Resistance to Fungicides and Antibiotics in *Phytophthora parasitica*: Genetic Nature and Use in Hybrid Determination

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Journal Series Paper No. 3453 of the Hawaii Institute of Tropical Agriculture and Human Resources.

This research was supported in part by the U.S. Department of Agriculture under Cooperative State Research Service (CSRS) Special Grant No. 85-CRSR-2-2661, managed by the Pacific Basin Advisory Group (PBAG). We thank the generous gift of chloroneb from Kincaid Enterprises, Nitro, WV.

Accepted for publication 13 July 1990.

ABSTRACT

Chang, T. T., and Ko, W. H. 1990. Resistance to fungicides and antibiotics in *Phytophthora parasitica*: Genetic nature and use in hybrid determination. Phytopathology 80:1414-1421.

Progeny derived from oospores produced through hormonal stimulation by each of four metalaxyl-resistant (M^r) mutants of *Phytophthora parasitica* segregated in a ratio of 3 resistant: 1 sensitive. Each of two chloroneb-resistant (Cn^r) mutants also gave rise to selfed progeny with a segregation ratio of 3 resistant: 1 sensitive. These results indicate that metalaxyl resistance and chloroneb resistance in these mutants are each conferred by a single dominant gene in the heterozygous condition. Progeny from the pairing between M^r A^1 carrying homozygous dominant resistance alleles and A^2 wild type consisted of selfs from each parent and hybrids carrying heterozygous dominant resistance alleles. Similar results were obtained when A^1 wild type was paired with M^r A^2 carrying homozygous dominant resistance alleles. Progeny from the pairing between homozygous Cn^r A^1 resistant to chloramphenicol and A^2 resistant to streptomycin consisted of hybrids resistant to either chloramphenicol or streptomycin, but not both. Similar results were obtained when A^1 resistant to chloramphenicol was paired with homozygous Cn^r A^2 resistant to streptomycin. These results suggest that chloramphenicol-resistance and streptomycin-resistance genes are present in the cytoplasm. Progeny from the pairing between homozygous M^r A^1 resistant to chloramphenicol and homozygous Cn^r A^2 resistant to streptomycin consisted of 4 selfs from A^1, 6 selfs from A^2, 46 hybrids resulting from the union of an A^1 oogonium with an A^2 antheridium, and 92 hybrids resulting from the union of an A^2 oogonium with an A^1 antheridium.

When A^1 and A^2 mating types of the same or different species of cross-inducing (heterothallic) *Phytophthora* are paired in cultures, sexual reproduction is initiated and oospores are produced (10,17). By pairing isolates of *Phytophthora* on the opposite sides of polycarbonate membranes, it was found that the opposite mating type is needed for production of a mating-type specific hormone to initiate sexual reproduction (11,14). However, this method does not preclude the possibility of hybridization in direct pairings. Genetic exchange in the pairings between different species of *Phytophthora* has been shown to be essentially nonexistent (3,4,6). Although it has been generally

accepted that progenies resulting from direct matings within the same species are always crossed (7,8,20), genetic evidence for this assumption has been difficult to obtain because of lack of genetic markers and difficulty in separating selfs from hybrids in direct pairings. Shattock et al (19) used isozyme banding patterns to distinguish selfs from hybrid progeny. However, the presumption of homozygous alleles in parental isolates has not been experimentally confirmed. Moreover, the method is time consuming and the number of isolates and species with isozyme banding patterns suitable for use as markers for the genetic studies is limited.

Ann and Ko (2) found that the characters of chloramphenicol resistance and streptomycin resistance in *Phytophthora parasitica* Dastur (*Phytophthora nicotianae* van Breda de Haan) were transmitted to all the asexual progeny as well as sexual progeny of uniparental origin induced by hormonal stimulation. When a chloramphenicol-resistant mutant was paired with a streptomycin-resistant mutant of the opposite mating type, all the progeny were resistant to either chloramphenicol or streptomycin, but not both, suggesting the absence of genetic exchange in the progeny. However, since nuclear Mendelian inheritance of both characters had not been demonstrated, the possibility that lack of recombination between chloramphenicol- and streptomycin-resistant markers is due to cytoplasmic inheritance could not be ruled out (2).

In this paper, we report the evidence for nuclear gene control of metalaxyl resistance and chloroneb resistance in *P. parasitica*. We also use these two markers to determine the genetic nature of chloramphenicol resistance and streptomycin resistance and to distinguish hybrids from selfs in direct pairings.

MATERIALS AND METHODS

Cultures. Isolates P991 (A^1) and P731 (A^2) of *P. parasitica* were supplied by G. A. Zentmyer. Isolates 6133Cp^r (A^1, ATCC 66743, CBS 207.90) and 6134S^r (A^2, ATCC 66744, CBS 208.90) of the same species which were resistant to chloramphenicol and streptomycin, respectively, were obtained in a previous study (2). These mutants were selected by growing the fungus on V-8 agar containing (per ml) 300 μg of streptomycin sulfate or 100 μg of chloramphenicol. Each was derived from a single zoospore.

