* on V-8 agar containing 25 ug/ml metalaxyl after eight successive subcultures

Isolate	Relative growth before subculture (%)[a]	Relative growth after 8 subcultures (%)
P991-9	98	0
P991-10	83	109
P991-12	102	65
P991-13	96	0
P991-8	98	0
P991-14	109	104
P991-19	98	104
P991-20	100	86
P991-21	90	0
P991-22	88	76
P991-23	90	110
P991-25	110	59
P991-29	128	0

[a] Relative growth (%) = growth on V-8 agar amended with 25 ug/ml metalaxyl divided by growth on V-8 agar in percentage.

Table 6. Colony types in single-zoospore cultures of *Phytophthora parasitica* obtained from isolates grown on V-8 agar containing 25 ug/ml metalaxyl at 24 C for 6 weeks

Isolate	Treatment	Colony type[a] and no. of cultures						
		A	B	C	D	E	F	Total
P991	Metalaxyl	43	17	2	35	2	1	100
P991	None	100	0	0	0	0	0	100
P731	Metalaxyl	54	32	2	7	4	1	100
P731	None	100	0	0	0	0	0	100

[a] Single-zoospore cultures grown on V-8 agar at 24 C for 5 days. Symbols: A, irregular zonal pattern, abundant aerial growth; B, uniform texture, abundant aerial growth; C, uniform fine texture, moderate aerial growth, usually slow or intermediate in growth; D, zonal pattern, little aerial growth, radiate; E, irregular mosaic pattern, moderate aerial growth, non-radiate, usually intermediate in growth; F, irregular pattern, little aerial growth, radiate, usually slow or intermediate in growth.

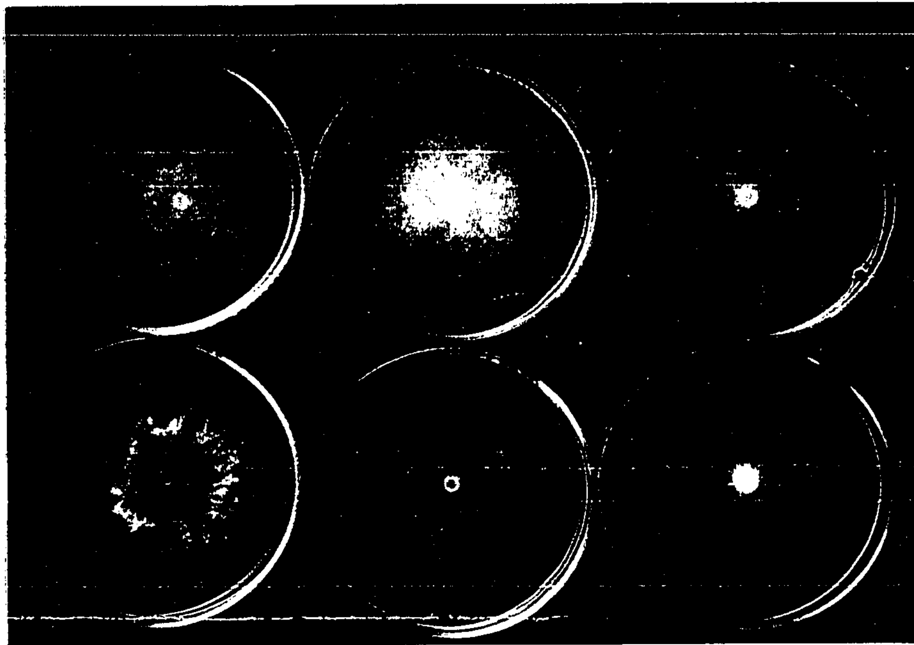

Fig. 4. Colony patterns displayed by single-zoospore cultures obtained from Phytophthora parasitica (P991) treated with metalaxyl. Cultures were grown on V-8 agar at 24 C for 5 days. Upper left, A type; upper center, B type; upper right, C type; lower left, D type; lower center, E type; lower right, F type.

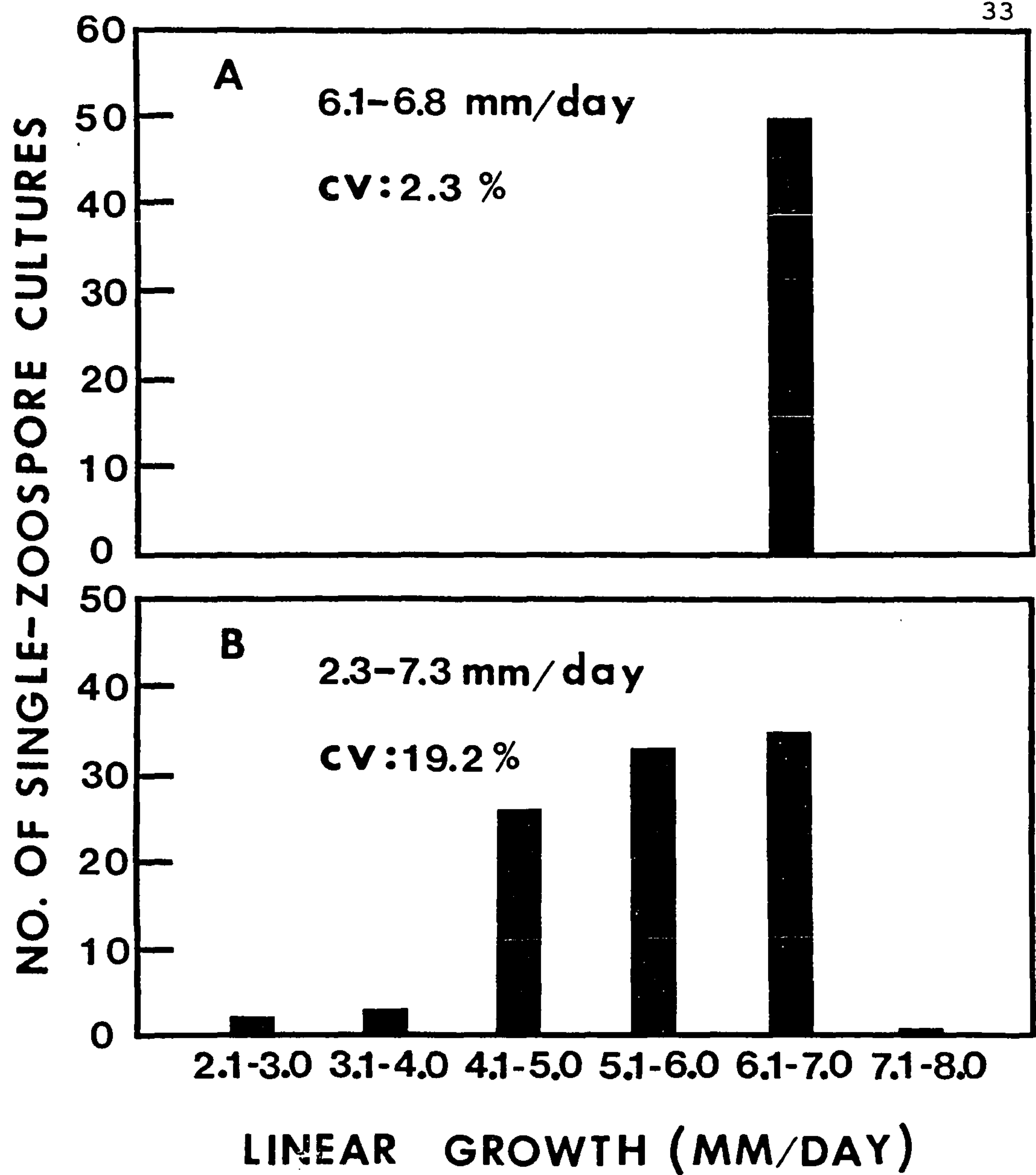

Fig. 5. Growth-rate distributions on V-8 agar of single-zoospore cultures obtained from *Phytophthora parasitica* (P991) grown for 6 weeks on V-8 agar with or without metalaxyl. A, single-zoospore cultures from untreated P991; B, single-zoospore cultures from P991 treated with metalaxyl. CV: coefficient of variation.

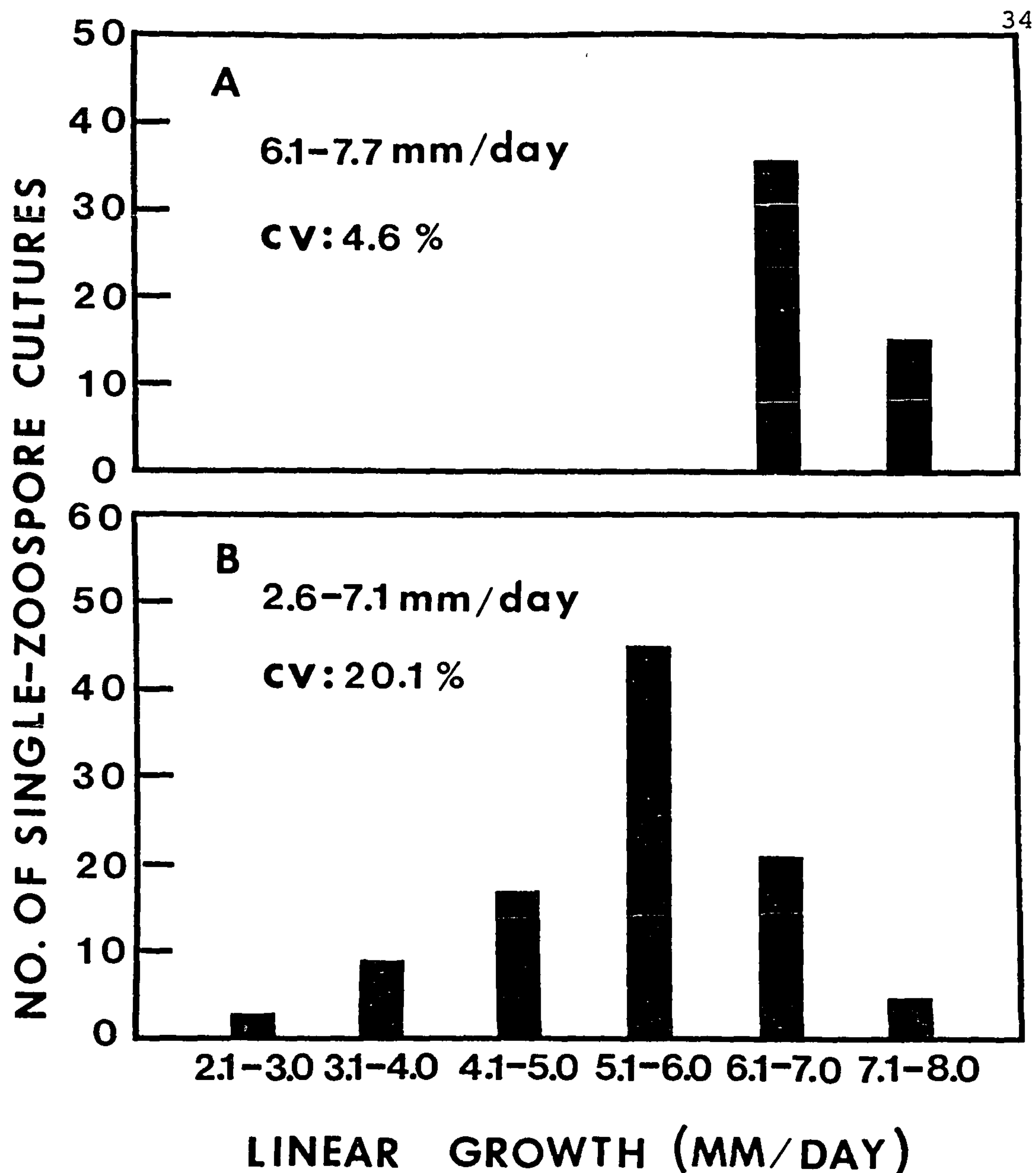

Fig. 6. Growth-rate distributions on V-8 agar of single-zoospore cultures obtained from <u>Phytophthora parasitica</u> (P731) grown for 6 weeks on V-8 agar with or without metalaxyl. A, single-zoospore cultures from untreated P731; B, single-zoospore cultures from P731 treated with metalaxyl. CV: coefficient of variation.

4). Control cultures from neither isolate showed any resistance to metalaxyl. Growth of the resistant mutant was not affected by metalaxyl up to 150 ug/ml, but was reduced at higher concentrations (Fig. 3). Growth of the wild type was completely inhibited by metalaxyl at a concentration of 10 ug/ml. After being successively subcultured on metalaxyl-free medium eight times, four of the 13 resistant mutants tested remained resistant, five became sensitive and four partially lost their resistance to metalaxyl (Table 5).

Permanent change of colony morphology by metalaxyl -- Colonies derived from zoospores of *P. parasitica* isolates P991 and P731 were uniform and similar in appearance. However, after exposure to metalaxyl for 6 weeks, each isolate produced zoospores which gave rise to six colony types, including the original type, on metalaxyl-free medium (Table 6, Fig. 4). When isolates of zoospore cultures each representing one of the six colony types from isolate P991 were successively subcultured on metalaxyl-free medium, none of them changed colony appearance after the 5th subculture.

Permanent change of growth rate by metalaxyl -- Growth rates on V-8 agar of single-zoospore cultures from *P. parasitica* isolates P991 and P731 were very uniform, ranging from 6.1-6.8 mm/day for the former and 6.1-7.7 mm/day for the latter. However, exposure of each isolate to metalaxyl for 6 weeks greatly increased the range of growth rates among their single-zoospore cultures; 2.3-7.3 mm/day for P991 (Fig. 5) and 2.6-7.1 mm/day for P731 (Fig. 6).

Metalaxyl caused a permanent change in growth rates of both metalaxyl-resistant and metalaxyl-sensitive zoospore cultures. After exposure to metalaxyl for 6 weeks, P991 produced zoospores giving rise to metalaxyl-resistant cultures. These exhibited growth rates on V-8 agar ranging from 2.5 to 6.4 mm/day and metalaxyl-sensitive cultures with growth rates ranging from 2.3 to 7.3 mm/day (Fig. 7). With the same treatment P731 produced zoospores giving rise to metalaxyl-resistant cultures with growth rates ranging from 2.3 to 6.7 mm/day and metalaxyl-sensitive cultures with growth rates ranging from 2.6 to 7.1 mm/day (Fig. 8). When isolates of zoospore cultures each representing one of the six colony types from P991 were successively subcultured on metalaxyl-free medium, their growth rates at the 5th subculture were not significantly different from the original rates. However, among these six isolates, five produced single-zoospore cultures with greater variation in growth rate than did the control isolate. The range in growth rate among single-zoospore cultures from isolate P991-2M^{S} was similar to that from the control isolate (Table 7).

