

# Selection for Fungicide Resistance Within a Growing Season in Field Populations of *Phytophthora infestans* at the Center of Origin

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## ABSTRACT

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The central highlands of Mexico should provide an optimal testing ground for evaluating the potential threat of selection for resistance to fungicides in the population of *Phytophthora infestans*. We evaluated the hypotheses that exposure to the fungicides azoxystrobin, cymoxanil, dimethomorph, fluazinam, mancozeb, metalaxyl, and propamocarb hydrochloride would lead to (i) a shift in the sensitivity distributions (i.e., selection) and (ii) a lower genotypic diversity of the population. We

compared populations from unsprayed plots with populations that had been exposed to several applications of each of the fungicides within a single field season. This study provides novel baseline data and shows that the Toluca valley *P. infestans* population has a wide range of sensitivities to the fungicides fluazinam, cymoxanil, dimethomorph, metalaxyl, and propamocarb. Directional selection toward resistance combined with a reduction in genetic diversity of the *P. infestans* population was observed only for the fungicide metalaxyl. The results obtained provide direct experimental support for continuing vigilance regarding further introductions of exotic strains of *P. infestans* into the United States.

*Additional keyword:* evolution.

The increasing frequency of resistance to pesticides by pests is a classical example of evolution at work on a time scale that we can experience directly. Evolution of resistance to a fungicide in populations of plant pathogens is a common phenomenon with important considerations for management of plant diseases (10, 23,31,45). A specific pathogen population can evolve resistance when frequency of resistant phenotypes increases as the result of a selection pressure imposed on the population by the use of the fungicide. A necessary condition for selection to occur is that variation in phenotype for resistance exists in the population and that resistant phenotypes exist in at least a low frequency. This selection pressure acts on phenotypes and, thus, on the inheritable genes or quantitative traits controlling the fungicide resistance phenotype. Selection pressure is higher for single genes than for traits controlled by either quantitative trait loci or multiple loci. Individuals in the pathogen population expressing the resistance phenotype increase in frequency when exposed to fungicides. The result of this mechanism of evolution, namely selection, is detected by a shift in the frequency distribution of a particular resistance phenotype coupled with a reduction in genetic diversity.

Evolution of fungicide resistance depends on many factors, including the genetics of resistance in the pathogen. Resistance to fungicides can be based on a single gene or can be polygenic in nature. A single, mitochondrial gene confers resistance to

strobilurins for the pathogens *Mycosphaerella fijiensis*, *Erysiphe graminis*, and *Plasmopara viticola* (9). In contrast, resistance to metalaxyl in *Phytophthora infestans* and triadimenol in *Pyrenophora teres* are controlled by a single chromosomal gene; however, these resistances are further modified by several additional loci (5,10,24,34,39,40).

Evolution to fungicide resistance also depends on potency, persistence and effect, distribution within a crop, and mode of action of a fungicide (1,4,8,22,25,30,31,41,44). Several fungicides belonging to diverse chemical classes are being deployed to control potato late blight. The fungicides included in this study included azoxystrobin, cymoxanil, dimethomorph, fluazinam, metalaxyl-M, and propamocarb hydrochloride. These fungicides are very diverse in terms of mode of action, efficacy, and chemical class. Three of these fungicides are from groups of fungicides considered to be systemic: carbamates (i.e., propamocarb), cymoxanil, and phenylamides (i.e., metalaxyl) (3). The mode of action of propamocarb hydrochloride is not known; however, it has some systemic activity being translocated upward in water-conducting tissues toward new plant growth. Cymoxanil and dimethomorph both are locally systemic and their modes of action are not known. Metalaxyl is a systemic phenylamide that specifically inhibits RNA polymerase. Azoxystrobin has translaminar activity and inhibits respiration (9). Fluazinam uncouples oxidative phosphorylation and is considered to be a protectant fungicide. Mancozeb is a protectant fungicide with multisite inhibitory activity that should result in little or no selection.

We hypothesize that the central highlands of Mexico, being the putative center of origin of *Phytophthora infestans* (Mont.) de Bary (15), provide a valuable testing ground for evaluating the potential threat of selection for resistance to fungicides. In the Toluca valley, this population reproduces sexually and both

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mating types exist in a 1:1 ratio (6,13,16). Genetic diversity for virulence (32,33,50,51), allozyme (13,49), restriction fragment length polymorphism (RFLP) (13,16), and amplified fragment length polymorphism (AFLP) (7) loci is higher in central Mexico than elsewhere in the world. Furthermore, the population has been exposed to many fungicides, including metalaxyl, propamocarb, cymoxanil, mancozeb, and, quite recently, azoxystrobin (18,19, 36,37). Given that the population is very diverse, has been exposed to fungicides, and is sexual in nature, the chance of detecting selection for resistance within a field season in this population would seem higher than in other locations (15).

