Sensitivities of Mexican Isolates of *Phytophthora infestans* to Chlorothalonil, Cymoxanil, and Metalaxyl

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ABSTRACT

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Seventy-five genetically diverse isolates of *Phytophthora infestans* from central and northwest Mexico were evaluated in vitro for their sensitivities to chlorothalonil, cymoxanil, and metalaxyl. Sensitivities were determined by measuring radial growth on agar medium amended with fungicide. Isolates were classified into five sensitivity categories based on their estimated EC_{50} (50% effective concentration) for each fungicide. For chlorothalonil and cymoxanil, the majority (83 and 70%, respectively) of isolates had EC_{50}s that fell between 0.1 and 1.0 µg/ml (ppm) and the mean EC_{50} values were 3.1 and 0.8 ppm, respectively. Three isolates had mean EC_{50} values for chlorothalonil in excess of 10 ppm. For metalaxyl, sensitivity phenotypes were more evenly distributed and the mean EC_{50} value was 52.5 ppm. Forty-four percent of the isolates had EC_{50} values in excess of 100 ppm. Variation in sensitivity phenotype was lowest for cymoxanil, also low for chlorothalonil, and higher for metalaxyl. For no fungicide were EC_{50} values correlated to mating type or allozyme genotype.

Fungicide resistance has created sometimes disastrous problems in disease management. This was certainly the case for *Phytophthora infestans* and the phenylamide fungicide, metalaxyl. Events were perhaps most completely described for the situation in Europe during the 1980s, but similar problems occurred in Mexico, the Far East, and the Middle East (2,6,7,9,10, 22,23). It wasn't until the 1990s that similar failures in disease control occurred in the U.S. and Canada (7,11,12,14).

Although the implicit expectation had been that resistance would arise from within a local population, we now know that in some locations resistance in *P. infestans* populations was introduced via immigration from other locations (20,21, 24). *Phytophthora infestans* may be unique in that populations once dominated by a single clonal lineage (16) are now being displaced by immigrant populations (21,28,29), and resistance to metalaxyl is often characteristic of the immigrant populations (13,20).

Given the predominant occurrence of a few widely distributed metalaxyl-resistant clonal lineages of *P. infestans* recently introduced into the U.S. (15,20), it seemed important to estimate the likelihood of resistance to other fungicides that will or might be important to future late blight management in the U.S. A survey of isolates from central Mexico (the location of the most diverse populations of *P. infestans*) (18,30) was important not only because of the diversity of the populations there, but also because these locations are the likely sources of future immigrations into the U.S.

The fungicides chlorothalonil, cymoxanil, and metalaxyl are all now widely used in Mexico to suppress late blight, but they have had different usage histories there. Chlorothalonil has been in use since the 1970s, but we are unaware of any reports of potato late blight control failures associated with resistance to chlorothalonil. Cymoxanil is relatively new to Mexico, with initial sales in 1991. Metalaxyl has been used at least since the early 1980s, and resistance is common (22).

Each of the fungicides has distinct performance characteristics. Chlorothalonil is broad spectrum, is not systemic, and inhibits all stages of fungal growth through contact activity (1). Chlorothalonil represents the broad class of protectant fungicides that have been used effectively for many years in late blight management without fungicide-resistance problems. Against oomycetes, cymoxanil exhibits local systemic activity and after-infection activity for the first half of the incubation period. Mycelial growth is more sensitive to inhibition than is zoospore germination (31). Cymoxanil represents a class of fungicides that had not been used in the U.S. for late blight management until 1995 (section 18 exemption in several states), but has been used in many European countries for up to 15 years without report of resistance problems. Metalaxyl is specific for oomycetes, is systemic, affects mycelial growth but not zoospore integrity, and may inhibit RNA polymerase (5). In practice, chlorothalonil is used exclusively as a protectant, but cymoxanil and metalaxyl have "post infection activity" (8,25).

The purpose of the present report was to obtain a preliminary estimate of the variability of response to chlorothalonil and cymoxanil in a genetically diverse collection of *P. infestans* isolates from Mexico. Sensitivity to metalaxyl was analyzed for comparison with previous assessments, and to investigate associations with other traits. The in vitro results reported here should provide an initial estimate of the variation in sensitivity to these fungicides in a genetically diverse collection of *P. infestans* isolates.