<u>Permanent change of other characteristics by metalaxyl</u> -- Single-zoospore isolates obtained from <u>P</u>. <u>parasitica</u> isolate P991 were uniform in their ability to produce sporangia in light and darkness (Table 8). However, after exposure to metalaxyl for 6 weeks, 6 of 7 single-zoospore isolates produced by P991 showed reduced ability to produce

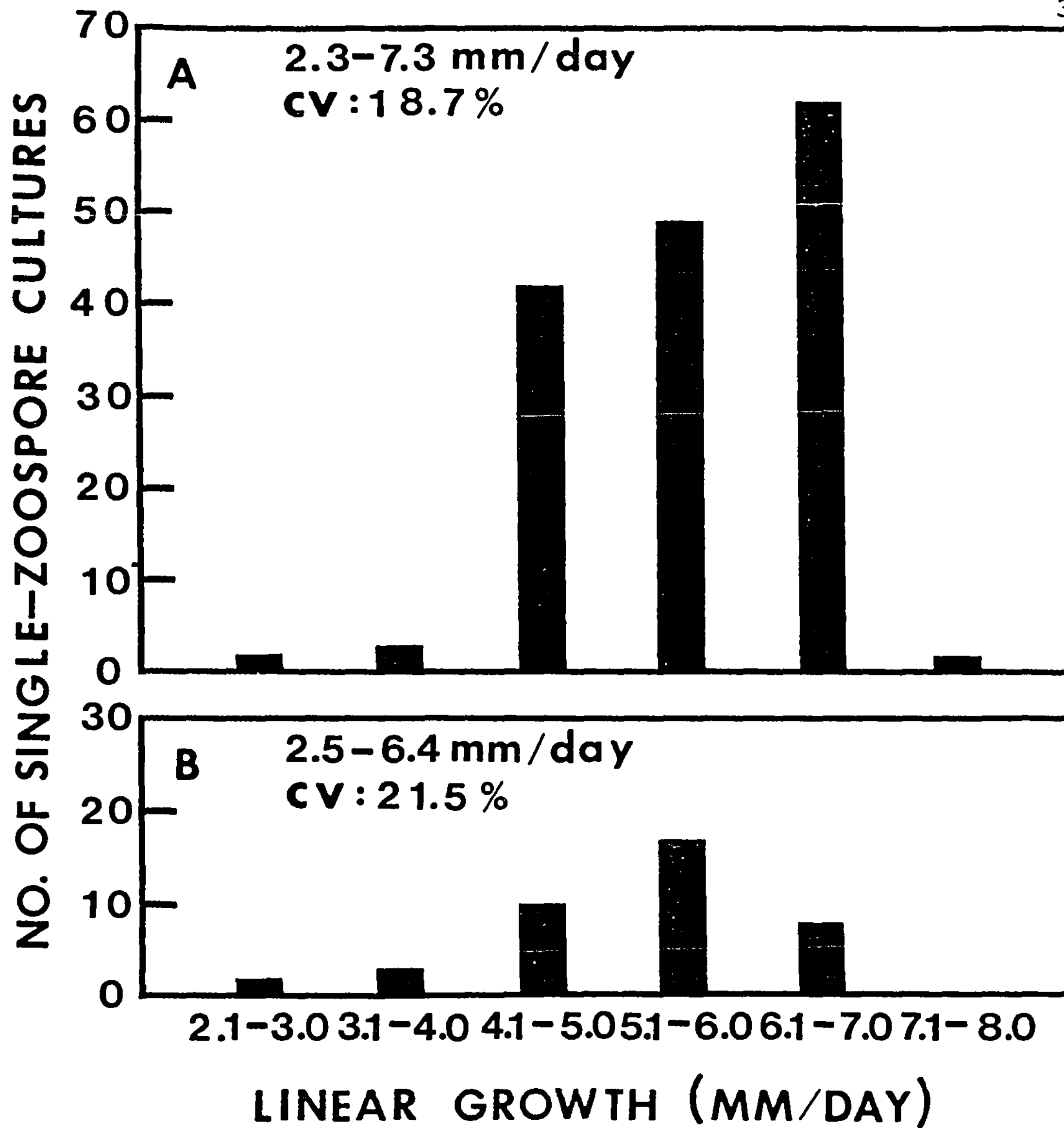

Fig. 7. Growth-rate distributions on V-8 agar of single-zoospore cultures obtained from *Phytophthora parasitica* (P991) grown for 6 weeks on V-8 agar with metalaxyl. A, single-zoospore cultures sensitive to metalaxyl. B, single-zoospore cultures resistant to metalaxyl. CV: coefficient of variation.

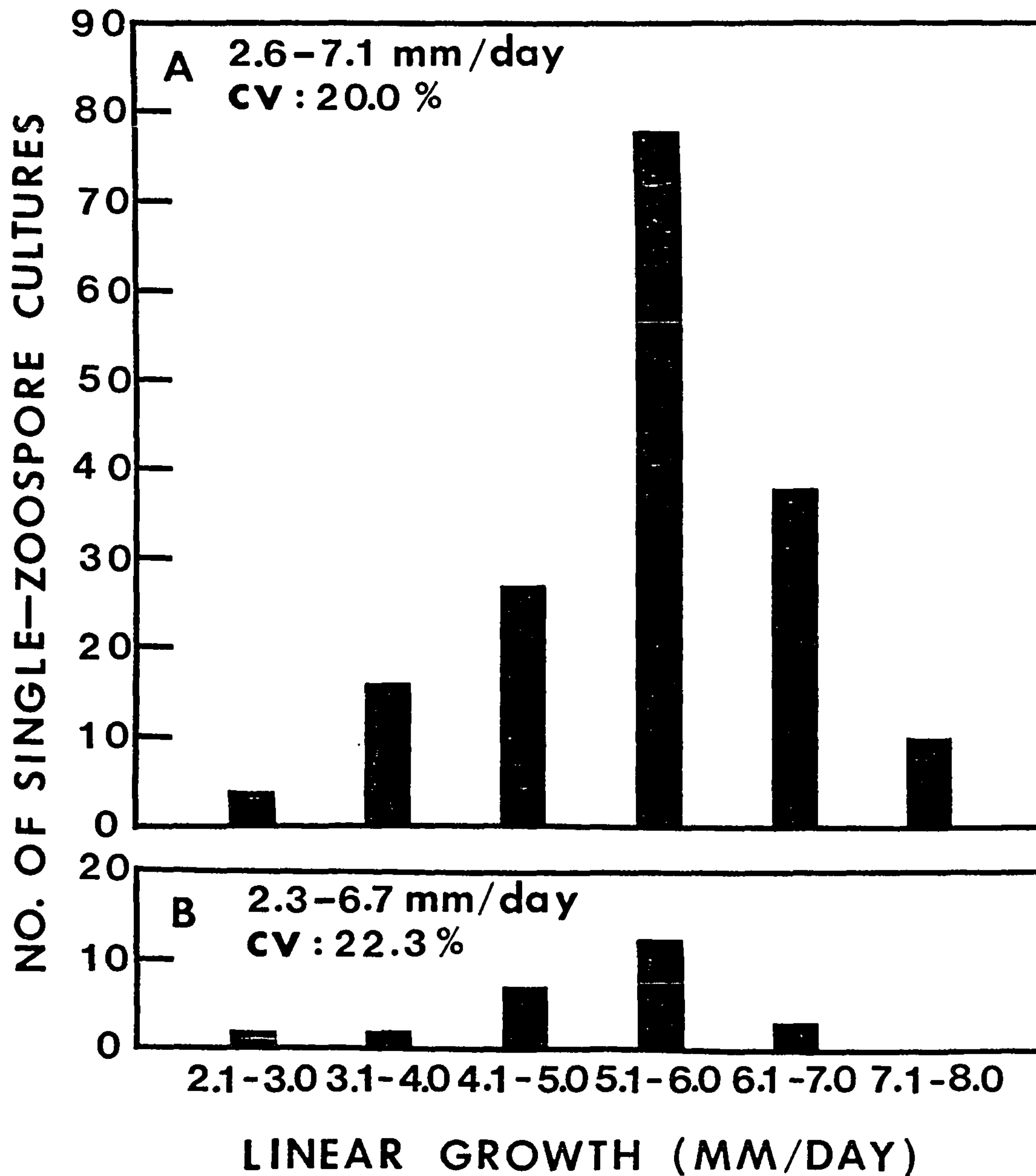

Fig. 8. Growth-rate distributions on V-8 agar of single-zoospore cultures obtained from *Phytophthora parasitica* (P731) grown for 6 weeks on V-8 agar with metalaxyl. A, single-zoospore cultures sensitive to metalaxyl. B, single-zoospore cultures resistant to metalaxyl. CV: coefficient of variation.

Table 7. Variation in growth rates among single-zoospore cultures from isolates each representing one of the six colony types from isolate P991 of *Phytophthora parasitica* grown for 6 weeks on V-8 agar containing 25 ug/ml metalaxyl

			Single-zoospore cultures		
Treatment and isolate	Colony type	Original growth rate (mm/day)	Range of growth rate (mm/day)	CV (%)[a]	Total no.
Metalaxyl					
P991-1M^{s}	C	2.5	0.4-3.8	40.0	60
P991-2M^{s}	A	5.6	5.3-6.4	4.7	60
P991-12M^{r}	E	5.6	4.0-6.8	7.5	60
P991-14M^{r}	B	6.3	3.0-6.6	18.1	75
P991-19M^{r}	F	4.9	1.0-6.1	43.6	60
P991-23M^{r}	D	5.0	1.3-5.9	53.3	72
Control					
P991-1	A	6.1	5.1-6.4	4.8	64

[a] CV, coefficient of variation.

Table 8. Variation in sporangial formation in the absence of metalaxyl among single-zoospore isolates obtained from isolate P991 of <u>Phytophthora</u> <u>parasitica</u> grown for 6 weeks on V-8 agar containing 25 ug/ml metalaxyl

Treatment and isolate	Colony type	No. of sporangia/cm^2	
		Under light	In darkness
Metalaxyl			
P991-2M^s	A	3000 A[a]	1220 B
P991-16M^s	D	350 DE	120 C
P991-24M^s	E	730 CD	200 C
P991-12M^r	E	520 D	500 C
P991-14M^r	B	1000 BC	130 C
P991-22M^r	B	1440 B	170 C
P991-23M^r	D	10 E	10 C
None			
P991-1	A	2880 A	2200 A
P991-2	A	3000 A	1850 A
P991-3	A	2790 A	2000 A
P991-4	A	2800 A	1650 A
P991-5	A	2580 A	1750 A

[a] Means followed by the same letter in each column are not significantly different at p=0.05 according to Duncan's multiple range test.

sporangia in the absence of metalaxyl. Only isolate P991-2M^{S} retained full ability to produce sporangia under light (Table 8). All tested single-zoospore isolates from P991 preexposed to metalaxyl for 6 weeks produced zoospores with unimpaired germination (Table 9). However, zoospores produced by three of these isolates displayed reduced ability to establish colonies on metalaxyl-free medium (Table 9).

Genetics of P. infestans

Oospore germination -- Oospores from the pairings of A^1 (533) and A^2 (550) mating types of *P. infestans* were used in this study. To determine the optimum concentration of $KMnO_4$ needed to activate oospores and the time required for activation, paired cultures were incubated at 19 C in darkness for 10 days for oospore formation and maintained under the same conditions for another 10 days for oospore maturation. After activation with $KMnO_4$, oospores were incubated on modified S+L medium at 19 C under light. Germination of oospores increased with increasing time of treatment with 0.25% $KMnO_4$ solution up to 15 min. After 30 min, germination decreased as time of treatment increased (Fig. 9). When oospores were treated for 15 min with $KMnO_4$ at the concentrations ranging from 0.125 to 2%, the optimum concentration for activation was 0.25 to 0.45% (Fig. 10). Activation of oospores with $KMnO_4$ at the concentration of 0.25% for 15 min was selected for the subsequent

Table 9. Variation in zoospore germination and ability of germinating zoospores to form colony among single-zoospore isolates obtained from isolate P991 of *Phytophthora parasitica* grown for 6 weeks on V-8 agar containing 25 ug/ml metalaxyl

Treatment and isolate	Colony type	Zoospore germination (%)	Formation of colony by germinating zoospores (%)
Metalaxyl			
P991-2M^{s}	A	94 A[a]	93 A
P991-16M^{s}	E	94 A	27 B
P991-24M^{s}	D	90 A	91 A
P991-12M^{r}	E	92 A	92 A
P991-14M^{r}	B	88 A	18 C
P991-22M^{r}	B	88 A	18 C
P991-23M^{r}	D	83 A	81 A
None			
P991-1	A	93 A	93 A
P991-2	A	91 A	90 A
P991-3	A	94 A	88 A
P991-4	A	92 A	92 A
P991-5	A	86 A	90 A

[a] Means followed by the same letter in each column are not significantly different at p=0.05 according to Duncan's multiple range test.

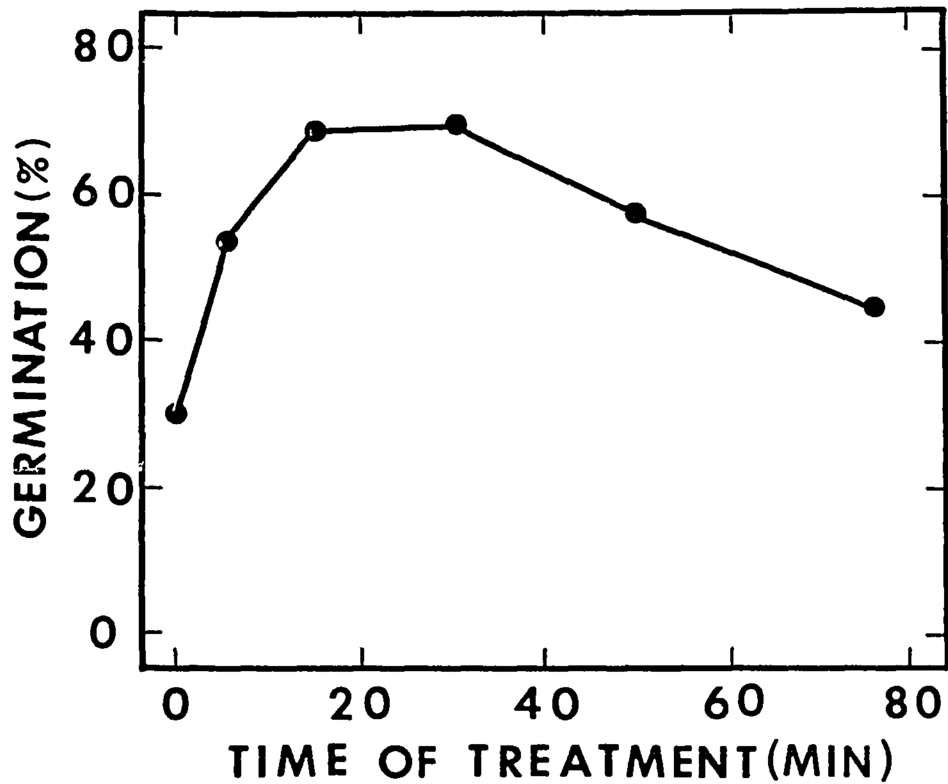

Fig. 9. Germination of oospores from pairing of A1 (533) and A2 (550) mating types of Phytophthora infestans after treatment with 0.25% $KMnO_4$ solution for 0, 5, 15, 30, 50, and 75 min. Germination was recorded after incubation on modified S+L medium at 19 C under light for 20 days.