The objective of our research was to determine the potential for fungicide resistance selection in the Toluca valley population of *P. infestans*. We specifically tested the hypotheses that exposure to the fungicide would lead to (i) a shift in the sensitivity distributions (i.e., selection) and (ii) a lower genotypic diversity of the population. Our approach was to compare sensitivity on a population that had not been exposed to the fungicide with populations that had been exposed to several applications of each of the fungicides within a single season.

## MATERIALS AND METHODS

**Cultural procedures.** Field experiments were conducted at the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) field station in Metepec, Toluca Valley, in 2000. Whole tubers of cv. Alpha were planted on 22 June. A private company provided Alpha seed tubers. Experimental plots were 10 by 5.5 m wide (six rows/plot and 16 plants/row). Plots were separated from each other by 4 m. Fertilization and non-experimental pesticide applications were applied according to standard grower practice in the region as described previously (18,19). Briefly, this practice is that tubers were treated with benomyl (Benlate; 1 kg a.i./ha) and captan (Captan 50 PH; 1 kg a.i./ha) in the planting hole with a hand sprayer. Fertilizer was applied at time of planting (N, 120 kg/ha; P, 180 kg/ha; K, 120 kg/ha; and Ca and Mg, 20 kg/ha) together with the granulated insecticide terbufos (Counter FZ-15; 1.05 kg a.i./ha). A second fertilizer application (60 kg of N) was applied at hilling on 28 July. The herbicide metribuzin (Lexone) was applied at a rate of 525 g a.i./ha as needed. Several insecticides were applied weekly in rotation as needed (methamidophos, dimethoate, oxamyl, monocrotophos, methomyl, and endosulfan).

**Fungicide treatments.** Potential for evolution of fungicide resistance within a single field season was evaluated for several active ingredients, including azoxystrobin, cymoxanil, dimethomorph, fluazinam, mancozeb, metalaxyl-M, and propamocarb hydrochloride. Each fungicide was applied to one experimental plot at the rates shown in Table 1 using a backpack sprayer. For all but one fungicide, applications were made at the lowest recommended rate to maximize potential for selection. The exception was the fungicide metalaxyl; we had experience from previous field seasons indicating that the epidemic would progress too rapidly at the lowest recommended rate, so that isolates could not be retrieved at the end of the field season. Thus, we used the

highest recommended rate for the metalaxyl treatment (Table 1). The fungicide dimethomorph was available in Mexico only in co-formulation with mancozeb (Table 1); thus, the effect of mancozeb and dimethomorph cannot be separated in that treatment. Fungicides were applied every 6 to 11 days (Fig. 1C; application dates are marked by downward-pointing triangles) to allow for a moderate yet sustained epidemic. For logistical reasons, sampling of isolates occurred over several days, which required that the mancozeb and azoxystrobin treatments received an extra application on September 26 (Fig. 1C) to allow sampling isolates that recently had been exposed to the fungicide.

**Microclimate and disease progress.** Disease severity (%) was assessed on a plot basis and was estimated visually once to twice per week as described previously (18,19). Air temperature and relative humidity were measured with a shielded thermistor and a sulfonated polystyrene humidity transducer, respectively (207 probe; Campbell Scientific, Logan, UT). The probe was placed at a height of 1.5 m. Rainfall was measured with an automated TE525 tipping bucket rain gage (Texas Instruments, Dallas, TX). The automated CR-10 weather station (Campbell Scientific) was located at approximately 100 m from the experiment. All sensor signals were sampled in 5-min intervals, and 60-min averages were calculated to enable estimation of hours during which relative humidity was >90%.