Induction of resistance to metalaxyl or chloroneb. Four pieces of culture blocks (ca. 10 × 10 × 3 mm) were placed on V-8 agar (10% V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar) containing (per ml) 0, 25, or 50 μg of metalaxyl (Subdue 2E, 25.11% active), or 0 or 100 μg of chloroneb (Terraneb sp, 65% active). 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 wk. Mutants, which appeared as fast-growing sectors, were transferred to V-8 agar containing (per ml) 25 μg of metalaxyl or 100 μg of chloroneb. Double mutants were similarly selected by growing antibiotic-resistant mutants on medium containing metalaxyl or chloroneb.

Isolation of single-zoospore cultures. Single-zoospore cultures were obtained using the method described by Ko (13). Zoospores were spread on 2% Bacto water agar, and those colonies originating from single zoospores were transferred to 5% V-8 agar (5% V-8 juice, 0.02% $CaCO_3$, and 2% Bacto agar) after incubation at 24 C for 2 days.

Determination of resistance to fungicides and antibiotics. The method described by Ann and Ko (2) 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 μg of metalaxyl per ml. 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 μg of chloroneb per ml. Resistance to chloramphenicol or streptomycin was determined by comparing growth on 5% clarified V-8 agar with and without 250 μg of chloramphenicol or 300 μg of streptomycin sulfate per ml. Two plates each with two culture disks were used for each single-spore culture. Symbols to represent phenotypes for chemical resistance were: Cp^r for resistance to chloramphenicol, S^r to streptomycin, Cn^r to chloroneb, and M^r to metalaxyl. For simplicity, phenotypes for chemical sensitivity (markers of original traits), including Cp^s, S^s, Cn^s, and M^s, were not mentioned.

Formation of oospores. Direct pairings were accomplished by placing two pieces (3 × 3 × 3 mm) of agar culture of the opposite mating type at 5 mm apart on a V-8 agar block (15 × 15 × 3 mm) in the center of a small Petri plate (60 mm). Plates were then sealed with two layers of Parafilm and incubated in darkness for 10 days at 24 C for oospore formation. Cultures were incubated for a total of 2–4 months under fluorescent light for oospore maturation (1). The polycarbonate membrane method described by Ko (11) was used to produce selfed oospores. A culture block (15 × 10 × 3 mm) of a 4-day-old A^1 or A^2 mating type was placed in the center of a Petri dish, covered with a sterile polycarbonate membrane (0.2 μm, 90 mm diameter; Nuclepore Corp., Pleasanton, CA), and chemically induced to produce oospores by a 1-day-old culture block of A^2 or A^1 type, respectively, on top. After incubation for 10 days at 24 C in darkness in a moist chamber, the membrane and the inducer on the top were removed, and the culture block containing oospores on the bottom was exposed to light for oospore maturation under the same conditions as described above.

Germination of oospores. The method of Ann and Ko (1) was used for germinating oospores of *P. parasitica*. Oospore suspension was obtained by grinding each culture containing oospores with 50 ml of distilled water in an Omni mixer (DuPont Instruments, Newtown, CT) at 4,500 rpm for 1 min. The suspension was filtered successively through a 53 μm and a 20 μm sieve. Oospores retained on the 20 μm sieve were washed with tap water and resuspended in 10 ml of sterile distilled water. Oospore suspension was mixed with equal volume of freshly prepared 0.5% $KMnO_4$ solution. After shaking for 20 min on a shaker, oospores were washed free of $KMnO_4$ on a 20 μm sieve with tap water. About 100–200 oospores were spread on S+L medium of Ruben

TABLE 1. Sensitivity to metalaxyl of single-zoospore and single-oospore cultures from metalaxyl-sensitive and metalaxyl-resistant isolates of *Phytophthora parasitica*

Isolate	Mating type	Single-zoospore cultures (no.)		Single-oospore cultures[b] (no.)			
						Expected ratio of 3:1	
		R[a]	S	R	S	χ^2	*P*
P991	A^1	0	50	0	50		
6133Cp^r	A^1	0	50	0	50		
P991-12M^r	A^1	50	0	37	13	0.00	0.99
P991-14M^r	A^1	55	0	40	10	0.43	0.50
P991-23M^r	A^2	50	0	76	32	1.00	0.30
6133Cp^{r}M^r	A^1	50	0	84	35	1.01	0.30

[a] R = resistant, S = sensitive.
[b] Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

et al (15) amended with 0.01% asparagine and 2% Bacto agar. After autoclaving, the medium was supplemented with 100 μg of ampicillin, 50 μg of nystatin, and 10 μg of pentachloronitrobenzene per ml to prevent growth of possible contaminants. After incubation at 24 C under light for 3–10 days, germinating oospores were individually transferred to V-8 agar using five oospores per plate.