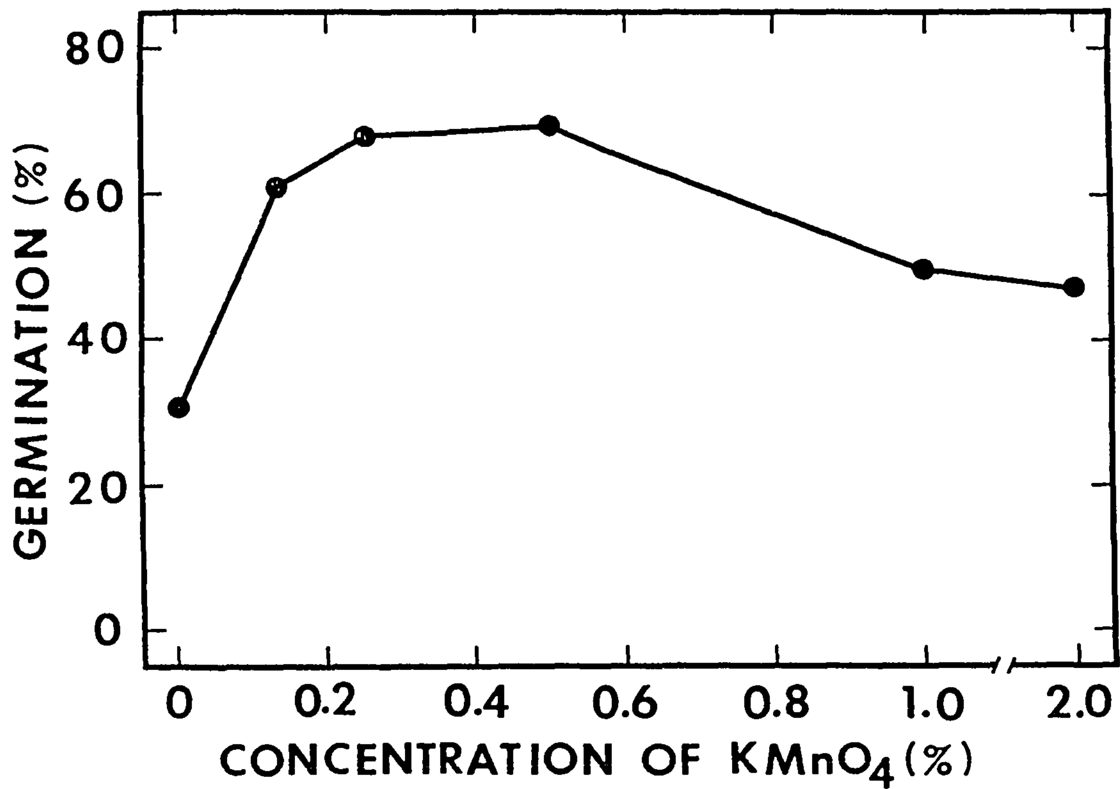

Fig. 10. Germination of oospores from pairing of A1 (533) and A2 (550) mating types of *Phytophthora infestans* after treatment for 15 min with various concentrations of KMnO4 solution. Germination was recorded after incubation on modified S+L medium at 19 C under light for 20 days.

experiments. Under these conditions, oospores of P. infestans germinated within 4 days and was as high as 70% after incubation for 20 days (Fig. 11).

Germ tubes produced by oospores emerged through oogonium wall or through the oogonial stalk and antheridium. Some of the germ tubes formed terminal sporangia, while others grew continuously (Fig. 12).

Light was applied to oospores during maturation and/or germination to determine its effect on germination rate. Results show that to obtain high germination light is required during germination but not during maturation (Fig. 13). The optimum maturation period for oospores of P. infestans was 20 to 30 days. Exposure of oospores to light during maturation did not affect germination during the short maturation period, but had adverse effect on germination when maturation period was extended beyond 50 days (Fig. 13). Some oospores germinated during maturation and the number of germinating oospores increased with increasing time of exposure to light.

Genetic markers used -- Isolates 533 (A^1) and 550 (A^2) of P. infestans and their progenies were used in this study. The information concerning origin, mating type and presumed genotypes at glucosephosphate isomerase (GPI-1) and peptidase (PEP) loci among isolates used is listed in Table 10. Isozyme patterns of GPI-1 and PEP of isolates 533 and 550 showed no differences among different ages of cultures ranging from 6 to 26 days indicating the stability of gene

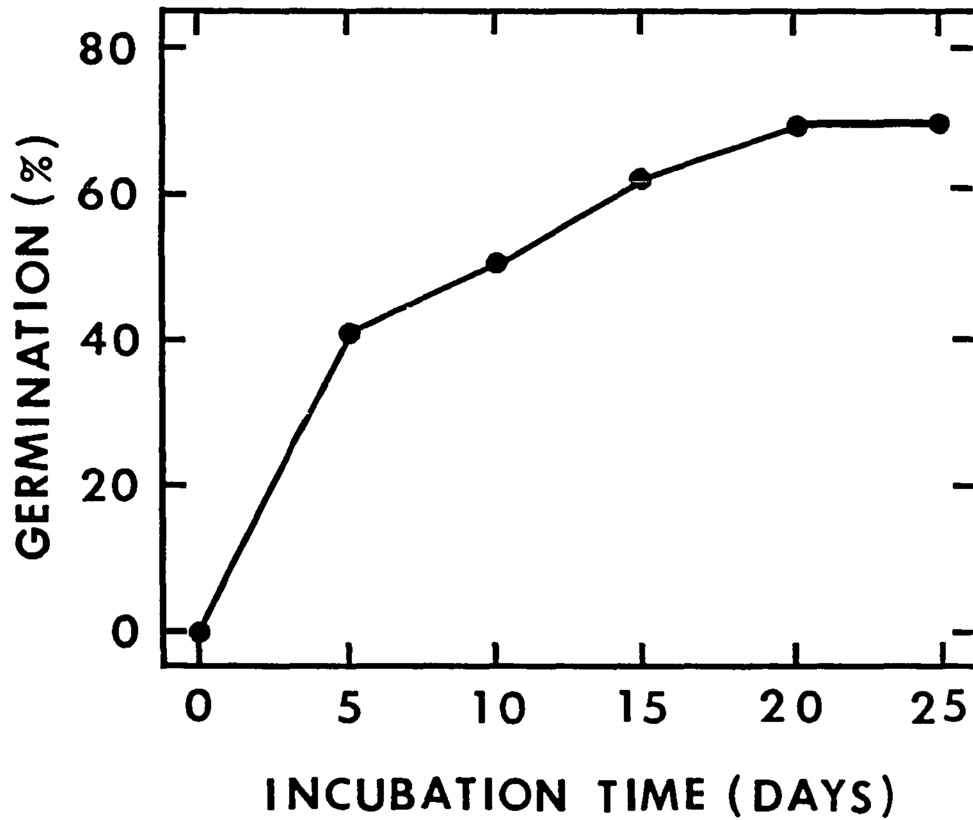

Fig. 11. Germination of oospores from paring of A1 (533) and A2 (550) mating types of *Phytophthora infestans* after treatment with 0.25% $KMnO_4$ solution for 15 min. Germination was recorded after incubation on modified S+L medium at 19 C under light for 5, 10, 15, 20, and 25 days.

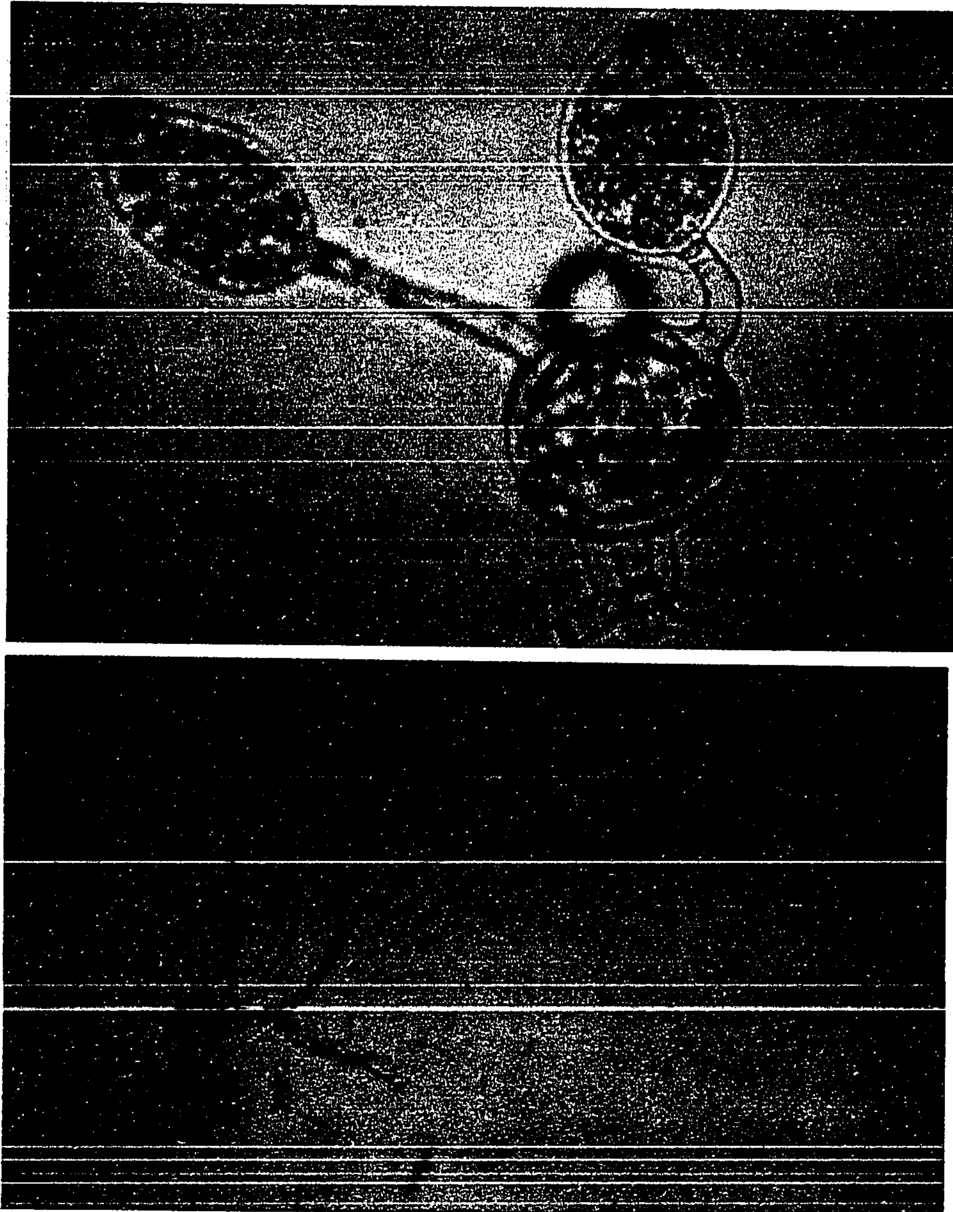

Fig. 12. Germination of oospores of Phytophthora infestans on modified S+L medium at 19 C under light. Top, a germinating oospore with two germ tubes each with a sporangium at the end (400X). bottom, an oospore germinating directly by producing mycelial filaments (100X).

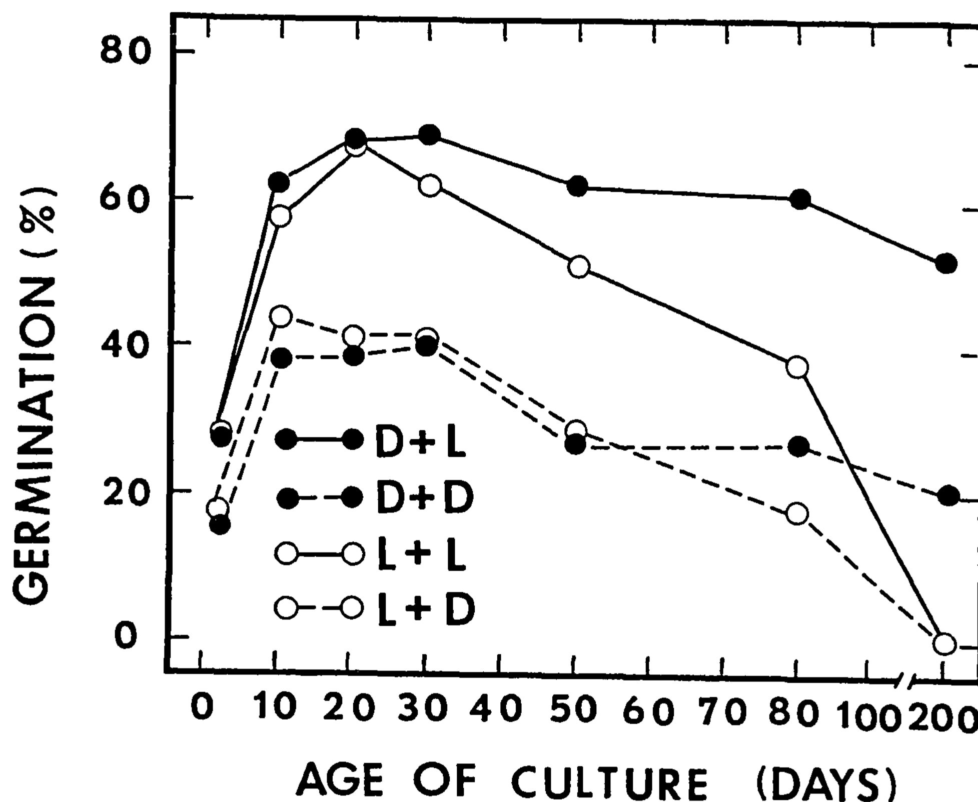

Fig. 13. Effect of age and light on germination of oospores from the pairing of A1 (533) and A2 (550) mating types of Phytophthora infestans. Oospores were treated with 0.25% KMnO4 for 15 min followed by incubation on modified S+L medium at 19 C for 20 days. L+L, oospores exposed to light during maturation and during germination; L+D, oospores exposed to light during maturation but kept in darkness during germination; D+L, oospores kept in darkness during maturation but exposed to light during germination; D+D, oospores kept in darkness during maturation and during germination.

expression in these two isozymes. All 50 single-zoospore cultures obtained from each isolate of 533 and 550 were identical with their respective parent isolates in mating type and isozyme patterns of GPI-1 and PEP.