**Isolates.** Isolates were sampled from single discrete lesions and isolated as described previously (16). By tagging each sampled plant, we avoided sampling the same plant twice. Isolates for the baseline sample before application were obtained on 29 July. Due to the fact that epidemics in fungicide-treated plots did not develop uniformly, isolates sampled after exposure to fungicides had to be collected between 10 September and 9 October as new infections were observed. Thus, all isolates were retrieved from plots that had received at least six fungicide applications. As mentioned above, fungicide applications continued while sampling occurred and all plots received one or two more applications until collections of isolates were complete for each treatment. A period of 24 h was observed after each spray before isolates were sampled. Isolates were clean cultured by placing a portion of a leaf lesion under a slice of potato tuber (≈0.5 cm thick) in a petri dish. Tuber slices were obtained from surface-sterilized, healthy tubers of cv. Alpha. Isolation from mycelial tufts on top of the tuber slice was performed 4 to 5 days later by transfer onto selective or regular Rye-A agar (2). Selective Rye A agar was regular Rye-A agar containing the following antibiotics and fungicides: rifampicin at 20 mg/liter, polymyxin B sulfate at 50 mg/liter, ampicillin at 200 mg/liter, pentachloronitrobenzene (PCNB) at 67 mg/liter, and benomyl at 100 mg/liter.

**Fungicide sensitivity.** Sensitivities, measured by estimating the effective concentration that inhibited growth by 50% (EC<sub>50</sub>), were obtained for the population before and after exposure to each fungicide. Each treatment population consisted of 40 to 60 isolates obtained from each experimental plot subjected to one of the fungicide treatments (Table 2). Two independent fungicide assays were conducted to assess the effect of each fungicide on each isolate. Data are expressed as the proportion of radial growth on

TABLE 1. Description of fungicide treatments applied to each 5-by-10-m plot planted with susceptible cv. Alpha<sup>a</sup>

| Treatment    | Active ingredients                | Product                | Experimental rate (a.i. ha <sup>-1</sup> ) | Recommended rates (a.i. ha <sup>-1</sup> ) |
|--------------|-----------------------------------|------------------------|--|--|
| No fungicide | ...                               | ...                    | ...  | ...  |
| Mancozeb     | Mancozeb (80%)                    | Manzate 200, DuPont    | 1.2 kg                                     | 0.8–3.2 kg                                 |
| Dimethomorph | Dimethomorph (9%), Mancozeb (60%) | Acrobat MZ, Cyanamid   | 0.18 kg, 1.2 kg                            | 0.18–0.27 kg, 1.2–1.8 kg                   |
| Cymoxanil    | Cymoxanil (60%)                   | Technical gr., DuPont  | 0.16 kg                                    | 0.16–0.24 kg                               |
| Propamocarb  | Propamocarb hydrochloride (36%)   | Previcur N, Agrevo     | 0.64 l                                     | 0.64 liter                                 |
| Metalaxyl    | Metalaxyl-M (46.2%)               | Ridomil Gold, Novartis | 0.15 kg                                    | 0.11–0.15 kg                               |
| Azoxystrobin | Azoxystrobin (22.9%)              | Quadris, Zeneca        | 0.05 kg                                    | 0.05–0.11 kg                               |
| Fluazinam    | Fluazinam (40.4%)                 | Shogun 500 FW, Zeneca  | 0.2 l                                      | 0.2–0.3 liter                              |

<sup>a</sup> Fungicides were applied at the experimental rate every 6 to 11 days to provide for a moderate but sustained epidemic.