MATERIALS AND METHODS

Selection of isolates. All isolates were obtained from the culture collection at Cornell University. They had been acquired from 1983 through 1989 primarily in central Mexico, but one isolate was collected from northwest Mexico in 1988. Following acquisition, the isolates were stored under oil, at –135°C, or in liquid nitrogen. The isolates had been characterized previously for mating type, genotypes at the two allozyme loci, glucose-6-phosphate isomerase (Gpi) and peptidase (Pep), and some had been analyzed for nuclear DNA fingerprint as determined by probe RG57 (17). A summary of these presumably selectively neutral characteristics is presented in Table 1 to illustrate the diversity of mating type, allozyme alleles, and collection sites and dates. Techniques for determining mating type, allozyme alleles, and DNA fingerprint were as described in Sujkowski et al. (29). Diversity of genotype, location, and year of

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collection was used to assure that there were no duplicate members of the same clone among the 75 isolates selected.

In vitro assessment of fungicide sensitivity. Fungicide sensitivity was determined by comparing the radial growth of each isolate on rye B agar (3) containing fungicide with the growth of the same isolate on medium without the fungicide. Fungicide was incorporated into the agar medium from a stock solution made in dimethyl sulfoxide (DMSO). Each stock solution was 1,000-fold more concentrated for fungicide than the final test solution, and test solutions were constructed by adding 1 ml of stock solution to 1 liter of agar medium. Control (zero fungicide) medium contained 0.1% DMSO with no fungicide. DMSO solutions with or without fungicide were added to molten, cooled agar (ca. 50°C) before the medium was dispensed into petri plates. The media were stirred constantly with a stir bar and the same amount of medium was distributed to each petri plate via a peristaltic pump. The final concentrations of fungicides in the media were 0.1, 1.0, 10.0, and 100.0 ppm (μg/ml). Chlorothalonil was obtained from a commercial formulation (Bravo 720); cymoxanil was provided by DuPont de Nemours and Co. as the 96% technical, and metalaxyl was provided by CIBA Crop Protection as the 91% technical grade.

Sensitivity assessments were standardized by using cultures that had been treated similarly. Disks of mycelium on agar (8 mm diameter) were obtained from the actively expanding margin of a 16- to 18-day-old colony of *P. infestans* growing on rye A agar (18°C) The agar disk was placed with the mycelium in contact with the test medium. Plates were incubated at 18°C for 10 to 21 days. Colony diameters (2 diameters at 90° angles ÷ 2) were measured on all plates when the control treatment (colony growing in the absence of fungicide) was at least 5 cm in diameter. All diameters were corrected for the size of the initial agar disk (8 mm).

Data are presented as relative values: the diameter of each colony on a fungicide-containing medium is presented relative to the diameter of the colony in the absence of fungicide. Individual values are given as percentage of control. There were two independent replications (separate source colonies) for each isolate, and the relative colony sizes (percentage of control) were averaged. Although previous studies with metalaxyl indicated very little variation in the assay technique over time (22), a subset of isolates was evaluated in a second independent experiment several months after the first evaluation to provide an additional independent test of potential variability.

Statistical analyses. The EC_{50} (effective concentration for 50% effect = concentration at which the relative growth is 50% of control), and EC_{90} values were estimated. Because the test concentrations often did not enable precise estimates for either EC_{50} or EC_{90} (often there was 100% growth at one concentration and no growth at 10 times that concentration), all EC_{50} or EC_{90} values were estimated and assigned to classes as follows: <0.1 μg/ml; 0.1 to <1 μg/ml; 1.0 to <10 μg/ml; 10 to <100 μg/ml; and ≥100 μg/ml. Calculation of mean values was done after assigning each value to the midpoint scale of the class. Standard deviations were calculated only on data transformed to $\log_{10}$ values because of the association between variance and mean when arithmetic values were used.

The association of mating type or allozyme genotype with fungicide sensitivity was tested. Initially, a chi-square analysis was attempted, but the large number of cells with zeros or very small numbers precluded the valid use of this test. Subsequently the analysis was conducted using nonparametric rank correlation analysis (27). Fifty-five isolates that had complete sets of data for mating types, allozymes, and all three fungicides were used for this analysis.