In addition to isozyme patterns, streptomycin resistance and chloramphenicol resistance were used as genetic characters.. When culture blocks of 50 single-oospore cultures from either selfing or cross of isolates 533 and 550 were incubated on V-8 rye agar containing 50 ug/ml streptomycin sulfate or 200 ug/ml chloramphenicol, two isolates (903 and 912) produced fast-growing sectors on the medium containing streptomycin and the other two isolates (947 and 950) formed fast-growing sectors on the medium containing chloramphenicol. The antibiotic resistant mutants were obtained from fast-growing sectors by single-zoospore isolation. The mating type of mutants was the same as the original isolates. The original isolate (903) was completely inhibited at 100 ug/ml streptomycin sulfate. However, the linear growth of streptomycin-resistant mutant ($903S^r$) on V-8 rye agar containing 0 and 500 ug/ml streptomycin-sulfate was about the same (Fig. 14). Chloramphenicol-resistant mutant ($947C^r$) was able to grow on V-8 rye agar amended with 100, 200, or 300 ug/ml chloramphenicol, although at slightly slower rates (Fig. 15). However, the original isolate (947) was completely inhibited at 100 ug/ml chloramphenicol. Antibiotic-resistant mutants remained resistant to their respective

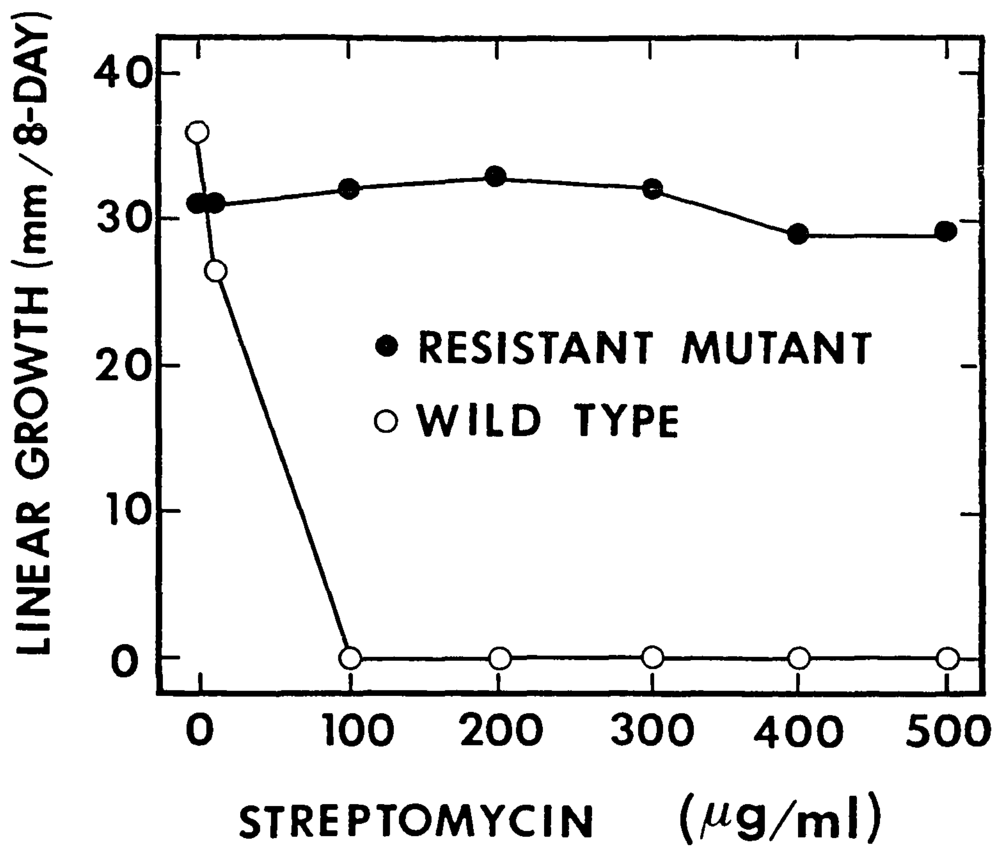

Fig. 14. Growth of the original isolate(903) and streptomycin-resistant mutant (903 S^r) of *Phytophthora infestans* on V-8 rye agar amended with various concentrations of streptomycin sulfate.

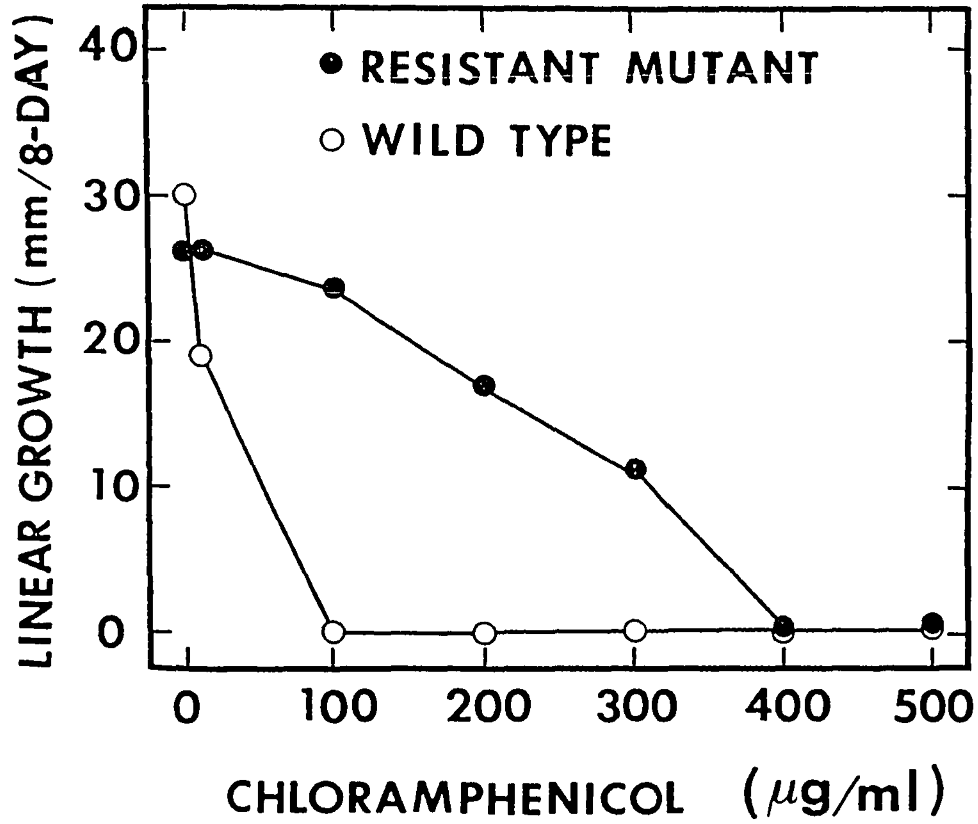

Fig. 15. Growth of the original isolate (947) and chloramphenicol-resistant mutant (947 Cp^r) of *Phytophthora infestans* on V-8 rye agar amended with various concentrations of chloramphenicol.

Table 10. Origin, mating type and presumed genotypes at glucosephosphate isomerase (GPI-1) and peptidase (PEP) loci among isolates of *Phytophthora infestans*

Isolate	Origin	Mating type	Genotype	
			GPI-1	PEP
550	wild type	A^2	G^1G^1	P^2P^2
533	wild type	A^1	G^2G^2	P^1P^2
912	550, selfing	A^1	G^1G^1	P^2P^2
903	533, selfing	A^1	G^2G^2	P^1P^1
908	533, selfing	A^1	G^2G^2	P^1P^2
925	533, selfing	A^1	G^2G^2	P^1P^1
926	533, selfing	A^2	G^2G^2	P^2P^2
928	533, selfing	A^1	G^2G^2	P^2P^2
932	533, selfing	A^1	G^2G^2	P^1P^1
936	533, selfing	A^1	G^2G^2	P^1P^1
948	533, selfing	A^2	G^2G^2	P^1P^1
901	533x550	A^1	G^1G^2	P^1P^2
915	533x550	A^2	G^1G^2	P^1P^2
930	533x550	A^1	G^1G^2	P^1P^2
994	533x550	A^2	G^1G^2	P^2P^2
947	550x928	A^2	G^1G^2	P^2P^2

antibiotics even after five consecutive subcultures in antibiotic-free medium and produced normal oospores when paired with an opposite mating type.

Isozyme patterns of progenies from selfing and cross of isolates 533 and 550 -- All single-oospore cultures from each of 5 isolates (533, 550, 928, 932 and 908) were either homozygous G^1G^1 (a slow-migrating band) or G^2G^2 (a fast-migrating band) and were identical with their respective parent at GPI-1 locus (Table 11). For example, all 92 single-oospore cultures originating from 533 produced a fast-migrating band (G^2G^2), and all 50 single-oospore cultures from isolate 550 produced a slow-migrating band (G^1G^1). At PEP locus, isolate 550 was presumed to be homozygous P^2P^2 because it produced a fast-migrating band. All 50 single-oospore cultures from 550 was P^2P^2 (Table 11). Isolate 533 which was heterozygous at PEP locus (P^1P^2) also produced selfed offspring with three-banded isozyme patterns (slow, intermediate and fast-migrating bands). Single-oospore cultures from 533 segregated 28 P^1P^1 : 42 P^1P^2 :22 P^2P^2 which fit a ratio of 1:2:1 (X^2=1.48, P=0.30) expected for a pair of codominant genes for dimeric enzymes in heterozygous condition (Table 11). The PEP locus of isolates 932, 908 and 928 originating from selfing of 533 were homozygous slow (P^1P^1), heterozygous (P^1P^2), and homozygous fast (P^2P^2), respectively. All single-oospore cultures from 932 and 928 were homozygous slow and homozygous fast, respectively (Table 11). Like isolate 533,

Table 11. Genotypes at glucosephosphate isomerase (GPI-1) and peptidase (PEP) loci of single-oospore cultures from selfing and cross of isolates 533 and 550 of *Phytophthora infestans*

Parent			single-oospore cultures				
Isolate	Genotype	Origin	Genotype	No.	Expected ratio	X^2	P
533	$G^2G^2P^1P^2$		$G^2G^2P^1P^1$	28	1:2:1	1.48	0.30
			$G^2G^2P^1P^2$	42			
			$G^2G^2P^2P^2$	22			
550	$G^1G^1P^2P^2$		$G^1G^1P^2P^2$	50			
928	$G^2G^2P^2P^2$	533, selfing	$G^2G^2P^2P^2$	40			
932	$G^2G^2P^1P^1$	533, selfing	$G^2G^2P^1P^1$	40			
908	$G^2G^2P^1P^2$	533, selfing	$G^2G^2P^1P^1$	21	1:2:1	0.15	0.90
			$G^2G^2P^1P^2$	42			
			$G^2G^2P^2P^2$	19			
533 X 550	$G^2G^2P^1P^2$ / $G^1G^1P^2P^2$		$G^1G^2P^1P^2$	122	1:1	0.11	0.70
			$G^1G^2P^2P^2$	116			
			$G^2G^2P^1P^2$	1[a]			

[a] This single-oospore cultures was from selfing of 533 in cross 533X550.

the single-oospore cultures from 908 also segregated 21 P^1P^1 : 42 P^1P^2 : 19 P^2P^2 which fit a ratio of 1:2:1 (X^2=0.15, P=0.90) expected for a pair of codominant genes for dimeric enzymes in heterozygous condition. In the cross 533X550, 238 of 239 single-oospore cultures were hybrids because they were heterozygous (G^1G^2) at GPI-1 locus and segregated 1:1 (X^2=0.11, P=0.70) at PEP locus (Table 11). One of 239 single-oospore cultures was from selfing of isolate 533.

<u>Behavior of isozyme patterns of a heterozygous offspring from the cross 533 X 550</u> -- Isolate 930 obtained from the cross 533X550 was heterozygous at both GPI-1 and PEP loci. The 180 single-oospore cultures from selfing of isolate 930 segregated 41 G^1G^1 : 96 G^1G^2 : 43 G^2G^2 at GPI-1 locus which fit the 1:2:1 ratio (X^2=0.84, P=0.50) expected for a codominant genes for dimeric enzymes in heterozygous condition, and 38 P^1P^1 : 94 P^1P^2 :48 P^2P^2 at PEP locus which also fit the 1:2:1 ratio (X^2=1.47, P=0.30) expected for a codominant genes for dimeric enzymes in heterozygous condition (Table 12). These cultures also segregated 7 $G^1G^1P^1P^1$: 16 $G^2G^2P^1P^1$: 15 $G^1G^2P^1P^1$: 25 $G^1G^1P^1P^2$: 55 $G^1G^2P^1P^2$: 14 $G^2G^2P^1P^2$: 26 $G^1G^2P^2P^2$: 9 $G^1G^1P^2P^2$: 13 $G^2G^2P^2P^2$ at both GPI-1 and PEP loci which fitted to the ratio of 1:1:2:2:4:2:2:1:1 (X^2=13.1, P=0.10) expected for two independent loci, indicating that genes in GPI-1 and PEP are not linked.

<u>Determination of hybrid and self in direct matings</u> -- To determine the ratio of hybrid : self in the cross, the

Table 12. Genotypes at glucosephosphate isomerase (GPI-1) and peptidase (PEP) loci of selfed oospore cultures of isolate 930 originated from the cross 533X550

Parent		Single-oospore cultures	
Isolate	Genotype	Genotype	No.
930	$G^1G^2P^1P^2$	$G^1G^1P^1P^1$	7[a]
		$G^2G^2P^1P^1$	16
		$G^1G^2P^1P^1$	15
		$G^1G^1P^1P^2$	25
		$G^1G^2P^1P^2$	55
		$G^2G^2P^1P^2$	14
		$G^1G^2P^2P^2$	26
		$G^1G^1P^2P^2$	9
		$G^2G^2P^2P^2$	13

[a] The X2 value for an expected ratio of 1:1:2:2:4:2:2:1:1 with eight degrees of freedom is 13.1 (P=0.10).

parental isolates with different homozygous genotypes at both GPI-1 and PEP loci were used. All selfed progenies obtained from each of five isolates used were the same as their respective parents at GPI-1 and PEP loci (Table 13). In the crosses 926X936, 550X928 and 550X932, the ratios of hybrids : selfs were 60 : 20, 75 : 0 and 74 : 1, respectively (Table 13). All progenies from the crosses 925X948 and 912X550 were with identical homozygous genotype at both GPI-1 and PEP loci like their parents.

Fifteen single-oospore cultures originating from selfing of the cross 926X936 were paired to determine if the ratio of self : hybrid can be increased. Seven of 10 crosses produced only hybrid offspring (Table 14). The other three crosses produced both hybrid and self. The ratios of hybrid : self were 60 : 16 (21% self) for the cross C1-2 X C1-1, 17 : 2 (10% self) for the cross C1-10 X C1-9 and 16 : 2 (11% self) for the cross C1-11 X C1-1.