RESULTS AND INTERPRETATION

Isolation of mutants. When culture blocks were incubated on medium containing 25 μg metalaxyl per ml, fast-growing sectors appeared within 6 wk. Among 100 single-zoospore cultures obtained from the sectors of P991, 23 cultures were resistant to metalaxyl. After being successively subcultured on metalaxyl-free medium five times, 10 of 23 resistant mutants remained resistant, 8 became sensitive, and 5 partially lost their resistance to metalaxyl. Among 200 single-zoospore cultures obtained from the sectors of 6133Cp^r, 18 cultures were resistant to metalaxyl. After being successively subcultured on metalaxyl-free medium five times, 8 of 18 resistant mutants remained resistant, 6 became sensitive, and 4 partially lost their resistance to metalaxyl. Fast-growing sectors also appeared within 6 weeks when culture blocks were incubated on medium containing 100 μg chloroneb per ml. Among 200 single-zoospore cultures derived from sectors of each isolate tested, 32 from 6133Cp^r and 21 from 6134S^r were resistant to chloroneb. All the mutants remained resistant to chloroneb after being successively subcultured on chloroneb-free medium five times. Only those stable mutants capable of producing large amounts of selfed oospores by hormonal stimulation were selected for this study.

TABLE 2. Sensitivity to metalaxyl of single-oospore cultures from randomly selected resistant progeny of selfed oospores of isolate P991-23M^r of *Phytophthora parasitica*

Isolate (23-No.)	R:S[a]	Expected ratio of 3:1 χ^2	P	Isolate (23-No.)	R:S	Expected ratio of 3:1 χ^2	P
01	15:4[b]	0.02	0.70	21	16:4	0.07	0.70
02	5:12	16.50	0.00	22	13:8	1.17	0.20
	(21:10	0.53	0.30)[c]	23	15:5	0.07	0.70
03	10:5	0.20	0.50	24	15:6	0.01	0.90
04	20:3	1.17	0.20	25	13:9	2.18	0.10
05	30:0			26	14:2	0.75	0.30
06	13:4	0.02	0.70	27	25:0		
07	5:10	11.80	0.00	28	13:5	0.00	0.99
	(25:10	0.07	0.70)[c]	29	75:0		
08	75:0			30	14:5	0.02	0.70
09	14:5	0.02	0.70	31	5:10	11.80	0.00
10	30:0			32	30:0		
11	16:5	0.01	0.90	33	15:5	0.07	0.70
12	20:0			34	20:4	0.05	0.30
13	12:6	0.30	0.50	35	18:5	0.01	0.90
14	17:0			36	22:10	0.38	0.50
15	10:10	6.10	0.01	37	17:6	0.01	0.90
16	8:13	13.40	0.00	38	17:5	0.00	0.99
17	16:7	0.13	0.70	39	20:7	0.01	0.90
18	25:0			40	14:5	0.02	0.70
19	18:3	0.78	0.30	41	16:6	0.00	0.99
20	27:0						

[a] R = resistant, S = sensitive.
[b] Number of single-oospore cultures.
[c] Additional batches of single-oospore cultures.

Genetic nature of metalaxyl resistance. All the single-zoospore and single-oospore cultures obtained from metalaxyl-sensitive isolates P991 and 6133Cp^r were sensitive to metalaxyl (Table 1). All the 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 1). 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 and suggest that sensitivity to metalaxyl in isolates P991 and 6133Cp^r to be used in pairing tests is conferred by a homozygous recessive gene.

Four metalaxyl-sensitive and 41 metalaxyl-resistant single-oospore isolates were randomly selected from oospore progeny of isolate P991-23M^r for further study. Eighteen to 24 single-oospore cultures obtained from each of four metalaxyl-sensitive isolates through hormonal stimulation were all sensitive to metalaxyl, 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 2), indicating that these isolates were homozygous for resistance to metalaxyl. Therefore, resistance to metalaxyl in these isolates, including isolates P991-2308M^r and P991-2329M^r to be used in the pairing tests, is conferred by a homozygous dominant gene. Selfed oospores produced by the other 31 metalaxyl-resistant isolates gave rise to both metalaxyl-resistant and metalaxyl-sensitive cultures (Table 2), 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 ($\chi^2 = 1.11$, $P = 0.20$) 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-sguare values ($\chi^2 < 3.84$, $P > 0.05$) 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 2). However, isolates 2, 7, 15, 16, and 31 produced progeny which did not fit to the expected ratio of 3 resistant: 1 sensitive ($\chi^2 > 3.84$, $P < 0.05$). 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. Both were consistent with the ratio of 3 resistant: 1 sensitive ($\chi^2 = 0.53$, $P = 0.30$ for isolate 2 and $\chi^2 = 0.07$, $P = 0.70$ for isolate 7) expected for a single dominant resistance gene in heterozygous condition.