Table 1. Characteristics of *Phytophthora infestans* used in fungicide sensitivity tests[a]

			Frequency of allozyme alleles							
			Gpi[b]					Pep[c]		
Collection	N	A1/A2	86	90	100	111	122	(70), (78), (83)[d]	92	(96), 100[d]
1983 Central Mexico	22	13/9	4	2	37	0	1	0	7	35
1986 Central Mexico	5	2/3	6	0	4	0	0	0	1	9
1987 Central Mexico	3	3/0	3	0	1	0	2	0	0	6
1988 NE Mexico	1	(H)[e]	0	0	1	0	1	0	0	2
1988 Central Mexico	15	–	4	2	11	0	8	1	7	20
1989 Central Mexico	29	17/10	17	0	30	2	6	3	5	46
Totals	75	35/22	34	4	84	2	18	4	20	118

[a] Not all data are complete for each isolate. Thus totals for mating type are less than 75 and totals for alleles of each allozyme are less than 150.
[b] Glucose-6-phosphate isomerase.
[c] Peptidase.
[d] Very rare alleles (in parentheses) are grouped with other alleles.
[e] Homothallic.

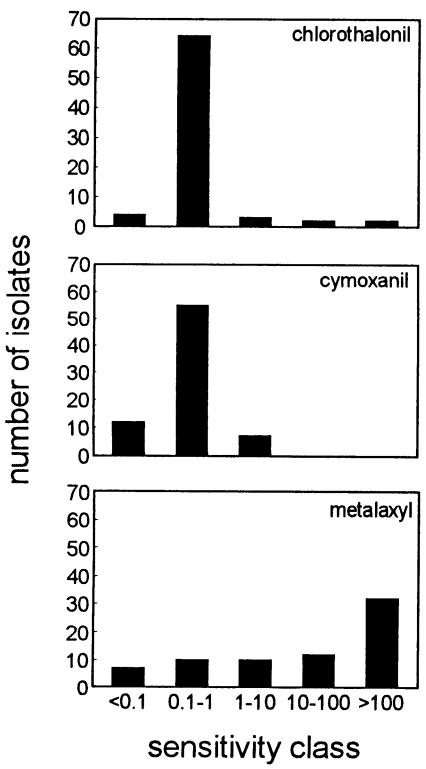

Fig. 1. EC_{50} values for 75 isolates of *Phytophthora infestans* in response to chlorothalonil, cymoxanil, and metalaxyl.

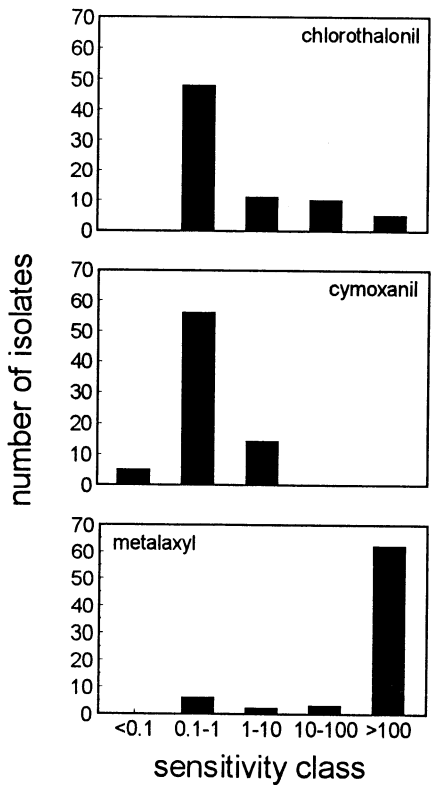

Fig. 2. EC_{90} values for 75 isolates of *Phytophthora infestans* in response to chlorothalonil, cymoxanil, and metalaxyl.

RESULTS

The reactions of the isolates to chlorothalonil and cymoxanil were generally similar to each other, but different from their reactions to metalaxyl (Fig. 1). For chlorothalonil the mean EC_{50} value was 3.1 ppm, and only three isolates had EC_{50}

values >10 ppm. For cymoxanil, the mean EC_{50} value was 0.8 ppm and no isolate had an EC_{50} value >10 ppm (Fig. 1). The vast majority of isolates (83% for chlorothalonil and 70% for cymoxanil) had EC_{50} values in the range of 0.1 to 1.0 ppm. The majority of EC_{90} values also occurred in this range for both chlorothalonil and cymoxanil (Fig. 2).

For metalaxyl, most (59%) isolates had EC_{50} values >10 ppm. This EC_{50} value has usually been identified as resistant (4,22). Forty-three percent (32/75) had EC_{50} values >100 ppm (Fig 1), and 87% (65/75) had EC_{90} values >100 ppm (Fig. 2).