Determination of genetic nature of antibiotic resistance -- Isolates 903 from selfing of 533, 947 from hybrid of the cross 550X928 and their antibiotic-resistant mutants were used in this study. All the 46 single-zoospore cultures and 54 single-oospore cultures from $903S^r$ were resistant to streptomycin, and all the 42 single-zoospore cultures and 54 single-oospore cultures from $947Cp^r$ were resistant to chloramphenicol (Table 15). All single-zoospore and single-oospore cultures from 903 and $903S^r$ which were homozygous at PEP locus and produced a slow-

Table 13. Genotypes at glucosephosphate isomerase (GPI-1) and peptidase (PEP) loci of single-oospore cultures from crosses and selfings of homozygotic isolates of *Phytophthora infestans*

Parent[a]		Single-oospore cultures	
Isolate	Genotype	Genotype	No.
926	$G^2G^2P^2P^2$	$G^2G^2P^2P^2$	45
925	$G^2G^2P^1P^1$	$G^2G^2P^1P^1$	44
936	$G^2G^2P^1P^1$	$G^2G^2P^1P^1$	43
948	$G^2G^2P^1P^1$	$G^2G^2P^1P^1$	41
912	$G^1G^1P^2P^2$	$G^1G^1P^2P^2$	48
926	$G^2G^2P^2P^2$	$G^2G^2P^2P^2$	5
x		$G^2G^2P^1P^1$	15
936	$G^2G^2P^1P^1$	$G^2G^2P^1P^2$	60
550	$G^1G^1P^2P^2$	$G^1G^1P^2P^2$	0
x		$G^2G^2P^2P^2$	0
928	$G^2G^2P^2P^2$	$G^1G^2P^2P^2$	75
550	$G^1G^1P^2P^2$	$G^1G^1P^2P^2$	1
x		$G^2G^2P^1P^1$	0
932	$G^2G^2P^1P^1$	$G^1G^2P^1P^2$	74
925	$G^2G^2P^1P^1$		
x		$G^2G^2P^1P^1$	49
948	$G^2G^2P^1P^1$		
912	$G^1G^1P^2P^2$		
x		$G^1G^1P^2P^2$	60
550	$G^1G^1P^2P^2$		

[a] Oospores produced by single isolate were obtained by hormonal stimulation using the polycarbonate membrane method.

Table 14. Genotypes at peptidase (PEP) locus of single oospore cultures from matings among progeny of cross 926X936

Parent[a]		Single-oospore cultures	
Pairing	Genotype	Genotype	No.
C1-2	P^1P^1	P^1P^1	6
X		P^2P^2	10
C1-1	P^2P^2	P^1P^2	60
C1-4	P^1P^1	P^1P^1	0
X		P^2P^2	0
C1-3	P^2P^2	P^1P^2	20
C1-6	P^1P^1	P^1P^1	0
X		P^2P^2	0
C1-5	P^2P^2	P^1P^2	17
C1-8	P^1P^1	P^1P^1	0
X		P^2P^2	0
C1-7	P^2P^2	P^1P^2	18
C1-10	P^1P^1	P^1P^1	1
X		P^2P^2	1
C1-9	P^2P^2	P^1P^2	17
C1-11	P^1P^1	P^1P^1	0
X		P^2P^2	2
C1-1	P^2P^2	P^1P^2	16
C1-12	P^1P^1	P^1P^1	0
X		P^2P^2	0
C1-3	P^2P^2	P^1P^2	21
C1-13	P^1P^1	P^1P^1	0
X		P^2P^2	0
C1-5	P^2P^2	P^1P^2	17
C1-14	P^1P^1	P^1P^1	0
X		P^2P^2	0
C1-7	P^2P^2	P^1P^2	15
C1-15	P^1P^1	P^1P^1	0
X		P^2P^2	0
C1-9	P^2P^2	P^1P^2	17

[a] All isolates used originated from selfing of the cross 926X936.

Table 15. Sensitivity to antibiotics and genotypes at peptidase (PEP) locus of single-zoospore cultures and single-oospore cultures from the original isolates of *Phytophthora infestans* (903, A1 and 947, A2) and their mutants resistant to streptomycin ($903S^r$, A1) or chloramphenicol ($947Cp^r$, A2)

	No. of single-spore cultures				
				PEP	
Isolate	Sensitive to chloramphenicol and streptomycin	Resistant to streptomycin	Resistant to chloramphenicol	Genotype	No.
Zoospore					
$903S^r$, P^1P^1	0	46	0	P^1P^1	46
$947Cp^r$, P^2P^2	0	0	42	P^2P^2	42
903, P^1P^1	48	0	0	P^1P^1	48
947, P^2P^2	54	0	0	P^2P^2	54
Oospore					
$903S^r$, P^1P^1	0	54	0	P^1P^1	54
$947Cp^r$, P^2P^2	0	0	54	P^2P^2	54
903, P^1P^1	50	0	0	P^1P^1	50
947, P^2P^2	50	0	0	P^2P^2	50

migrating band (P^1P^1) were P^1P^1 (Table 15). All single-zoospore and single-oospore cultures from 947 and 947Cp^r which were also homozygous at PEP locus but produced a fast-migrating band (P^2P^2) were P^2P^2.

All progenies from crosses 903S^{r}X947Cp^r, 903S^{r}X947, 903X947Cp^r and 903X947, were hybrids with heterozygous PEP pattern (P^1P^2) (Table 16). When 903S^r was paired directly with 947Cp^r, all the single-oospore cultures from the cross were resistant to either chloramphenicol or streptomycin, but not both or neither (Table 16). All single-oospore cultures from the cross 903S^{r}X947 were either resistant to streptomycin or sensitive to streptomycin and the single-oospore cultures from the cross 903X947Cp^r were either resistant to chloramphenicol or sensitive to chloramphenicol. All single-oospore cultures from the cross 903X947 were sensitive to chloramphenicol and streptomycin. These results suggest that genes controlling resistance to streptomycin and chloramphenicol are located in cytoplasm.

Two F1 cultures (C2-1S^r A^1 and C2-2Cp^r A^1) obtained from the cross 903S^{r}X947Cp^r were used for analysis of F2 generation. C2-1S^r was resistant to streptomycin and C2-2Cp^r was resistant to chloramphenicol. All 80 single-oospore cultures obtained from C2-1S^r were resistant to streptomycin but segregated 23 P^1P^1 : 45 P^1P^2 : 12 P^2P^2 at PEP locus with the expected ratio of 1:2:1 (X^2=4.28, P=0.10) (Table 17). All 80 single-oospore cultures obtained from C_2-2Cp^r were resistant to chloramphenicol but segregated 17

Table 16. Sensitivity to antibiotics and genotypes at peptidase (PEP) locus of progenies from the pairings of wild type and antibiotic resistant mutants of 903, A1 and 947, A2 of <u>Phytophthora</u> <u>infestans</u>

Pairing	Progeny (No. of single-oospore cultures)				
	Sensitive to chloramphenicol and streptomycin	Resistant to streptomycin	Resistant to chloramphenicol	PEP	
				Genotype	No.
903S^rx947Cp^r p^1p^1 p^2p^2	0	48	58	P^1P^2	106
903S^rx947 p^1p^1 p^2p^2	37	51	0	P^1P^2	88
903x947Cp^r p^1p^1 p^2p^2	35	0	56	P^1P^2	91
903x947 p^1p^1 p^2p^2	78	0	0	P^1P^2	78

Table 17. Sensitivity to antibiotics and genotypes at peptidase (PEP) locus of selfed oospore cultures from two of the progeny of the cross $903S^r$ X $947Cp^r$ of *Phytophthora infestans*

Parent		Progeny (No. of single-oospore cultures)					
Isolate	Genotype	Resistant to streptomycin	Resistant to chloramphenicol	Genotype (No.)	Expected ratio	X^2	P
$C2\text{-}1S^r$	P^1P^2	80	0	P^1P^1 (23)	1:2:1	4.28	0.10
				P^1P^2 (45)			
				P^2P^2 (12)			
$C2\text{-}2Cp^r$	P^1P^2	0	80	P^1P^1 (17)	1:2:1	2.48	0.20
				P^1P^2 (37)			
				P^2P^2 (26)			

P^1P^1 : 37 P^1P^2 : 26 P^2P^2 at PEP locus with the expected ratio of 1:2:1 (X^2=2.48, P=0.20).

Mating types in progenies from selfing and crosses -- Both A^1 and A^2 mating types were present in the selfed progenies of 11 isolates tested (Table 18). Eight of these isolates also produced A^1A^2 type. Selfed progeny of the A^2 isolate 948 consisted of only A^2 type. A^1, A^2 and A^1A^2 types were present in the progenies from all seven crosses tested.

Colony morphology and growth rate in progenies of selfing and cross -- Oospore progenies from individual isolates and cross of 533 and 550 of *P. infestans* segregated into five colony types designated A, B, C, D and E (Table 19, Fig. 16). The colony of isolates 533 and 550 belonged to type A with uniform texture and abundant aerial growth. All the zoospore progeny of both isolates are similar to their parents in colony appearance. However, colony morphology among oospore progenies varied greatly (Table 19). All five colony types were present in the oospore progeny of isolate 533 and the cross 533X550, while colonies among oospore progeny of isolate 550 included type C in addition to the parental type A.

Rates of linear extension of mycelia of 50 single-zoospore cultures of isolate 533 of *P. infestans* were very similar ranging from 22.7 to 35.2 mm/8 days and with a CV (coefficient of variation) of 8.3% (Fig. 17, Table 20). However, the growth rate of 86 cultures from selfed oospores

Table 18. Mating type distribution in single-oospore cultures from selfings and crosses of Phytophthora infestans

Parent[a]	Mating type	No. of single-oospore cultures		
		A1	A2	A1A2
533	A1	90	2	2
550	A2	1	39	0
928	A1	38	1	1
932	A1	39	1	0
908	A1	77	4	1
926	A2	3	41	1
925	A1	39	4	1
936	A1	34	7	2
948	A2	0	48	0
912	A1	42	6	0
901	A1	55	5	2
930	A1	151	23	6
533X550		36	68	16
928X550		45	24	6
932X550		30	30	15
936X926		23	35	22
925X948		28	14	7
912X550		27	26	7
901X550		35	18	22

[a] Oospores produced by single isolate were obtained by hormonal stimulation using the polycarbonate membrane method.

Table 19. Colony types in single-zoospore and single-oospore cultures from selfing and cross of isolates 533 A1 and 550 A2 of *Phytophthora infestans* on V-8 rye agar at 19 C for 8-10 days

Isolate	Mating type	Origin of cultures	Colony type[a] and no. of cultures					
			A	B	C	D	E	Total
533	A[1]	Zoospores	50	0	0	0	0	50
		Oospores	45	13	12	6	10	86
550	A[2]	Zoospores	48	0	0	0	0	48
		Oospores	26	0	14	0	0	40
533X550		Oospores	34	32	15	16	9	106

[a] Symbols: A, uniform texture, abundant aerial growth; B, uniform fine texture, abundant aerial growth, intermediate in growth; C, uniform texture, little aerial growth; D, irregular pattern, little to moderate aerial growth, usually slow in growth; E, irregular pattern, little to moderate aerial growth, slight radiate, with white spots, usually slow in growth.

Fig. 16. Five colony patterns of _Phytophthora infestans_ on rye V-8 agar at 19 C for 8 days. Upper left, A type; upper right, B type; lower right, C type; lower center, D type; lower left, E type.

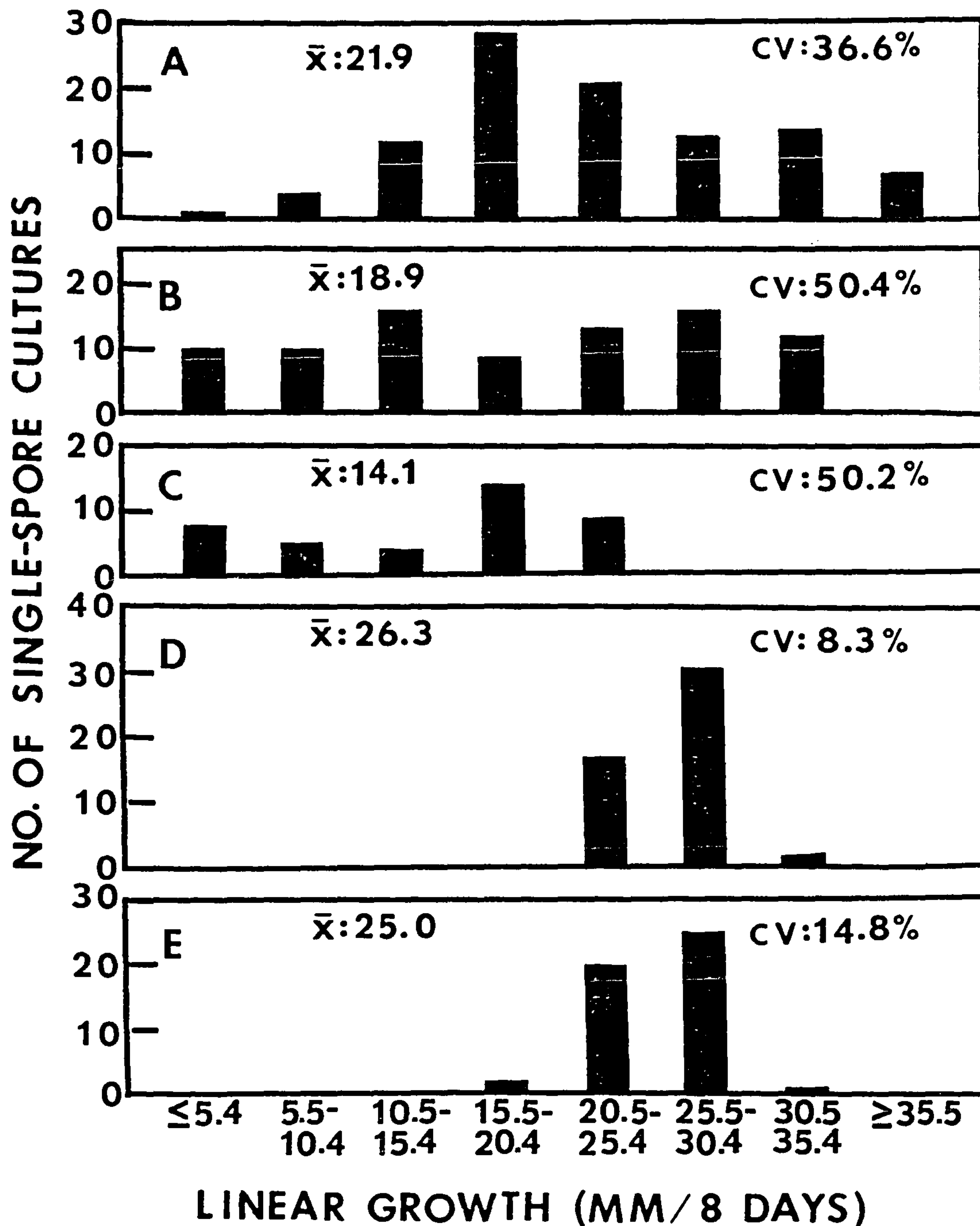

Fig. 17. Linear growth distributions of single-spore cultures of *Phytophthora infestans* on V-8 rye agar at 19 C. A, single-oospores cultures from pairing of isolates 533 and 550. B, single selfed oospore cultures of isolate 533. C, single selfed oospore cultures of isolate 550. D, single-zoospore cultures of isolate 533. E, single-zoospore cultures of isolate 550. x, mean. CV, coefficient of variation.