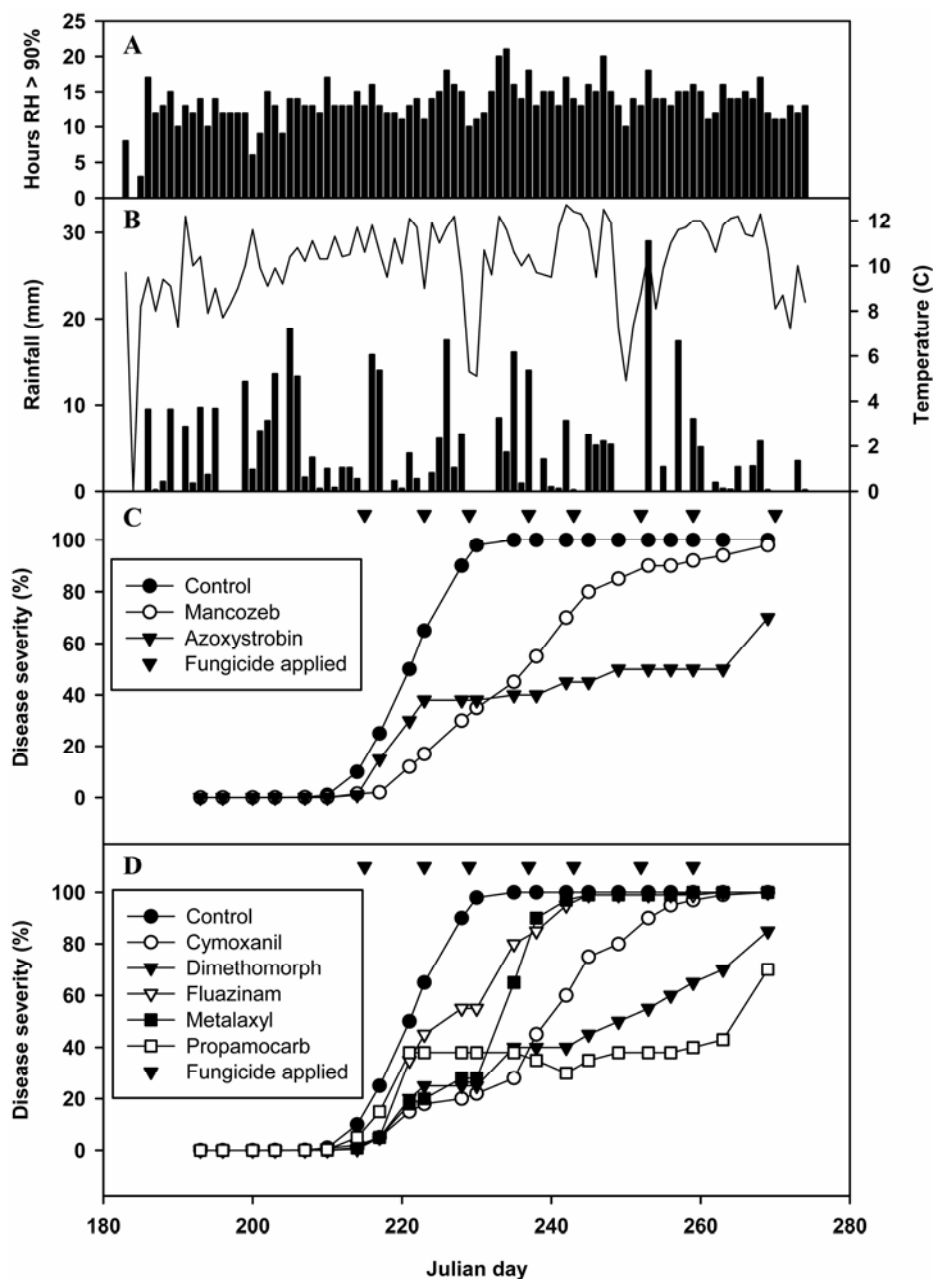
fungicide-amended medium relative to growth on nonamended medium. Fungicide concentrations used included 0.0, 0.1, 1.0, 10, and 100  $\mu\text{g a.i. ml}^{-1}$ . The  $\text{EC}_{50}$  was estimated for each isolate from a regression of relative mycelial growth versus the logarithm of fungicide concentration.

**Genotypic diversity.** Genotypic diversity was estimated by defining a multilocus genotype for each isolate by combining data for mating type and two isozyme loci (16,17). Mating type was determined by pairing each isolate with known A1 and A2 tester strains on Rye-A agar. Oospores forming in a cross of the unknown paired with the A1 tester strain were of the A2 mating type and vice versa. Allozyme genotypes for *Glucose-6-phosphate-isomerase (Gpi)* (EC 5.3.1.9) and *Peptidase (Pep)* (EC 3.4.3.1) were determined using cellulose acetate membranes as described previously (12,16).

**Statistical analyses.** Frequencies of fungicide resistance before and after exposure for each fungicide treatment were compared

using Monte Carlo approximations to the two-sided Fisher's exact test, which is appropriate for frequency distributions that include small or zero cell counts (47). Analyses of frequency distributions were performed separately for each fungicide assay and, after determining that the distributions were not significantly different, data were combined for final presentation. Multilocus genotypic diversity analysis for each population distinguished richness, evenness, and diversity as described previously (16,17). Genotypic diversity was calculated as Shannon-Wiener's index  $H' = -\sum p_i \times \ln(p_i)$  (38), where  $p_i$  is the observed frequency of the  $i$ th genotype. The significance of differences between genotypic diversities was calculated for  $H'$  using a  $t$  test (27) with a Bonferroni correction for multiple comparisons. The evenness index  $E_5$  (26) was calculated as

$$E_5 = \frac{(1/\hat{\lambda}) - 1}{e^{H'} - 1}$$



**Fig. 1.** Dynamics observed during the growing season for **A**, hours at which relative humidity was above 90% (Hours RH > 90%), **B**, rainfall (mm) and temperature (°C), and **C** and **D**, disease progress. Dates at which fungicides were applied are marked with downward-pointing triangles; in **C**, treatments received one more fungicide application than treatments in **D**. The control did not receive any fungicide application.