Ten of the 84 metalaxyl-resistant single-oospore cultures from 6133Cp^{r}M^r (Table 1) were further studied by selfing. Eight isolates produced oospore progenies segregated in a ratio of 3 resistant: 1 sensitive ($\chi^2 = 0$–0.47, $P = 0.30$–0.99), indicating that resistance to metalaxyl in these isolates is conferred by a heterozygous dominant gene. The other two isolates (6133-1Cp^{r}M^r and 6133-

TABLE 3. Sensitivity to chloroneb of single-zoospore and single-oospore cultures from chloroneb-sensitive and chloroneb-resistant isolates of *Phytophthora parasitica*

Isolate	Mating type	Single-zoospore cultures (no.) R[a]	S	Single-oospore cultures[b] (no.) R	S	Expected ratio of 3:1 χ^2	P
6133Cp^r	A^1	0	50	0	50		
6134S^r	A^2	0	50	0	50		
6133Cp^{r}Cn^r	A^1	50	0	70	30	1.08	0.20
6134S^{r}Cn^r	A^2	50	0	71	33	2.17	0.10

[a] R = resistant, S = sensitive.
[b] Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

8Cp^{r}M^r) produced oospore progenies all resistant to metalaxyl, indicating that metalaxyl resistance in these isolates is conferred by a homozygous dominant gene. An additional 75 single-oospore cultures were obtained from 6133-1Cp^{r}M^r to be used in the pairing tests. All of them were resistant to metalaxyl and chloramphenicol, indicating that in addition to the homozygous dominant gene for metalaxyl resistance, 6133-1Cp^{r}M^r also carries a chloramphenicol-resistant trait which can be passed to all the selfed progeny.

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 3). All the single-zoospore cultures from chloroneb-resistant mutants 6133Cp^{r}Cn^r and 6134S^{r}Cn^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 3). These results indicate that resistance to chloroneb in isolates 6133Cp^{r}Cn^r and 6133S^{r}Cn^r is conferred by a single dominant gene which is in heterozygous condition and suggest that sensitivity to chloroneb in isolates 6133Cp^r and 6134S^r to be used in the pairing tests is conferred by a homozygous recessive gene.

Two chloroneb-sensitive and 11 chloroneb-resistant single-oospore isolates were randomly selected from oospore progeny of isolate 6133Cp^{r}Cn^r for further study. Nineteen to 23 cultures derived from selfed oospores produced by each chloroneb-sensitive isolates were all sensitive to chloroneb, consistent with the earlier test 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 4), indicating that the allele for resistance to chloroneb in these isolates is homozygous. Therefore, resistance to chloroneb in these isolates, including isolate 6133-1Cp^{r}Cn^r to be used in the pairing tests, is conferred by a homozygous dominant gene. Selfed oospore progeny produced by the other 8 chloroneb-resistant progeny segregated for chloroneb-resistance and chloroneb-sensitivity, indicating that resistance to chloroneb in these progeny 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 ($\chi^2 = 0.01$, $P = 0.90$) expected for a single dominant resistant gene in heterozygous condition in the parent 6133Cp^{r}Cn^r.

Among the eight isolates which were heterozygous for resistance to chloroneb, six produced progeny with a good fit to a 3:1 ratio (Table 4). The other two progenies had an excess of sensitive phenotypes.

Characteristics of progenies from pairings between wild types and metalaxyl-resistant mutants. Mutants P991-2308M^r (A^1) and P991-2329M^r (A^2) are known to carry a homozygous dominant gene for resistance to metalaxyl from the previous tests. All the single-oospore cultures from the pairing between these two isolates were resistant to metalaxyl (Table 5) expected for both parents carrying a homozygous dominant gene.

All the single-oospore cultures produced by selfing wild type P731 (A^2) were sensitive to metalaxyl (Table 5), indicating that this isolate is homozygous for alleles determining sensitivity to metalaxyl. Oospore progeny from the pairing between P991-

TABLE 4. Sensitivity to chloroneb of single-oospore cultures from randomly selected resistant progeny of selfed oospores of isolate 6133Cp^{r}Cn^r of *Phytophthora parasitica*

Isolate (6133-No.)	Single-oospore cultures[b] (no.)		Expected ratio of 3:1	
	R[a]	S	χ^2	P
1	100	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		

[a]R = resistant, S = sensitive.
[b]Oospore produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

TABLE 5. Sensitivity to metalaxyl of progenies of pairings between metalaxyl-resistant mutants and wild types of *Phytophthora parasitica*

Method of pairing	Single-oospore cultures (no.)	
	R[a]	S
Separated by polycarbonate membrane		
P731, A^2 (stimulated by P991, A^1)	0	50
Direct pairing		
P991-2308M^r, A^1 × P731, A^2	99	21
P991-2329M^r, A^2 × P991, A^1	64	11
P991-2308M^r, A^1 × P991-2329M^r, A^2	60	0

[a]R = resistant, S = sensitive.