Table 20. Growth rate of zoospore and oospore progenies of *Phytophthora infestans*

Parent		Progeny			
				Linear growth (mm/ 8 days)	
Isolate	Mating type	Spores[a]	No. of cultures	Range	CV (%)
533	A1	Zoospores	50	22.7-35.2	8.3
		Oospores	86	3.4-34.2	50.4
550	A2	Zoospores	48	16.6-33.8	14.8
		Oospores	40	2.8-25.3	50.2
533X550		Oospores	106	5.0-34.4	36.6

[a] Oospore production by single isolates was chemically induced using the polycarbonate membrane method.

of the same isolate varied greatly ranging from 3.4 to 34.2 mm/8 days and with a CV of 50.4%. Similar results were obtained when isolate 550 was used. The range of growth rate of the 48 zoospore progeny was 16.6 to 33.8 mm/ 8 days and the CV was 14.8% (Fig. 17, Table 20). For the 40 oospore progeny, growth rate was highly variable extending from 2.8 to 25.3 mm/ 8 days and with a CV of 50.2%. The range of growth rate of the 106 oospore progeny from the cross 533X550 was 5.0 to 34.4 mm/8 days and with the CV was 36.6% (Table 20, Fig. 17).

Genetics of *P. parasitica*

Genetic nature of metalaxyl resistance -- All single-zoospore and single-oospore cultures obtained from metalaxyl-sensitive isolates P991 and 6133Cp^r were sensitive to metalaxyl (Table 21). All single-zoospore cultures from metalaxyl-resistant mutants tested were resistant to metalaxyl. However, progeny derived from oospores produced by each resistant mutant through hormonal stimulation segregated in a ratio of 3 resistant : 1 sensitive (Table 21). These results indicate that resistance to metalaxyl in isolates P991-12M^r, P991-14M^r, P991-23M^r and 6133Cp^{r}M^r is conferred by a single dominant gene in heterozygous condition.

Four metalaxyl-sensitive and 41 metalaxyl-resistant isolates were randomly selected from oospore progeny of isolate P991-23M^r for further study. Single-oospore

Table 21. Sensitivity to metalaxyl of single-zoospore and single-oospore cultures from metalaxyl-sensitive and metalaxyl-resistant isolates of *Phytophthora parasitica*

		No. of single-zoospore cultures		No. of single-oospore cultures[a]				
Isolate	Mating type	Resistant	Sensitive	Resistant	Sensitive	Expected ratio	X^2	P
P991	A^1	0	50	0	50			
$6133Cp^r$	A^1	0	50	0	50			
$P991\text{-}12M^r$	A^1	50	0	37	13	3:1	0.00	0.99
$P991\text{-}14M^r$	A^1	55	0	40	10	3:1	0.43	0.50
$P991\text{-}23M^r$	A^2	50	0	76	32	3:1	1.00	0.30
$6133Cp^rM^r$	A^1	50	0	84	35	3:1	1.01	0.30

[a] Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

cultures obtained from metalaxyl-sensitive isolates through hormonal stimulation were all sensitive to metalaxyl (Table 22), consistent with the earlier test that sensitivity to metalaxyl in these isolates is recessive. All single-oospore cultures obtained from 10 of 41 metalaxyl-resistant isolates through hormonal stimulation were resistant to metalaxyl (Table 22) indicating that these isolates were homozygous for resistance to metalaxyl. Selfed oospores produced by the other 31 metalaxyl-resistant isolates gave rise to both metalaxyl-resistant and metalaxyl-sensitive cultures indicating that these isolates were heterozygous for resistance to metalaxyl. The ratio of 10 homozygous resistant : 31 heterozygous resistant is consistent with 1:2 ratio ($X^2 = 1.11$, $P = 0.20$) expected for a single dominant resistance gene in heterozygous condition in the parent P991-23M^r.

Among the 31 isolates which were heterozygous for resistance to metalaxyl, 26 produced progeny with acceptable chi-square values ($X^2<3.84$, $P>0.05$) for testing the goodness of fit to a ratio of 3 resistant : 1 sensitive expected for a single dominant resistance gene in heterozygous condition (Table 22). However, isolates 2, 7, 15, 16 and 31 produced progeny which did not fit the expected ratio of 3 resistant : 1 sensitive ($X^2>3.84$, $P<0.05$). Additional batches of single-oospore cultures were obtained from isolates 2 and 7. The new progeny consisted of 21 resistant : 10 sensitive for isolate 2 and 25 resistant : 10 sensitive for isolate 7.

Table 22. Sensitivity to metalaxyl of single-oospore cultures from randomly selected progeny of selfed oospore of isolate P991-23M[r] of *Phytophthora parasitica*

Isolate	No. of single-oospore cultures[a]		Expected ratio of 3:1	
	Resistant	Sensitive	X^2	P
Resistant				
1	15	4	0.02	0.70
2	5	12	16.50	0.00
3	10	5	0.20	0.50
4	20	3	1.17	0.20
5	30	0		
6	13	4	0.02	0.70
7	5	10	11.80	0.00
8	75	0		
9	14	5	0.02	0.70
10	30	0		
11	16	5	0.01	0.90
12	20	0		
13	12	6	0.30	0.50
14	17	0		
15	10	10	6.10	0.01
16	8	13	13.40	0.00
17	16	7	0.13	0.70
18	25	0		

Table 22. (Continued) Sensitivity to metalaxyl of single-oospore cultures from randomly selected progeny of selfed oospore of isolate P991-23M^r of <u>Phytophthora</u> <u>parasitica</u>

Isolate	No. of single-oospore cultures[a]		Expected ratio of 3:1	
	Resistant	Sensitive	X^2	P
19	18	3	0.78	0.30
20	27	0		
21	16	4	0.07	0.70
22	13	8	1.17	0.20
23	15	5	0.07	0.70
24	15	6	0.01	0.90
25	13	9	2.18	0.10
26	14	2	0.75	0.30
27	25	0		
28	13	5	0.00	0.99
29	75	0		
30	14	5	0.02	0.70
31	5	10	11.80	0.00
32	30	0		
33	15	5	0.07	0.70
34	20	4	0.50	0.30
35	18	5	0.01	0.90
36	22	10	0.38	0.50
37	17	6	0.01	0.90

Table 22. (Continued) Sensitivity to metalaxyl of single-oospore cultures from randomly selected progeny of selfed oospore of isolate P991-23M[r] of <u>Phytophthora parasitica</u>

	No. of single-oospore cultures[a]		Expected ratio of 3:1	
Isolate	Resistant	Sensitive	X^2	P
38	17	5	0.00	0.99
39	20	7	0.01	0.90
40	14	5	0.02	0.70
41	16	6	0.00	0.99
Sensitive				
1	0	20		
2	0	18		
3	0	22		
4	0	24		

[a] Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

Both were consistent with the ratio of 3 resistant : 1 sensitive (X^2=0.53, P=0.30 for isolate 2 and X^2=0.07, P=0.70 for isolate 7) expected for a single dominant resistance gene in heterozygous condition.

Genetic nature of chloroneb resistance -- All the single-zoospore and single-oospore cultures obtained from chloroneb-sensitive isolates 6133Cp^r and 6134S^r were sensitive to chloroneb (Table 23). All the single-zoospore cultures from chloroneb-resistant mutants 6133Cp^rCn^r and 6134S^rCn^r were resistant to chloroneb. However, progeny derived from selfed oospores produced by each resistant mutant segregated in a ratio of 3 resistant : 1 sensitive (Table 23). These results indicate that resistance to chloroneb is conferred by a single dominant gene which is in heterozygous condition in these two mutants.

Two chloroneb-sensitive and 11 chloroneb-resistant isolates were randomly selected from oospore progeny of isolate 6133Cp^rCn^r for further study. Cultures derived from selfed oospores produced by chloroneb-sensitive isolates were all sensitive to chloroneb (Table 24), indicating that sensitivity to chloroneb in these isolates is recessive. All single-oospore cultures obtained from 3 of 11 chloroneb-resistant isolates through hormonal stimulation were resistant to chloroneb (Table 24), indicating that the gene for resistance to chloroneb in these isolates is homozygous dominant alleles. Selfed oospores produced by the other 8 chloroneb-resistant isolates gave rise to both chloroneb-

Table 23. Sensitivity to chloroneb of single-zoospore and single-oospore cultures from chloroneb-sensitive and chloroneb-resistant isolates of <u>Phytophthora parasitica</u>

		No. of single-zoospore cultures		No. of single-oospore cultures[a]				
Isolate	Mating type	Resistant	Sensitive	Resistant	Sensitive	Expected ratio	X^2	P
$6133Cp^r$	A^1	0	50	0	50			
$6134S^r$	A^2	0	50	0	50			
$6133Cp^rCn^r$	A^1	50	0	70	30	3:1	1.08	0.20
$6134S^rCn^r$	A^2	50	0	71	33	3:1	2.17	0.10

[a] Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

Table 24. Sensitivity to chloroneb of single-oospore cultures from randomly selected progeny of selfed oospore of isolate 6133Cp^{r}Cn^r of *Phytophthora parasitica*

Isolate	No. of single-oospore cultures[a]		Expected ratio of 3:1	
	Resistant	Sensitive	X^2	P
Resistant				
1	25	0		
2	17	8	0.33	0.50
3	12	8	1.67	0.10
4	20	0		
5	10	10	5.40	0.01
6	18	5	0.01	0.90
7	14	6	0.07	0.70
8	15	5	0.07	0.70
9	13	11	4.50	0.01
10	25	10	0.09	0.70
11	22	0		
Sensitive				
1	0	19		
2	0	23		

[a] Oospore produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

resistant and chloroneb-sensitive cultures, indicating that resistance to chloroneb in these isolates is controlled by a dominant gene in heterozygous condition. The ratio of 3 homozygous resistant : 8 heterozygous resistant is consistent with a 1:2 ratio (X^2 = 0.01, P = 0.90) expected for a single dominant resistant gene in heterozygous condition in the parent 6133Cp^rCn^r.

Among the eight isolates which were heterozygous for resistance to chloroneb, six produced progeny with acceptable chi-square values for testing the goodness of fit to a ratio of 3 resistant : 1 sensitive expected for a single dominant resistant gene in heterozygous condition (Table 24). The other two isolates produced progeny which did not fit the expected ratio of 3 resistant : 1 sensitive.

Characteristics of progenies from crosses between wild types and metalaxyl-resistant mutants -- All single-oospore cultures produced by wild types P991 and P731 through hormonal stimulation were sensitive to metalaxyl, while all single-oospore cultures produced by metalaxyl-resistant P991-2308M^r and P991-2329M^r, originated from single-oospore cultures of P991-23M^r shown in Table 22, were resistant to metalaxyl (Table 25). These results suggest that sensitivity to metalaxyl in the wild types is controlled by a pair of homozygous recessive alleles, while resistance to metalaxyl in these two mutants is controlled by a pair of homozygous dominant alleles. All single-oospore cultures from the pairing between the metalaxyl-resistant P991-2308M^r

Table 25. Sensitivity to metalaxyl of progenies of pairings between metalaxyl-resistant mutants and wild types of *Phytophthora parasitica*

Method of pairing	No. of single-oospore cultures	
	Resistant	Sensitive
Separated by polycarbonate membrane		
P991, A^1 (stimulated by P731, A^2)	0	50
P731, A^2 (stimulated by P991, A^1)	0	50
P991-2308M^r, A^1 (stimulated by P731, A^2)	75	0
P991-2329M^r, A^2 (stimulated by P991, A^1)	75	0
Direct pairing		
P991-2308M^r, A^1 X P731, A^2	99	21
P991-2329M^r, A^2 X P991, A^1	64	11
P991-2308M^r, A^1 X P991-2329M^r, A^2	60	0

(A^1) and P991-2329M^r (A^2) were resistant to metalaxyl (Table 25). This is consistent with the expected result when resistance to metalaxyl in both parents is homozygous. The ratio of selfs : hybrids in the progeny can not be determined from this test.

Oospore progeny from the pairing between metalaxyl-resistant A^1 (P991-2308M^r) and A^2 wild type (P731) consisted of 99 metalaxyl-resistant and 21 metalaxyl-sensitive cultures (Table 25). Five metalaxyl-sensitive and 40 metalaxyl-resistant isolates were randomly selected from the oospore progeny for further study. Cultures derived from selfed oospores produced by metalaxyl-sensitive isolates were all sensitive to metalaxyl (Table 26), indicating that sensitivity to metalaxyl in these isolates is conferred by a pair of homozygous recessive alleles. Therefore, these isolates are the result of selfing of the wild type parent (P731). All single-oospore cultures obtained from 1 of 40 metalaxyl-resistant isolates through hormonal stimulation were resistant to metalaxyl (Table 26), indicating that resistance to metalaxyl in this isolate is controlled by a pair of homozygous dominant alleles and the isolate is, therefore, originated from selfing of the metalaxyl-resistant parent (P991-2308M^r). Selfed oospores produced by the other 39 metalaxyl-resistant isolates gave rise to both metalaxyl-resistant and metalaxyl-sensitive cultures, indicating that these isolates carry dominant resistant gene in heterozygous condition and are, therefore, hybrids.

Table 26. Sensitivity to metalaxyl of single-oospore cultures from randomly selected progeny of the direct pairing between metalaxyl-resistant mutant (P991-2308M^r, A1) and wild type (P731, A2) of *Phytophthora parasitica*

	No. of single-oospore cultures[a]		Expected ratio of 3:1	
Isolate	Resistant	Sensitive	X^2	P
Resistant				
1	79	39	3.67	0.05
2	22	12	1.41	0.20
3	41	11	0.23	0.50
4	30	20	5.23	0.01
5	25	13	1.26	0.20
6	37	21	3.31	0.05
7	10	10	5.40	0.01
8	17	9	0.82	0.30
9	23	30	26.60	0.00
10	18	7	0.01	0.90
11	15	6	0.01	0.90
12	52	0		
13	20	7	0.01	0.90
14	17	4	0.14	0.20
15	18	5	0.01	0.90
16	17	8	0.33	0.70
17	32	18	2.67	0.10

Table 26. (Continued) Sensitivity to metalaxyl of single-oospore cultures from randomly selected progeny of the direct pairing between metalaxyl-resistant mutant (P991-2308M^{r}, A1) and wild type (P731, A2) of <u>Phytophthora parasitica</u>

	No. of single-oospore cultures[a]		Expected ratio of 3:1	
Isolate	Resistant	Sensitive	X^2	P
18	19	9	0.43	0.50
19	20	6	0.00	1.00
20	27	11	0.14	0.70
21	65	32	2.89	0.05
22	33	15	0.69	0.30
23	34	16	0.96	0.30
24	36	17	1.06	0.30
25	18	9	0.60	0.30
26	32	8	0.30	0.50
27	28	9	0.01	0.90
28	17	9	0.82	0.30
29	15	11	3.28	0.05
30	16	4	0.07	0.70
31	14	5	0.02	0.50
32	14	12	5.13	0.01
33	16	8	0.50	0.30
34	18	9	0.60	0.30
35	24	7	0.01	0.90
36	21	6	0.01	0.90

Table 26. (Continued) Sensitivity to metalaxyl of single-oospore cultures from randomly selected progeny of the direct pairing between metalaxyl-resistant mutant (P991-2308M^r, A1) and wild type (P731, A2) of Phytophthora parasitica

	No. of single-oospore cultures[a]		Expected ratio of 3:1	
Isolate	Resistant	Sensitive	X^2	P
37	17	8	0.33	0.50
38	16	5	0.01	0.90
39	24	10	0.16	0.50
40	30	12	0.13	0.70
Sensitive				
1	0	26		
2	0	30		
3	0	27		
4	0	38		
5	0	30		

[a] Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

Among the 39 isolates which were heterozygous for resistance to metalaxyl, 35 produced progeny with acceptable chi-square values for testing the goodness of fit to a 3 resistant : 1 sensitive expected for a single dominant resistance gene in heterozygous condition (Table 26). The other four isolates produced progeny which did not fit the expected ratio of 3 resistant to 1 sensitive.