where  $\lambda$  corresponds to Simpson's index (43):

$$\hat{\lambda} = \frac{\sum_{i=1}^g n_i(n_i - 1)}{n(n-1)}$$

and  $n_i$  equals the number of observations of the  $i$ th genotype and  $n$  equals sample size. Genotypic richness expresses the number of expected genotypes in a sample and was estimated using rarefaction curves based on the sample size of the smallest population using the algorithm <Rarefac.C> (16,17). Bootstrapping of genotypes observed within fungicide-treated populations was conducted using the SAS macro <jackboot.sas> (available online from the SAS Institute, Cary, NC) to calculate confidence intervals for  $H'$  (16,17). Bootstrapping was conducted using 2,000 resamples at a confidence interval of 95% using the normal "standard" confidence interval option.

## RESULTS

Environmental conditions were favorable for potato late blight development. Most days had at least 10 h of relative humidity above 90%, indicating the presence of extended hours of leaf wetness (Fig. 1A). Similarly, average daily temperature was low, ranging between 6 and 12°C, and rainfall occurred on 68% of days (Fig. 1B). Disease in the unsprayed control progressed rapidly following an s-shaped pattern without apparent interruptions to the epidemic progress (Fig. 1C and D). The first fungicide application occurred on 2 August (Fig. 1C and D, downward pointing triangles) right after late blight was first observed in the experimental area. Fungicides were applied every 6 to 11 days thereafter at the discretion of the principal investigator (Fig. 1C and D). Disease in all fungicide-treated plots progressed more slowly than in the untreated control. All fungicide-treated plots ended up with >60% final disease severity (Fig. 1C and D).

For five of the seven fungicides tested, isolates showing considerable levels of resistance could be detected. With the exceptions of azoxystrobin and mancozeb, isolates able to grow at  $>10^2$   $\mu\text{g a.i./ml}$  were detected in plots treated with the fungicides cymoxanil, dimethomorph, fluazinam, metalaxyl, and propamocarb hydrochloride (Fig. 2).

Shifts in sensitivity profiles to each of the fungicides after exposure were detected based of Fisher's exact test for four of the seven fungicides (Fig. 2). In case of the fungicides metalaxyl and cymoxanil, the frequency distributions significantly ( $P < 0.0011$  and 0.0001, respectively) shifted toward insensitivity as reflected by the median for each population (Fig. 2), indicating directional selection toward resistance. Distributions for the fungicides azoxystrobin and propamocarb hydrochloride also were significantly different ( $P < 0.0001$ ); however, the median of the distributions

shifted to the left, making the population after exposure more sensitive to these fungicides. For the fungicides dimethomorph, fluazinam, and mancozeb, no changes in frequency distribution could be detected ( $P > 0.05$ ).

We tested the hypothesis that genotypic diversity would be lowered in a population exposed to the fungicide compared with the unexposed population. Although genotypic diversity based on Shannon-Wiener's index  $H'$  between fungicide-exposed and baseline populations was significantly different for both the metalaxyl- and the dimethomorph-exposed populations, bootstrap confidence intervals support significant differences for only the metalaxyl-exposed population (Table 2). The baseline sample was somewhat clonal, where 18 genotypes were detected out of 50 isolates evaluated (Table 2). The metalaxyl-exposed population had the lowest expected number of genotypes as estimated by rarefaction, the lowest diversity, and intermediate evenness. The metalaxyl-exposed population became the most clonal, with only nine genotypes recovered from 50 isolates evaluated. However, the evenness in genotype distribution of  $E_5 = 0.73$  was similar to that of the baseline population at  $E_5 = 0.78$ , reflecting that several of the more resistant genotypes were selected.