TABLE 6. Sensitivity to metalaxyl of single-oospore cultures from randomly selected resistant progeny of the direct pairing between metalaxyl-resistant mutant (P991-2308M^r, A^1) and wild type (P731, A^2) of *Phytophthora parasitica*

Isolate	R:S[a]	Expected ratio of 3:1		Isolate	R:S	Expected ratio of 3:1	
		χ^2	P			χ^2	P
1	79:39[b]	3.67	0.05	21	65:32	2.89	0.05
2	22:12	1.41	0.20	22	33:15	0.69	0.30
3	41:11	0.23	0.50	23	34:16	0.96	0.30
4	30:20	5.23	0.01	24	36:17	1.06	0.30
5	25:13	1.26	0.20	25	18:9	0.60	0.30
6	37:21	3.31	0.05	26	32:8	0.30	0.50
7	10:10	5.40	0.01	27	28:9	0.01	0.90
8	17:9	0.82	0.30	28	17:9	0.82	0.30
9	23:30	26.60	0.00	29	15:11	3.28	0.05
10	18:7	0.01	0.90	30	16:4	0.07	0.70
11	15:6	0.01	0.90	31	14:5	0.02	0.5
12	52:0			32	14:12	5.13	0.01
13	20:7	0.01	0.90	33	16:8	0.50	0.30
14	17:4	0.14	0.20	34	18:9	0.60	0.30
15	18:5	0.01	0.90	35	24:7	0.01	0.90
16	17:8	0.33	0.70	36	21:6	0.01	0.90
17	32:18	2.67	0.10	37	17:8	0.33	0.50
18	19:9	0.43	0.50	38	16:5	0.01	0.90
19	20:6	0.00	1.00	39	24:10	0.16	0.50
20	27:11	0.14	0.70	40	30:12	0.13	0.70

[a]R = resistant, S = sensitive.
[b]Number of single-oospore cultures.

TABLE 7. Sensitivity to metalaxyl of single-oospore cultures from randomly selected resistant progeny of the direct pairing between metalaxyl-resistant mutant (P991-2329M^r, A^2) and wild type (P991, A^1) of *Phytophthora parasitica*

Isolate	Single-oospore cultures[a] (no.)		Expected ratio of 3:1	
	R[b]	S	χ^2	P
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		

[a]Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.
[b]R = resistant, S = sensitive.

TABLE 8. Phenotypes of progenies of pairing between a chloramphenicol- and chloroneb-resistant mutant (6133-1Cp^{r}Cn^r, A^1) and a streptomycin-resistant mutant (6134S^r, A^2) of *Phytophthora parasitica*

Method of pairing	Single-oospore cultures (no.)		
	Resistant to Cp[a] and Cn	Resistant to S	Resistant to S and Cn
Separated by polycarbonate membrane			
6133-1Cp^{r}Cn^r, A^1 (stimulated by 6134S^r, A^2)	75	0	0
6134S^r, A^2 (stimulated by 6133-1Cp^{r}Cn^r, A^1)	0	100	0
Direct pairing			
6133-1Cp^{r}Cn^r, A^1 × 6134S^r, A^2	95	0	38

[a]Cp = chloramphenicol, Cn = chloroneb, S = streptomycin.

2308M^r and P731 consisted of 99 metalaxyl-resistant and 21 metalaxyl-sensitive cultures (Table 5). Five metalaxyl-sensitive and 40 metalaxyl-resistant isolates were randomly selected from the oospore progeny for further study by selfing. Twenty-six to 38 single-oospore cultures obtained from each of five metalaxyl-sensitive isolates were all sensitive to metalaxyl, indicating that sensitivity to metalaxyl in these isolates is conferred by a pair of homozygous 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 6), indicating that resistance to metalaxyl in this isolate is controlled by a pair of homozygous dominant alleles and that the isolate, 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 (Table 6), indicating that these isolates are heterozygous and carry a dominant resistant allele from the resistant parent (P991-2308M^r) and a recessive sensitive allele from the sensitive parent (P731). The result also indicates that the homozygous alleles determining sensitivity to metalaxyl in P731 are recessive. Segregation in 35 of the 39 presumed hybrids fit a 3:1 ratio ($\chi^2 < 3.84$, $P > 0.05$). In four progenies, an excess of sensitive phenotypes gave significant deviation from the expected 3:1 ratio (Table 6).

Wild type P991 (A^1) is known to carry a homozygous recessive gene for sensitivity to metalaxyl from the previous tests. Oospore progeny from the pairing between P991 and P991-2329M^r consisted of 64 metalaxyl-resistant and 11 metalaxyl-sensitive cultures (Table 5). Two metalaxyl-sensitive and six metalaxyl-resistant isolates were randomly selected from the oospore progeny for further study. Twenty to 24 single-oospore cultures obtained from each of the metalaxyl-sensitive isolates were all sensitive to metalaxyl, 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 (P991). All single-oospore cultures obtained from 1 of 6 metalaxyl-resistant isolates by selfing were resistant to metalaxyl (Table 7), indicating that resistance to metalaxyl in this isolate is controlled by a pair of homozygous dominant alleles and that the isolate, therefore, originated from selfing of the metalaxyl-resistant parent (P991-2329M^r). Selfed oospores produced by the other 5 metalaxyl-resistant isolates gave rise to both metalaxyl-resistant and metalaxyl-sensitive cultures (Table 7), indicating that these isolates are hybrids carrying a dominant resistant allele from the resistant parent (P991-2329M^r) and a recessive sensitive allele from the sensitive parent (P991). Segregation in all the 5 presumed hybrids fit a 3:1 ratio (Table 7).