Oospore progeny from the pairing between A^1 wild type (P991) and metalaxyl-resistant A^2 (P991-2329M^r) consisted of 64 metalaxyl-resistant and 11 metalaxyl-sensitive cultures (Table 25). Two metalaxyl-sensitive and six metalaxyl-resistant isolates were randomly selected from the oospore progeny for further study. Based on the analysis of sensitivity to metalaxyl of the progeny from selfed oospores produced by each isolate as described above (Table 27), the two sensitive isolates were from selfing of the wild type parent (P991), one resistant isolate was from selfing of the resistant parent (P991-2329M^r) and the other five resistant isolates were hybrids. All hybrids which were heterozygous for resistance to metalaxyl segregated in a ratio of 3 resistant : 1 sensitive (Table 27), confirming that resistance to metalaxyl in isolate P991-2329M^r is conferred by a pair of homozygous dominant alleles.

Characteristics of progeny from cross between 6133-1Cp^rCn^r, A^1 and 6134S^r, A^2 -- All single-oospore progenies of 6133-1Cp^rCn^r and 6134S^r have the same characteristic of chemical resistance as their parents (Table 28). This

Table 27. Sensitivity to metalaxyl of single-oospore cultures from randomly selected progeny of the direct pairing between metalaxyl-resistant mutant (P991-2329M[r], A2) and wild type (P991, A1) of <u>Phytophthora parasitica</u>

	No. of single-oospore cultures[a]		Expected ratio of 3:1	
Isolate	Resistant	Sensitive	X^2	P
Resistant				
1	17	8	0.33	0.50
2	22	6	0.05	0.70
3	14	6	0.07	0.70
4	16	6	0.00	0.99
5	12	8	1.67	0.10
6	28	0		
Sensitive				
1	0	20		
2	0	24		

[a] Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

suggests that 6133-1Cp^rCn^r carries a dominant gene for chloroneb resistance in homozygous condition and 6134S^r carries a pair of homozygous recessive alleles. Oospore progeny from the pairing between 6133-1Cp^rCn^r and 6134S^r consisted of 95 cultures resistant to chloramphenicol and chloroneb and 38 cultures resistant to streptomycin and chloroneb (Table 28). The latter represent hybrids with the streptomycin-resistant character from the parent 6134S^r and the chloroneb-resistant character from the parent 6133-1Cp^rCn^r. Forty of 95 cultures resistant to chloroneb and chloramphenicol were randomly selected for further study. Progeny from selfed oospores produced by three of these cultures were all resistant to chloramphenicol and chloroneb (Table 29), indicating that they are from selfing of parent 6133-1Cp^rCn^r. Progeny from selfed oospores produced by each of the other 37 cultures consisted of cultures resistant to chloramphenicol and chloroneb and cultures resistant to chloramphenicol but sensitive to chloroneb (Table 29). This result suggests that these 37 cultures are hybrids with a dominant gene resistant to chloroneb from the parent 6133-1Cp^rCn^r and a recessive gene sensitive to chloroneb from the parent 6134S^r. Results show that hybrids from the pairing between chloramphenicol-resistant 6133-1Cp^rCn^r and streptomycin-resistant 6134S^r are resistant to either chloramphenicol or streptomycin but not both, suggesting that chloramphenicol-resistant and streptomycin-resistant genes are present in cytoplasm.

Table 28. Phenotypic behavior of progenies of pairing between mutant resistant to chloramphenicol and chloroneb ($6133\text{-}1Cp^rCn^r$, A1) and streptomycin-resistant mutant ($6134S^r$, A2) of <u>Phytophthora parasitica</u>

Method of pairing	No. of single-oospore cultures		
	Resistant to chloramp.and chloron.	Resistant to strept.	Resistant to strept. and chloron.
Separated by polycarbonate membrane			
$6133\text{-}1Cp^rCn^r$, A^1 (stimulated by $6134S^r$, A^2)	75	0	0
$6134S^r$, A^2 (stimulated by $6133\text{-}1Cp^rCn^r$, A^1)	0	100	0
Direct pairing			
$6133\text{-}1Cp^rCn^r$, A^1 X $6134S^r$, A^2	95	0	38

Table 29. Phenotypic behavior of single-oospore cultures from randomly selected progeny[a] of the direct pairing between mutant resistant to chloramphenicol and chloroneb (6133-1Cp^rCn^r, A1) and streptomycin resistant mutant (6134S^r, A2) of <u>Phytophthora parasitica</u>

Isolate	No. of single-oospore cultures[b]		Expected ratio of 3:1	
	Resistant to chloramp. and chloron.	Resistant to chloramp. but sensitive to chloron.	X^2	P
1	25	0		
2	11	9	3.27	0.05
3	18	4	0.24	0.50
4	15	5	0.07	0.70
5	15	7	0.24	0.50
6	17	3	0.60	0.30
7	20	0		
8	14	6	0.07	0.70
9	14	8	0.97	0.30
10	18	3	0.78	0.30
11	14	5	0.02	0.70
12	17	3	0.60	0.30
13	15	5	0.07	0.70
14	17	8	0.33	0.50
15	15	6	0.01	0.90

Table 29. (Continued) Phenotypic behavior of single-oospore cultures from randomly selected progeny[a] of the direct pairing between mutant resistant to chloramphenicol and chloroneb (6133-1$Cp^r Cn^r$, A1) and streptomycin resistant mutant (6134S^r, A2) of *Phytophthora parasitica*

	No. of single-oospore cultures[b]		Expected ratio of 3:1	
Isolate	Resistant to chloramp. and chloron.	Resistant to chloramp. but sensitive to chloron.	X^2	P
16	18	2	1.67	0.10
17	18	2	1.67	0.10
18	17	6	0.01	0.90
19	16	4	0.07	0.70
20	11	10	4.58	0.01
21	19	0		
22	16	6	0.00	0.99
23	16	4	0.07	0.70
24	20	5	0.12	0.70
25	18	3	0.78	0.30
26	14	10	2.72	0.05
27	13	7	0.60	0.30
28	14	10	2.72	0.05
29	16	5	0.01	0.90
30	15	3	0.30	0.50
31	13	7	0.60	0.30
32	10	10	5.40	0.01

Table 29. (Continued) Phenotypic behavior of single-oospore cultures from randomly selected progeny[a] of the direct pairing between mutant resistant to chloramphenicol and chloroneb (6133-1Cp^{r}Cn^r, A1) and streptomycin resistant mutant (6134S^r, A2) of Phytophthora parasitica

Isolate	No. of single-oospore cultures[b]		Expected ratio of 3:1	
	Resistant to chloramp. and chloron.	Resistant to chloramp. but sensitive to chloron.	X^2	P
33	11	14	15.66	0.00
34	18	4	0.24	0.50
35	13	7	0.60	0.30
36	15	9	1.39	0.20
37	12	8	1.67	0.10
38	15	5	0.07	0.70
39	14	8	0.97	0.30
40	12	8	1.67	0.10

[a] Selection was made only from those progeny which were resistant to both chloramphenicol and chloroneb.

[b] Oospore produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

Among the 37 isolates which were heterozygous for resistance to chloroneb, 34 produced progeny with acceptable chi-square values for testing the goodness of fit to a 3 resistant : 1 sensitive expected for a single dominant resistance gene in heterozygous condition (Table 29). The other three isolates produced progeny which did not fit the expected ratio of 3 resistant : 1 sensitive.

Characteristics of progeny from cross between $6133Cp^r$, A^1 and $6134\text{-}1S^rCn^r$, A^2 -- All the single-oospore progeny of $6133Cp^r$ and $6134\text{-}1S^rCn^r$ also have the same characteristic of chemical resistance as their parent (Table 30), indicating that $6134\text{-}1S^rCn^r$ carries a dominant gene for chloroneb resistance in homozygous condition and $6133Cp^r$ carries a pair of homozygous recessive alleles. Oospore progeny from the pairing between $6133Cp^r$ and $6134\text{-}1S^rCn^r$ consisted of 4 cultures resistant to chloramphenicol but sensitive to chloroneb, 17 cultures resistant to streptomycin and chloroneb, and 102 cultures resistant to chloramphenicol and chloroneb (Table 30). The 4 cultures resistant to chloramphenicol but sensitive to chloroneb represent selfs of the parent $6133Cp^r$, while the 102 cultures resistant to chloramphenicol and chloroneb represent hybrids with chloramphenicol-resistant character from parent $6133Cp^r$ and chloroneb-resistant character from parent $6134\text{-}1S^rCn^r$. Eight of 17 cultures resistant to streptomycin and chloroneb were randomly selected for further study. Progeny from selfed oospores produced by one of these cultures were all

Table 30. Phenotypic behavior of progenies of pairing between chloramphenicol-resistant mutant (6133Cp^r, A1) and mutant resistant to streptomycin and chloroneb (6134-1S^{r}Cn^r, A2) of *Phytophthora parasitica*

Method of pairing	No. of single-oospore cultures		
	Resistant to chloramp.	Resistant to strept. and chloron.	Resistant to chloramp. and chloron.
Separated by polycarbonate membrane			
6133Cp^r, A^1 (stimulated by 4134-1S^{r}Cn^r, A^2)	100	0	0
6134-1S^{r}Cn^r, A^2 (stimulated by 6133Cp^r, A^1)	0	75	0
Direct pairing			
6133Cp^r, A^1 X 6134-1S^{r}Cn^r, A^2	4	17	102

resistant to streptomycin and chloroneb (Table 31), indicating that the culture is from selfing of the parent 6134-1$S^r$$Cn^r$. Progeny from selfed oospores produced by each of the other 7 cultures consisted of cultures resistant to streptomycin and chloroneb and cultures resistant to streptomycin but sensitive to chloroneb (Table 31), suggesting that they are hybrids with a dominant gene resistant to chloroneb from the parent 6134-1$S^r$$Cn^r$ and a recessive gene sensitive to chloroneb from parent 6133Cp^r. Results show that hybrids from the cross between chloramphenicol-resistant 6133Cp^r and streptomycin-resistant 6134-1$S^r$$Cn^r$ are resistant to either chloramphenicol or streptomycin but not both, again suggesting that chloramphenicol-resistant and streptomycin-resistant genes are present in cytoplasm. All hybrids which were heterozygous for resistance to chloroneb segregated in a ratio of 3 resistant : 1 sensitive (Table 31), confirming that resistance to chloroneb in isolate 6134-1$S^r$$Cn^r$ is conferred by a pair of homozygous dominant alleles.

Characteristics of progeny from cross between 6133-1$Cp^r$$M^r$, A^1 and 6134-1$S^r$$Cn^r$, A^2 -- All single-oospore progenies of 6133-1$Cp^r$$M^r$ and 6134-1$S^r$$Cn^r$ were similar to their respective parent in chemical resistance (Table 32), indicating control of metalaxyl resistance in 6133-1$Cp^r$$M^r$ by a dominant gene in homozygous condition and control of chloroneb resistance in 6134-1$S^r$$Cn^r$ also by a dominant gene in homozygous condition. Oospore progeny from the pairing

Table 31. Phenotypic behavior of single-oospore cultures from randomly selected progeny[a] of the direct pairing between chloramphenicol-resistant mutant (6133Cp^r, A1) and mutant resistant to streptomycin and chloroneb (6134-1S^{r}Cn^r, A2) of *Phytophthora parasitica*

Isolate	No. of single-oospore cultures[b]		Expected ratio of 3:1	
	Resistant to strept. and chloron.	Resistant to strept. but sensitive to chloron.	X^2	P
1	14	6	0.07	0.70
2	14	8	0.97	0.30
3	17	5	0.00	0.99
4	23	0		
5	13	5	0.00	0.99
6	18	6	0.06	0.70
7	13	9	2.18	0.10
8	21	4	0.65	0.30

[a] Selection was made only from those progeny which were resistant to both streptomycin and chloroneb.

[b] Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

Table 32. Phenotypic behavior of progenies of pairing between mutant resistant to chloramphenicol and metalaxyl (6133-1Cp^rM^r, A1) and mutant resistant to streptomycin and chloroneb (6134-1S^rCn^r, A2) of *Phytophthora parasitica*

Method of pairing	No. of single-oospore cultures			
	Resistant to chloramp. and metal.	Resistant to strept., metal. and chloron.	Resistant to chloramp., metal. and chloron.	Resistant to strept. and chloron.
Separated by polycarbonate membrane				
6133-1Cp^rM^r, A^1 (stimulated by 6134-1S^rCn^r, A^2)	75	0	0	0
6134-1S^rCn^r, A^2 (stimulated by 6133-1Cp^rM^r, A^1)	0	0	0	75
Direct pairing				
6133-1Cp^rM^r, A^1 X 6134-1S^rCn^r, A^2	4	92	46	6

between 6133-1Cp^{r}M^r and 6134-1S^{r}Cn^r consisted of 4 cultures resistant to chloramphenicol and metalaxyl, 92 resistant to streptomycin, metalaxyl and chloroneb, 46 resistant to chloramphenicol, metalaxyl and chloroneb, and 6 resistant to streptomycin and chloroneb (Table 32). The 4 cultures resistant to chloramphenicol and metalaxyl are from selfing of the parent 6133-1Cp^{r}M^r and the 6 cultures resistant to streptomycin and chloroneb are from selfing of the parent 6134-1S^{r}Cn^r. The 46 cultures resistant to chloramphenicol, metalaxyl and chloroneb are hybrids with chloramphenicol- and metalaxyl-resistant characters from parent 6133-1Cp^{r}M^r and the chloroneb-resistant character from parent 6134-1S^{r}Cn^r. The 92 cultures resistant to streptomycin, metalaxyl and chloroneb are also hybrids but with metalaxyl-resistant character from parent 6133-1Cp^{r}M^r and streptomycin- and chloroneb-resistant characters from parent 6134-1S^{r}Cn^r.

DISCUSSION

Physiological and morphological effects of metalaxyl on Phytophthora

Results of this study show that ability of mating type conversion by metalaxyl varies among isolates of the same species of Phytophthora. Because of the limited supply of P. infestans from different sources for the present study, population diversity was achieved by using cultures derived from oospores. Mating type conversion by metalaxyl in P. infestans was observed among these cultures.