## DISCUSSION

This study provides novel baseline fungicide sensitivity data for a sexual population of *P. infestans* evaluated in the field at the center of origin under natural growing conditions. The oomycete *P. infestans* has a wide range of sensitivities to the fungicides fluazinam, cymoxanil, dimethomorph, metalaxyl, and propamocarb, with  $EC_{50}$ s ranging from  $10^{-2}$  to  $10^3$   $\mu\text{g ml}^{-1}$ , indicating that these fungicides might result in more selection when compared with azoxystrobin and mancozeb, which had comparatively narrow distributions (Fig. 2).

Comparison of our work to previously published reports reveals interesting similarities and differences. The data available from previous studies for the fungicides cymoxanil and metalaxyl showed similar distributions of  $EC_{50}$  values or sensitivities (11,16, 28,35,48). Resistance to mancozeb and cymoxanil had similar distributions in a Brazilian population of *P. infestans* (35). The distribution of resistance to dimethomorph in our study overlapped with that of another recent study by Stein and Kirk (46). In contrast, resistance to azoxystrobin in our work ranged between  $10^{-3}$  and  $10^{-1.5}$   $\mu\text{g ml}^{-1}$ , whereas Gisi et al. (11) found a range of  $10^{-2}$  to  $10$   $\mu\text{g ml}^{-1}$  for isolates collected in Switzerland. Both the Mexican and the Swiss population (11) most likely had not been exposed previously to azoxystrobin. Both studies used a similar range of fungicide concentrations. Our assay relied on relative growth on fungicide amended agar, whereas Gisi et al. (11) used

TABLE 2. Frequencies of multilocus genotypes based on mating type, *glucose-6-phosphate isomerase* (*Gpi*) allozyme, and *peptidase* (*Pep*) allozyme patterns of isolates of *Phytophthora infestans*

| Population                | Sample size <sup>a</sup> | $g^b$ | $g_{30}^c$ | $H'^d$ | CI of $H'^e$ | $E_5^f$ |
|---------------------------|--------------------------|-------|------------|--------|--------------|---------|
| Baseline (nonexposed)     | 50                       | 18    | 14.2       | 2.60   | 2.31–2.89    | 0.78    |
| Mancozeb                  | 50                       | 17    | 13.8       | 2.55   | 2.25–2.84    | 0.95    |
| Propamocarb hydrochloride | 51                       | 20    | 15.8       | 2.78   | 2.51–3.05    | 0.83    |
| Cymoxanil                 | 49                       | 15    | 11.8       | 2.21   | 1.85–2.57    | 0.63    |
| Metalaxyl                 | 50                       | 9     | 7.9        | 1.82** | 1.51–2.12    | 0.73    |
| Dimethomorph              | 30                       | 11    | 10.8       | 2.15** | 1.88–2.41    | 0.82    |
| Azoxystrobin              | 60                       | 15    | 11.6       | 2.37   | 2.09–2.66    | 0.79    |
| Fluazinam                 | 32                       | 13    | 12.6       | 2.30   | 1.99–2.58    | 0.76    |

<sup>a</sup> Sample size was considerable smaller in the dimethomorph and fluazinam treatments because isolates were lost in storage before the assays could be completed.

<sup>b</sup> Number of multilocus genotypes observed.

<sup>c</sup> Expected number of multilocus genotypes calculated for a sample size of  $n = 30$  isolates per population estimated using the rarefaction method (17,42).

<sup>d</sup> Shannon-Wiener diversity index ( $H'$ ) for multilocus genotypes (17,38). Values followed by \*\* indicate that  $H'$  in this population is significantly different ( $P < 0.01$ ) from  $H'$  of the baseline population according to pairwise, Bonferroni corrected  $t$  tests.