Characteristics of progeny from pairing between 6133-1Cp^{r}Cn^r, A^1 and 6134S^r, A^2. 6133-1Cp^{r}Cn^r and 6134S^r are known to carry a homozygous dominant gene for chloroneb resistance and a homozygous recessive gene for chloroneb sensitivity, respectively, from the previous tests. Moreover, the former carries a chloramphenicol-resistant trait which appeared in all the selfed progeny, and the latter carries a streptomycin-resistant trait which also appeared in all the selfed progeny (Table 8). Oospore progeny from the pairing between 6133-1Cp^{r}Cn^r and 6134S^r consisted of 95 cultures resistant to chloramphenicol and chloroneb and 38 cultures resistant to streptomycin and chloroneb (Table 8). The concomitant presence of streptomycin-resistant and chloroneb-resistant traits suggests that these 38 cultures are hybrids with the streptomycin-resistant character from the parent 6134S^r and the chloroneb-resistant character from the parent 6133-1Cp^{r}Cn^r. Forty of 95 cultures resistant to chloroneb and chloramphenicol were randomly selected for further study to determine if they are self or hybrid. Progeny from selfed oospores produced by three of these cultures were all resistant to chloramphenicol and chloroneb (Table 9), indicating that they are from selfing of the parent 6133-1Cp^{r}Cn^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 9). This result suggests that these 37 cultures are hybrids with a dominant gene resistant to chloroneb from the parent 6133-1Cp^{r}Cn^r and a recessive gene sensitive to chloroneb from the parent 6134S^r. The results show that hybrids from the pairing between chloramphenicol-resistant 6133-1Cp^{r}Cn^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 cytoplasmic.

Among the 37 isolates which were heterozygous for resistance to chloroneb, 34 produced progeny with a good fit to a 3:1 ratio ($\chi^2 < 3.84$, $P > 0.05$). The other three isolates did not fit the 3:1 ratio due to an excess of sensitive phenotypes (Table 9).

TABLE 9. Phenotypes of single-oospore cultures from chloramphenicol- and chloroneb-resistant progeny of the direct pairing between a chloramphenicol- and chloroneb-resistant mutant (6133-1Cp^{r}Cn^r, A^1) and a streptomycin resistant mutant (6134S^r, A^2) of *Phytophthora parasitica*

Isolate	R:S[a]	Expected ratio of 3:1		Isolate	R:S	Expected ratio of 3:1	
		χ^2	P			χ^2	P
1	25:0[b]			21	19:0		
2	11:9	3.27	0.05	22	16:6	0.00	0.90
3	18:4	0.24	0.50	23	16:4	0.07	0.70
4	15:5	0.07	0.70	24	20:5	0.12	0.70
5	15:7	0.24	0.50	25	18:3	0.78	0.30
6	17:3	0.60	0.30	26	14:10	2.72	0.05
7	20:0			27	13:7	0.60	0.30
8	14:6	0.07	0.70	28	14:10	2.72	0.50
9	14:8	0.97	0.30	29	16:5	0.01	0.90
10	18:3	0.78	0.30	30	15:3	0.30	0.50
11	14:5	0.02	0.70	31	13:7	0.60	0.30
12	17:3	0.60	0.30	32	10:10	5.40	0.01
13	15:5	0.07	0.70	33	11:14	15.66	0.00
14	17:8	0.33	0.50	34	18:4	0.24	0.50
15	15:6	0.01	0.90	35	13:7	0.60	0.30
16	18:2	1.67	0.10	36	15:9	1.39	0.20
17	18:2	1.67	0.10	37	12:8	1.67	0.10
18	17:6	0.01	0.90	38	15:5	0.07	0.70
19	16:4	0.07	0.70	39	14:8	0.97	0.30
20	11:10	4.58	0.01	40	12:8	1.67	0.10

[a]R = resistant to chloroneb and chloramphenicol, S = sensitive to chloroneb but resistant to chloramphenicol.
[b]Number of single-oospore cultures.

TABLE 10. Phenotypes of progenies of pairing between a chloramphenicol-resistant mutant (6133Cp^r, A^1) and a streptomycin- and chloroneb-resistant mutant (6134-1S^{r}Cn^r, A^2) of *Phytophthora parasitica*

Method of pairing	Single-oospore cultures (no.)		
	Resistant to Cp[a]	Resistant to S and Cn	Resistant to Cp and Cn
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 × 6134-1S^{r}Cn^r, A^2	4	17	102

[a]Cp = chloramphenicol, Cn = chloroneb, S = streptomycin.