Mexico had been considered to be the only location having A^2 mating type of P. infestans (19, 56) until several years ago when Hohl and Iselin (21) reported the discovery of A^2 isolates in Switzerland. Subsequently A^2 isolates were also found in Scotland (31), England and Wales (59) and Japan (33). The discovery of A^2 isolates of P. infestans in Europe appeared to follow closely the commercial application of metalaxyl to control late blight of potato and tomato in this region (22). Therefore, the conversion of A^1 to A^2 mating type by metalaxyl in infected host tissues is postulated as a possible origin of A^2 isolates of P. infestans in Europe. However, the possibility that some or all A^2 isolates found in Europe and Japan were due to mating type conversion by other fungicides or due to direct or indirect import from Mexico has not been ruled out.

Based on hormone production and responsiveness to hormones, 16 sexuality types among members of *Phytophthora* were postulated by Ko (26, 28). When the mating type of *P. parasitica* was changed from A^1 to A^2 by ethazol (29), the sexuality type was changed from S^1 to S^4. This study shows that metalaxyl can change the sexuality type of P991 of *P. parasitica* and 902 of *P. infestans* from S^1 to S^4 and S^5. When the mating type of *P. parasitica* was changed from A^2 to A^1 by chloroneb (29), the sexuality type was changed from S^4 to S^1. However, when A^2 mating type of *P. infestans* was changed to A^1 by metalaxyl, the sexuality type was changed from S^4 to S^1 and S^2. S^5 of *P. parasitica* and S^2 of *P. infestans* have not been reported previously (28).

As in previous study with *P. parasitica* (28), the finding of mating type change from A^1 to A^2 in *P. infestans* does not support the hypothesis that A^1 is homozygous recessive aa, and that A^2 is heterozygous Aa (40), because the homozygous recessive character should not segregate. Ko (27) postulated the presence of a repressor in *Phytophthora* which represses the expression of A^1 mating type with one molecular configuration, and A^2 type with another configuration. Results from this study suggest that metalaxyl may have changed the mating type by reversing the function of the repressor. The unstable nature of A^1A^2 culture of *P. infestans* is similar to that of *P. parasitica* (27) and is also considered to be a transitional state in the process of mating type change.

Results show that exposure of _P_. _parasitica_ to metalaxyl for 6 weeks caused many of the asexual progeny to change their colony morphology, growth rate, ability to produce sporangia and ability of zoospores produced by them to form colonies. Morphological and physiological changes occurred in both metalaxyl-sensitive and metalaxyl-resistant isolates. Altered morphological and physiological characters were expressed in the absence of metalaxyl, indicating that the changes are permanent and suggesting the mutagenic nature of metalaxyl. Since asexual progeny produced by these mutants displayed further segregation in growth rates, genes affected by metalaxyl are probably located in the cytoplasm. Whether metalaxyl is also mutagenic to other fungi remains to be investigated. When one isolate of _P_. _infestans_ was grown for 6 weeks on medium containing metalaxyl, colony morphology and growth rate of many of its asexual progeny were changed when cultured on metalaxyl-free medium (Chang and Ko, unpublished).

Incubation of _P_. _parasitica_ on medium containing metalaxyl also induced resistance to metalaxyl among its asexual progeny. Nine of the mutants became either sensitive to or less resistant to metalaxyl, while four others retained their resistance to metalaxyl after eight consecutive subcultures on metalaxyl-free medium. This is in agreement with previous reports (12, 66). Selection has been suggested to be the mechanism for the appearance of metalaxyl-resistant mutants (12). However, results of this

study suggest that the mutagenic effect of metalaxyl may also play an important role in creating mutants resistant to metalaxyl.

Genetics of P. infestans

Results from this research show that activation of oospores with $KMnO_4$ and exposure of spores to light during germination are important treatments for obtaining consistent high germination rate of oospores of P. infestans. Under such conditions oospores of P. infestans germinated about 70% within 20 days. Exposure to light during oospore maturation is not required for high germination rate of P. infestans oospores. However, for obtaining high germination rate of P. parasitica such treatment is essential (3).

Isolate 533 of P. infestans was heterozygous at PEP locus and produced a three-banded isozyme pattern. All asexual cultures were identical to the parent at PEP locus, but progenies from selfing segregated 1:2:1. Isolates 932, 908 and 928 originating from 533 were homozygous slow (P^1P^1), heterozygous (P^1P^2) and homozygous fast (P^2P^2) at PEP locus, respectively. All selfed progenies derived from 932 and 928 were homozygous slow and homozygous fast, respectively. Like the parental isolate 533, selfed progenies of 908 also segregated 1:2:1 at PEP locus. These results indicate that P. infestans is diploid in somatic nuclei. Greater variation in growth rate and colony

morphology of selfed-oospore cultures than that of zoospore cultures of the same isolate is also an indirect evidence for diploidy in somatic nuclei of P. infestans. Shattock et al. (48) reported that an isolate of P. infestans was heterozygous at PEP locus because segregation occurred when 9 selfed-oospore cultures were analyzed. However, one of the three expected phenotypes did not appear due to the small sample size. Using improved squashing techniques, Sansome provided cytological evidence suggesting that species of Phytophthora are diploid (40). Studies with DNA specific stains and fluorochromes also provided evidence for diploidy of Phytophthora (51).

Boccas (7) reported that growth rates of homothallic P. syringae were relatively uniform among single-zoospore cultures but were variable among single-oospore cultures. Ann (2) also found that amylase activity, colony morphology and growth rate of heterothallic P. parasitica were relatively uniform among single zoospore cultures but not selfed oospore cultures. These results suggest that these characters are controlled by heterozygous genes present in diploid organisms.

Based on the electrophoretic patterns of GPI-1, 238 of 239 progeny from the cross 533X550 were hybrids. Only one of them was a selfed oospore. When isolates with homozygous genes at GPI-1 and PEP loci were paired, the ratios of hybrids : selfs were 60 : 20 for the cross 926X936, 75 : 0 for 550X928 and 74:1 for 550X932. Selfing appeared to be

inhibited during direct matings. Based on electrophoretic patterns of GPI-1, Shattock et al. (47) also reported that less than 2% of the F1 progeny from 533X550 and 533X511 were selfs, but up to 10% from a cross between 550 and a Dutch metalaxyl-resistant 1100 were selfs.

Streptomycin-resistant mutants and chloramphenicol-resistant mutants have been isolated from different species of Phytophthora (4, 24, 46, 54, 61). Both streptomycin-resistant and chloramphenicol-resistant mutants were also isolated from P. infestans in this study. All single-zoospore cultures and single-oospore cultures were identical to their respective parents in antibiotic resistance. These results are consistent with the results of P. parasitica reported by Ann and Ko (4) and P. cactorum reported by Shaw and Elliott (54). When A^1 isolate $903S^r$, resistant to streptomycin, was paired directly with A^2 isolate $947Cp^r$, resistant to chloramphenicol, all single-oospore cultures from the cross were resistant to either chloramphenicol or streptomycin, but not both or neither, suggesting that all single-oospore cultures from direct pairing were of uniparental origin or that antibiotic-resistant characters were located in cytoplasm. Since both A^1 and A^2 mating types are capable of serving as female (oogonium) and male (antheridium) (27, 28), a reciprocal test is not suitable for determining if cytoplasmic inheritance is involved in antibiotic-resistance. In this study, electrophoretic patterns of PEP was used in combination with antibiotic-

resistant characters to determine the origin of each progeny in direct matings. Although all the progeny from the cross 903S^r (P^1P^1) X 947Cp^r (P^2P^2) were hybrids (P^1P^2), they were resistant to either streptomycin or chloramphenicol but not both or neither. This suggests that genes responsible for resistance to streptomycin and chloramphenicol were located in cytoplasm.

Sansome (40) suggested that mating type is controlled by two alleles of a single gene and that A^1 mating type is homozygous recessive (aa), while A^2 type is heterozygous (Aa). In the present study, the selfed progenies of both A^1 and A^2 types of P. infestans consisted of A^1, A^2 and self-fertile types, similar to results obtained for P. parasitica by Ann and Ko (4). These results do not support the hypothesis suggested by Sansome because homozygous recessive A^1 should not segregate during selfing. Sansome (41) also suggested that self-fertile (A^1A^2) isolates of heterothallic Phytophthora are due to the trisomic condition (Aaa) of the mating type locus, but this is not compatible with the occurrence of self-fertile isolates from selfed homozygous A^1 parents. However, self-fertile did appear in the selfed-oospore cultures obtained from both A^1 and A^2 parents in P. infestans as well as P. parasitica (4). Shaw (52) and Shattock et al. (47) reported that A^1 did not segregate in selfed progeny, although selfing of A^2 of P. infestans gave rise to both A^1 and A^2 progenies. This could be due to insufficient number of progenies tested. Ko (28) postulated

the coexistence of genes for A^1 and A^2 in Phytophthora and suggested that genes for hormone production and reception are linked in both A^1 and A^2 isolates and that transcription of such linked genes is regulated by a repressor that represses the expression of A^1 with one molecular configuration and A^2 with another configuration. The appearance of A^1, A^2 and self-fertile types in the selfed progeny from both A^1 and A^2 types can be easily explained based on this hypothesis.

Caten and Jinks (13) found that zoospore progeny of P. infestans displayed an extensive variation in growth rate and colony morphology and suggested that these characters were under cytoplasmic control. In this study, zoospore progenies of isolates 533 and 550 were uniform and similar to their respective parents in the rate of linear extension and colony appearance, suggesting that these characters were probably controlled by nuclear genes in these isolates.

Contrary to zoospore progenies, progenies of selfed oospores of isolates 533 and 550 of P. infestans displayed a continuous quantitative variation in growth rate. This suggested that heterozygous polygenes probably were involved in determining the rate of linear growth. Release of variation in growth rate in progenies of selfed oospores but not zoospores of P. infestans is consistent with the hypothesis that species of Phytophthora are diploid in vegetative state (40). These results show that inheritance

of growth rate of P. infestans is similar to that of P. parasitica (2) and P. syringae (7).

The character of colony pattern in both isolates of P. infestans tested also showed extensive variation in progenies of selfed oospores but not zoospores. Since more than one new colony types appeared as a result of sexual reproduction, it is considered possible that heterozygous polygenes also are involved in determining the appearance of colony in these fungi. Ann (2) also found variation in colony morphology among progenies of selfed oospores but not zoospores of P. parasitica.

Genetics of P. parasitica

The results suggest that both chloroneb resistance and metalaxyl resistance in P. parasitica are controlled by single dominant genes which are located in the nucleus because they are inherited in regular Mendelian fashion. The uniparental inheritance of chloramphenicol-resistant and streptomycin-resistant characters in P. parasitica suggests that these characters are controlled by cytoplasmic genes inherited solely through the maternal parent (58). The use of fungicide-resistant genes in the nucleus in conjunction with antibiotic-resistant genes in the cytoplasm in the pairings between different mating types of cross-inducing Phytophthora not only enables one to distinguish hybrids from selfs, but also to know the parental origin of oogonium and antheridium for each hybrid oospore (Fig. 18). For

example, hybrids resistant to chloramphenicol, metalaxyl and chloroneb from the pairing between 6133-1Cp^{r}M^r and 6134-1S^{r}Cn^r are from oospores resulting from the union of oogonium from 6133-1Cp^{r}M^r with antheridium from 6134-1S^{r}Cn^r, and hybrids resistant to streptomycin, metalaxyl and chloroneb are from the union of oogonium from 6134-1S^{r}Cn^r with antheridium from 6133-1Cp^{r}M^r (Fig. 18).

Isolates P991-23M^r and 6133Cp^{r}Cn^r were heterozygous for resistance to metalaxyl and chloroneb, respectively (Table 21, 23). In principle their selfed progeny carrying heterozygous gene for resistance should all produced a second generation of selfed offspring segregating in a ratio of 3 resistant : 1 sensitive expected for a single dominant resistance gene in heterozygous condition. Although most of them produced offspring which fit the expected ratio, offspring produced by 5 of 31 progeny from isolate P991-23M^r and 2 of 8 progeny from isolate 6133Cp^{r}Cn^r did not fit (Table 22, 24). The reason for the inconsistent behavior of these progeny is still unknown. When new batches of single-oospore cultures were obtained from two of these progeny, both segregated in a ratio of 3 resistant : 1 sensitive as expected. It is, therefore, considered possible that the discrepancy may be due to biological variation as numbers used were relatively small. Isolates P991-2308M^r and 6133-1Cp^{r}Cn^r were homozygous for resistance to metalaxyl and chloroneb, respectively (Table 25, 28). In principle their progeny heterozygous for chemical resistance, from the

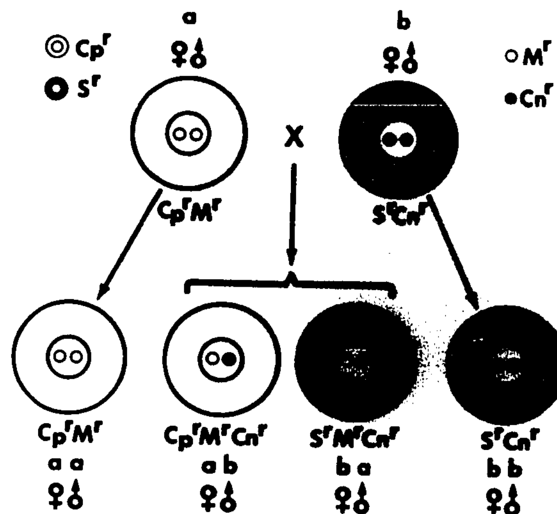

Fig. 18. Diagrammatic representation of results of the pairing between a carrying homozygous metalaxyl-resistant (M^r) alleles in the nucleus and chloramphenicol-resistant (Cp^r) gene in the cytoplasm and b carrying homozygous chloroneb-resistant (Cn^r) alleles in the nucleus and streptomycin-resistant (S^r) gene in the cytoplasm. Both parents produce oogonia ( ) and antheridia ( ) in Phytophthora. Sex symbols of the progeny represent the origins of gametangia for the formation of each type of offspring.

pairing with sensitive isolates, should all produced a second generation of selfed offspring segregating in a ratio of 3 resistant : 1 sensitive expected for a single dominant gene in heterozygous condition. Although most of them produced offspring which fit the expected ratio, offspring produced by 4 of 39 progeny from isolate P991-2308M^r and 3 of 37 progeny from isolate 6133-1Cp^rCn^r did not fit (Table 26, 29). Biological variation is also considered to the possible factor contributing to the inconsistent behavior of these progenies.

Chloroneb resistance in *Ustilago maydis* has also been reported to be controlled by a single gene (61). Contrary to the results reported here, metalaxyl resistance in *Bremia lactucae* (15) and *Phytophthora infestans* (45) was considered to be controlled by a single gene exhibiting incomplete dominance. Streptomycin resistance in *Chlamydomonas reinhardtii* has been found to be controlled by nuclear genes in some strains and cytoplasmic genes in others (39). Both nuclear inheritance and cytoplasmic inheritance of chloramphenicol resistance have also been observed in *Aspergillus nidulans* (20).

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