The standard deviations (log_{10} scale) for reactions of the isolates as a group were smallest for cymoxanil, slightly larger for chlorothalonil, and largest for metalaxyl. The standard deviations (log_{10} scale) for the mean EC_{50}s were 0.6, 0.3, and 1.2 for chlorothalonil, cymoxanil, and metalaxyl, respectively. Thus, in these isolates there was least diversity in reaction to cymoxanil, somewhat more diversity for reaction to chlorothalonil, and much more diversity for reaction to metalaxyl.

The in vitro assay was generally consistent in assessing sensitivity to cymoxanil and chlorothalonil over time. Six isolates were re-assessed at the conclusion of the study for their sensitivity to chlorothalonil; one isolate was assessed for consistency of reaction to cymoxanil. Four of the isolates had been regarded as very sensitive to chlorothalonil, but two had appeared to be less sensitive. For five of six of the isolates tested for sensitivity to chlorothalonil, the individual EC_{50}s of the early and late tests were within 0.5 log units. The sixth isolate (one of the two in the subset that had an EC_{50} >10 ppm for chlorothalonil) gave a variable response. In the first of the later tests it appeared sensitive (EC_{50} of <1.0 ppm), but in a repetition it again appeared less sensitive (EC_{50} of ca. 10 ppm). The EC_{50}s for reaction to cymoxanil were within 0.5 log units in early and late tests.

No significant correlations were found among mating type, allozyme genotypes, and sensitivities to chlorothalonil, cymoxanil, or metalaxyl. However, a significant but weak correlation was found between sensitivities to chlorothalonil and cymoxanil ($R = 0.34$ at $P = 0.05$).

DISCUSSION

The vast majority of isolates had low EC_{50}s in response to chlorothalonil and to cymoxanil, but higher EC_{50}s in response to metalaxyl. The average EC_{50}s were 3.1 and 0.8 ppm to chlorothalonil and cymoxanil, respectively. In contrast, the average EC_{50} in response to metalaxyl was 52.5 ppm. These results conform to expectation developed from known genetics of resistance, modes of action, and usage histories. Resistance to metalaxyl is generally regarded to be simply inherited (26) and the mode of action is thought to be quite specific (5), leading to the conclusion that resistance might be more readily developed if the fungicide is used extensively (as it has been in Mexico). Certainly the majority of isolates were resistant to metalaxyl based on commonly used criteria (4, 7,22). In contrast, chlorothalonil presumably affects many cellular processes; resistance and the genetics of resistance are not described even though this compound also has been used extensively in Mexico. Cymoxanil had been only little used in Mexico before our sampling, so selection for resistance had not been tested there. Little is known about mode of action and genetics of resistance (if it occurs).

The diversity of responses to chlorothalonil appeared slightly greater than the diversity of responses to cymoxanil. This may reflect different selection pressures or different inherent diversity. The isolates were collected before cymoxanil was available commercially in Mexico, so any insensitive isolates would not have been selected, and our sample size was too small to detect any rare traits. However, the uniform responses to cymoxanil in these diverse isolates is consistent with the absence of reported resistance to cymoxanil in Europe, where the fungicide has been used intensively since the late 1970s. Even in a population of *P. infestans* collected from Mexico in 1993, there was no EC_{50} >10 ppm.

The lack of association between fungicide sensitivity and any of the presumably neutral markers (mating type, allozyme alleles) suggests that none of these alleles is linked to metalaxyl insensitivity. This contrasts markedly with the situation in the U.S. in the early 1990s when the vast majority of A2 individuals were highly insensitive to metalaxyl. The population of *P. infestans* in the U.S. during the early 1990s was very strongly clonal, with only four genotypes widely detected (15,16,19, 20), and the two major A2 clonal lineages were both insensitive to metalaxyl. The population of *P. infestans* from central Mexico has characteristics of a randomly mating sexual population, in which association among markers would only be maintained for those that are very tightly linked, and it appears from this study that the association between mating type and metalaxyl-sensitivity in the U.S. during the 1990s results from the strongly clonal population structure of this organism in the U.S., and not from genetic linkage.

The detection of three individuals with EC_{50}s >10 ppm for chlorothalonil needs to be further investigated. Because resistance to chlorothalonil has not been demonstrated to be a problem in practice, we suspect that these in vitro reactions do not fully predict results in the field. However, the in vitro assay provides one measure of response to fungicides that might be a necessary but not sufficient factor in conditioning epidemiologically important resistance.

The fact that there was a weak association between cymoxanil and chlorothalonil sensitivity also needs to be further investigated. There are at least two possibilities. One is that the association is real, but the other is that some trait typical of these isolates causes them to appear less sensitive to these fungicides in these in vitro tests.

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