Characteristics of progeny from pairing between 6133Cp^r, A^1 and 6134-1S^{r}Cn^r, A^2. 6133Cp^r and 6134-1S^{r}Cn^r are known to carry a homozygous recessive gene for sensitivity to chloroneb and a homozygous dominant gene for resistance to chloroneb, respectively, from the previous tests. Moreover, the former carries a chloramphenicol-resistant trait which appeared in all the selfed progeny, and the latter carries a streptomycin-resistant trait which also appeared in all the selfed progeny (Table 10). Oospore progeny from the pairing between 6133Cp^r and 6134-1S^{r}Cn^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 10). The 4 cultures resistant to chloramphenicol are presumed selfs of the parent 6133Cp^r. The concomitant presence of chloramphenicol-resistant and chloroneb-resistant traits suggests that the 102 cultures are hybrids with the chloramphenicol-resistant character from the parent 6133Cp^r and the chloroneb-resistant character from the parent 6134-1S^{r}Cn^r. Eight of 17 cultures resistant to streptomycin and chloroneb were randomly selected for further study to determine if they are self or hybrid. Progeny from selfed oospores produced by one of these cultures were all resistant to streptomycin and chloroneb (Table 11), indicating that the culture is from selfing of the parent 6134-1S^{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 11), suggesting that they are hybrids with a dominant gene resistant to chloroneb from the parent 6134-1S^{r}Cn^r and a recessive gene sensitive to chloroneb from the parent 6133Cp^r. All the hybrids which were heterozygous for resistance to chloroneb segregated in a ratio of 3 resistant: 1 sensitive (Table 11), confirming that the resistance to chloroneb in isolate 6134-1S^{r}Cn^r is conferred by a pair of homozygous dominant alleles and that sensitivity to chloroneb in isolate 6133Cp^r is conferred by a pair of homozygous recessive alleles. The results show that hybrids from the pairing between chloramphenicol-resistant 6133Cp^r and streptomycin-resistant 6134-1S^{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.

Characteristics of progeny from pairing between 6133-1Cp^{r}M^r, A^1 and 6134-1S^{r}Cn^r, A^2. 6133-1Cp^{r}M^r is known from the previous tests to carry a homozygous dominant gene for metalaxyl resistance and a chloramphenicol-resistant gene passable to all the selfed progeny and presumably cytoplasmic. From the previous tests, 6134-1S^{r}Cn^r is also known to carry a homozygous dominant gene for resistance to chloroneb and a cytoplasmic streptomycin-resistant gene. Oospore progeny from the pairing between 6133-1Cp^{r}M^r and 6134-1S^{r}Cn^r consisted of four cultures resistant to chloramphenicol and metalaxyl; 92 resistant to streptomycin, metalaxyl, and chloroneb; 46 resistant to chloramphenicol, metalaxyl, and chloroneb; and six resistant to streptomycin and chloroneb (Table 12). The 46 cultures containing the chloramphenicol-resistant and metalaxyl-resistant characters from the parent 6133-1Cp^{r}M^r and the chloroneb-resistant character from the parent 6134-1S^{r}Cn^r are hybrids, and so are the 92 cultures containing the metalaxyl-resistant character from the parent 6133-1Cp^{r}M^r and the streptomycin-resistant and chloroneb-resistant characters from the parent 6134-1S^{r}Cn^r. All the hybrids are resistant to either chloramphenicol or streptomycin but not both, confirming that chloramphenicol-resistant gene in 6133-1Cp^{r}M^r and streptomycin-resistant gene in 6134-1S^{r}Cn^r are cytoplasmic. The four cultures resistant to chloramphenicol and metalaxyl and the six cultures resistant to streptomycin and chloroneb contain resistance characters only from one parent, and are, therefore, selfs of 6133-1Cp^{r}M^r and 6134-1S^{r}Cn^r, respectively.

TABLE 11. Phenotypes of single-oospore cultures from streptomycin- and chloroneb-resistant progeny of the direct pairing between a chloramphenicol-resistant mutant (6133Cp^r, A^1) and a mutant resistant to streptomycin and chloroneb (6134-1S^{r}Cn^r, A^2) of *Phytophthora parasitica*

Isolate	Single-oospore cultures[b] (no.)		Expected ratio of 3:1	
	Resistant to Cn[a] and S	Sensitive to Cn but resistant to S	χ^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]S = streptomycin, Cn = chloroneb.
[b]Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method.

DISCUSSION

Our 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. When these nuclear genes were used as markers, both selfs and hybrids were found in the progenies from the pairings between different mating types of *P. parasitica*. Hybrids, recognized by the presence of recombinant genes for fungicide resistance, produced by parents carrying genes for resistance to different antibiotics, were resistant to either chloramphenicol or streptomycin but not both, indicating that chloramphenicol resistance and streptomycin resistance in *P. parasitica* are controlled by cytoplasmic genes inherited solely through the maternal parent (21). Therefore, lack of recombination between chloramphenicol-resistant and streptomycin-resistant markers in the direct pairings between opposite mating types of *P. parasitica* is not due to absence of hybridization as originally conceived (2,14).