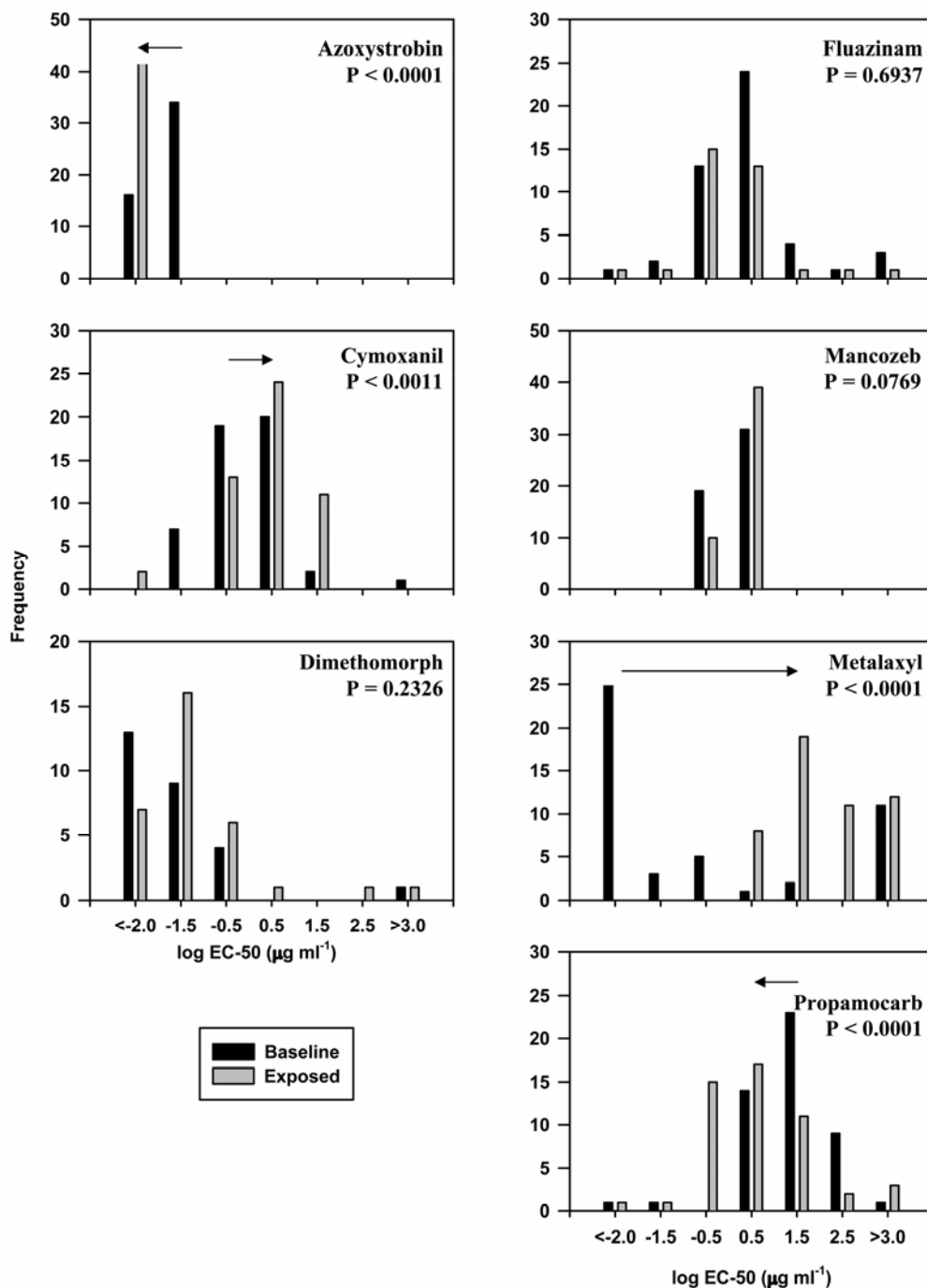
<sup>e</sup> Bootstrap confidence intervals of  $H'$  based on sample size of 30, using 2,000 resamples, assuming a normal distribution of the sample statistic and a 95% confidence interval (17).

<sup>f</sup> Index of evenness for multilocus genotypes (17,26).

leaf discs from fungicide-treated plants. Thus, the differences between the two studies might relate to a difference in methodology. Another study by Hamlen et al. (20) shows a narrow distribution of resistance to cymoxanil, ranging mostly between  $10^{-2}$  to  $10^{-1}$   $\mu\text{g ml}^{-1}$ . Our frequency distribution ranged between  $10^{-2}$  and  $10^3$  (Fig. 2). The study by Hamlen et al. (20) used a global population, including isolates from Mexico. Although the abstract mentions both in vitro and in vivo studies, according to the methodology described, their study relied on greenhouse experiments and does not describe in detail how experiments were conducted and how  $\text{EC}_{50}$  values were calculated (20). Thus, the differences observed between our results and those by Hamlen et

al. again might be due to methodological differences. Based on their results, Hamlen et al. (20) concluded that, worldwide, the population of *P. infestans* is sensitive to cymoxanil. Our results indicate that the resistance spectrum is broad and includes isolates resistant to cymoxanil.