Heterothallic species of *Phytophthora* are unique in that sexual reproduction readily occurs even when opposite mating types of morphologically and physiologically distinct species are paired in cultures (14). Results obtained from pairing isolates of *Phytophthora* on the opposite sides of polycarbonate membranes indicate that the opposite mating types are needed for production

TABLE 12. Phenotypes of progenies from pairing a mutant resistant to chloramphenicol and metalaxyl (6133-1Cp^{r}M^r, A^1) and a mutant resistant to streptomycin and chloroneb (6134-1S^{r}Cn^r, A^2 of *Phytophthora parasitica*

Method of pairing	Single-oospore cultures[a] (no.)			
	Resistant to Cn[b] and MT	Resistant to S, Mt, and Cn	Resistant to Cp, Mt, and Cn	Resistant to S and Cn
Separated by polycarbonate membrane				
6133-1Cp^{r}M^r, A^1 (stimulated by 6134-1S^{r}Cn^r, A^2)	75	0	0	0
6134-1S^{r}Cn^r, A^2 (stimulated by 6133-1Cp^{r}M^r, A^1)	0	0	0	75
Direct pairing				
6133-1Cp^{r}M^r, A^1 × 6134-1S^{r}Cn^r, A^2	4	92	46	6

[a]Oospores produced by single isolates were obtained by hormonal stimulation using the polycarbonate membrane method and direct pairing.
[b]S = streptomycin, Cn = chloroneb, Cp = chloramphenicol, Mt = metalaxyl.

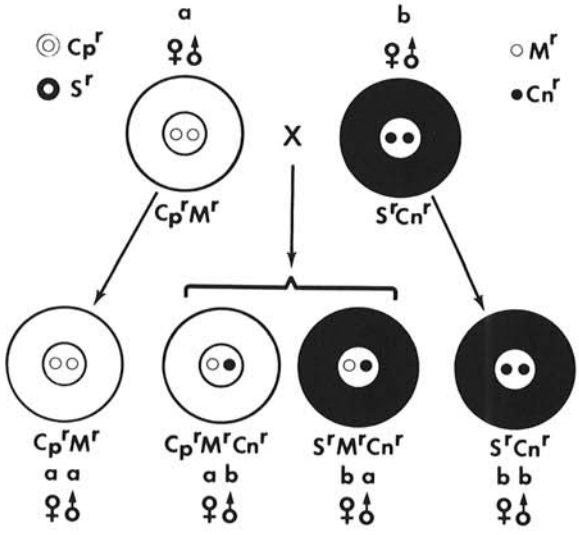

Fig. 1. Diagrammatic representation of results of the pairing between **a**, carrying homozygous dominant metalaxyl-resistant (M^r) alleles in the nucleus and chloramphenicol-resistant (Cp^r) gene in the cytoplasm and **b**, carrying homozygous dominant chloroneb-resistant (Cn^r) alleles in the nucleus 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.

of mating-type-specific hormones to initiate sexual reproduction (11,14) and suggest that sexual reproduction in homothallic *Phytophthora* is controlled by the same α hormones produced by themselves (12). Based on hormone production and reception, 16 types of hormonally regulated sexuality among species of *Phytophthora* were postulated. These sexuality types were divided into three groups, i.e., cross-induction (heterothallic), self-induction (homothallic), and neuter (12,14). Since genetic exchange in the pairings between different species of *Phytophthora* has been shown to be essentially nonexistent (3,4,6), sexual reproduction under this condition appears to be of total hormonal heterothallism (14). Current results showed that both selfs and hybrids were produced when different mating types of *P. parasitica* were paired directly. Therefore, in the intraspecific pairings of cross-inducing *Phytophthora*, in addition to hormonal heterothallism, the conventional biological heterothallism is also involved.

The use of different homozygous dominant genes for fungicide resistance and different cytoplasmic genes for antibiotic resistance in each parent in the pairings between opposite mating types of the same species of *Phytophthora* will enable one to distinguish hybrids from selfs in a simple test (Fig. 1). It will also enable one to know the parental origin of oogonium and antheridium for each hybrid oospore. 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. 1).

Isolates P991-23M^r and 6133Cp^{r}Cn^r were heterozygous for resistance to metalaxyl and chloroneb, respectively (Tables 1 and 3). In principle, their selfed progeny heterozygous for resistance should all produce a second generation of selfed offspring segregating in a ratio of 3 resistant: 1 sensitive. Although most segregations fit the expected ratio, several did not (Tables 2 and 4). When new batches of single-oospore cultures were obtained from two of these progenies, both segregated 3 resistant: 1 sensitive as expected. Similar deviations from an expected 3:1 segregation were found in some of the second generations of pairings P991-2308M^{r}XP731 and 6133-1Cp^{r}Cn^{r}X6134S^r homozygous for resistance to metalaxyl and chloroneb, respectively (Tables 6 and 9). The reason for the inconsistent behavior of these progenies remains to be investigated.

Chloroneb resistance in *Ustilago maydis* (DC.) Corda has also been reported to be controlled by single gene (22). Contrary to the results reported here, metalaxyl resistance in *Bremia lactucae* Regel (5) and *Phytophthora infestans* (Mont.) de Bary (18) was considered to be controlled by a single gene exhibiting incomplete dominance. The discrepancy may be due to difference in isolates used. Streptomycin resistance in *Chlamydomonas reinhardi* Dangeard has been found to be controlled by nuclear genes in some mutant strains and cytoplasmic genes in others (16). Both nuclear inheritance and cytoplasmic inheritance of chloramphenicol resistance have also been observed in *Aspergillus nidulans* (Eidam) Winter (9).

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