We detected directional selection toward higher resistance only for the fungicides metalaxyl and cymoxanil. Only in the case of metalaxyl did we also detect a significant reduction in genotypic diversity based on Bonferroni adjusted *t* tests. The significant shifts in the distributions of azoxystrobin and propamocarb toward more sensitivity were not expected. We expected to detect either positive selection toward more resistance, as was the case



**Fig. 2.** Distribution of the logarithm of effective concentration that inhibited growth by 50% ( $\text{EC}_{50}$ ) values before and after exposure of the *Phytophthora infestans* population to different fungicides. Classes of sensitivity were based on the logarithm of  $\text{EC}_{50}$  values and defined as follows:  $\leq -2.0$ ,  $> -2.0$  to  $\leq -1.0$ ,  $> -1.0$  to  $\leq 0.0$ ,  $> 0.0$  to  $\leq 1.0$ ,  $> 1.0$  to  $\leq 2.0$ ,  $> 2.0$  to  $\leq 3.0$ , and  $> 3.0$   $\mu\text{g ml}^{-1}$ . The two-sided probability values given were determined using a Monte Carlo approximation to the Fisher's exact test for  $s \times r$  tables contrasting the frequency distributions for baseline and exposed populations for each fungicide. The arrows indicate the direction (and approximate magnitude) of a statistically significant shift in median from distributions before and after exposure of the population to a fungicide.

for metalaxyl and cymoxanil, or no selection, as was the case for three of the fungicides, including fluazinam, mancozeb, and dimethomorph. Several explanations are possible for these observations. In both cases, the frequency distributions before and after exposure were compared using a Fisher's exact test to detect shifts in the median (and overlap of the range of values). In the case of azoxystrobin, the distribution of EC<sub>50</sub> values was located at the lower end of the detection threshold; thus, our assay was at the limit of the range at which we could reasonably expect to describe a distribution of fungicide resistance. It is apparent that, for the methodology used, azoxystrobin sensitivity needs to be evaluated using fungicide concentrations lower than 0.1 µg a.i. ml<sup>-1</sup>. In the case of propamocarb, the situation is quite different, and no straightforward explanation comes to mind. The distributions before and after exposure cover the full range of EC<sub>50</sub> values and both distributions overlap over the whole range.

The fact that selection for fungicide resistance could not be detected within a field season for azoxystrobin, dimethomorph, fluazinam, and propamocarb does not mean that resistance could not evolve. Evolutionary forces such as mutation, migration, and random drift (31) cannot be expected to be detected within a single field season in small experimental plots and with a very small sample size (≈50 isolates) unless these forces are very strong. In analogy to the durability of resistance of a host to a pathogen, "durability" of a fungicide can be evaluated only over long periods of time and large areas (14,21). This also is reflected in the unexpected result that we actually detected a shift toward susceptibility in the dimethomorph-treated populations relative to baseline samples.

Experiments were performed in experimental plots and, thus, the baseline population sampled would be expected to be less diverse relative to a sample representing the diversity found in the valley overall. A comparison to our previous work using the exact same methodology for determining multilocus genotypes based on mating type, *Gpi*, and *Pep* loci reveals that the genotypic diversity in the baseline sample was indeed lower ( $H' = 2.6$ ) relative to a larger, hierarchical population sample representative of the Toluca Valley overall ( $H' = 2.8$  to 3.1) (16,17).

Given that the Mexican population of *P. infestans* contains individuals at frequencies >2% (i.e., 1 in 50 isolates) that have been shown to have EC<sub>50</sub> values >100 µg a.i. ml<sup>-1</sup> to cymoxanil, dimethomorph, fluazinam, metalaxyl, and propamocarb (Fig. 2), further introductions of novel clones from Mexico should be avoided. The situation is quite different for azoxystrobin, where all isolates evaluated were sensitive. Expected risk of evolution of fungicide resistance is determined by the evolutionary potential of *P. infestans* (29). Given that *P. infestans* has a mixed reproductive system, a high potential for genotype flow (through human activity), a moderate to large effective population size, and baseline populations that already have insensitive isolates, it has to be considered to be a high-risk pathogen for evolution of fungicide resistance (29). Establishment and clonal reproduction of strains tolerant to cymoxanil, dimethomorph, fluazinam, and propamocarb could lead to a situation analogous to that of metalaxyl. The results obtained provide direct experimental support for continuing vigilance regarding further introductions of exotic strains of *P. infestans* into the United States.

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