Disease Control and Pest Management

Use of Two- and Three-Way Mixtures to Prevent Buildup of Resistance to Phenylamide Fungicides in *Phytophthora* and *Plasmopara*

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The authors acknowledge the technical assistance of E. Rimbach.

Accepted for publication 31 March 1987.

ABSTRACT

Samoucha, Y., and Gisi, U. 1987. Use of two- and three-way mixtures to prevent buildup of resistance to phenylamide fungicides against *Phytophthora* and *Plasmopara*. Phytopathology 77:1405-1409.

Oxadixyl, mancozeb, and cymoxanil were tested, singly and as two- or three-way mixtures, for their potential to control phenylamide-sensitive and -resistant strains of *Phytophthora infestans* on tomato or potato and *Plasmopara viticola* on grape under growth chamber and plastic house conditions. Fungicide mixtures provided better disease control of phenylamide-resistant strains than either one of the components alone. Two- and three-way mixtures were compared in terms of selection pressure against mixed populations of sporangium suspensions of both pathogens over a period of eight generations of sporangia. Applications were made three to seven times at 7–14-day intervals. For inoculation, a sporangium suspension with an initial proportion of resistant strains of 0.01, 0.1, 1, 10, 20, or 50% was used. When initial amounts of resistance in the mixed population were 1, 0.1, and 0.01%, the two-way mixture oxadixyl/mancozeb did not select for more than about 15% resistance after two, three, and four applications, respectively, each application made on a new sporangium generation. If initial percentage of resistance was 10% or higher, 100% resistant strains were present after four to five sporangium generations. In contrast, mixed populations containing as high as 50% resistance remained fairly stable, when treated with the three-way mixture oxadixyl/mancozeb/cymoxanil; the proportion of the resistant subpopulation even decreased in tendency during a period of eight sporangium generations, each treated with a new fungicide application.

Additional key word: fungicide resistance.

The type of fungicide used for disease control greatly determines the likelihood of the development of resistance by target organisms (4,8). Failure in disease control caused by fungicide resistance occurs most often after the introduction of single-site fungicides (3). Resistance problems have their origin in resistant strain subpopulations existing at very low levels, before the exposure of the entire population to the new fungicide starts (2,7). Once a population is exposed to a new fungicide, resistant strains are selected, and their frequency may increase until disease control may become inefficient.

In recent years, prepacked mixtures of single-site and contact fungicides have been formulated. Such mixtures were expected to delay the buildup of resistant strains in fungal populations (13). Theoretical models of the buildup of fungicide resistance support the use of mixtures for delaying resistance problems (5,12,19). Under growth chamber conditions and starting with 0.01% resistant sporangia of *Phytophthora infestans* (Mont. de Bary) in the population, 100% resistance occurred after three applications of metalaxyl alone, compared with 50% resistance when a mixture of metalaxyl and mancozeb was used (20). Sanders et al (18) showed that full-rate mixtures of metalaxyl and mancozeb slightly delayed the buildup of resistance in *Pythium* and that reduced-rate mixtures had improved delaying effect. Nevertheless, mixtures of metalaxyl with mancozeb failed to control resistant populations of *Phytophthora infestans* in potatoes (1) or *Pseudoperonospora cubensis* (Berk. & Curt.) Rostow in cucumbers (15) under field conditions.

In the present study, the potential of two- and three-way mixtures of oxadixyl, mancozeb, and cymoxanil to prevent the selection of phenylamide resistant strains in mixed populations of *Phytophthora infestans* and *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni was investigated over a period of eight generations of sporangia or for as many as eight fungicide applications.

MATERIALS AND METHODS

Fungicides and fungicide application. The three tested fungicides, each with a different mode of action (9), were the systemic phenylamide fungicide oxadixyl (25WP), the systemic acetamide fungicide cymoxanil (50WP), and the dithiocarbamate contact fungicide mancozeb (80WP). All preparations were used as aqueous suspensions that were sprayed singly or in mixtures directly on the plants until near runoff. All concentrations are given as amount of active ingredient per volume.

Fungus strains. All fungus strains were obtained from the collection of Sandoz Witterswil. Potato (*Solanum tuberosum*) tubers, cultivar Bintje, were cut into 0.5-cm slices, washed with water, dried with paper towel, and put into 18-cm-diameter petri dishes. After 1 hr, the slices were sprayed with a suspension of sporangia (about 5×10^5/ml) of *Phytophthora infestans*, strain 58 (sensitive to oxadixyl), using a glass atomizer, and incubated at 15 C in the dark. After 7 days abundant quantities of sporangia could be harvested from the slices to give final concentrations of 1×10^5/ml. Because the strain 13 (resistant to oxadixyl) of *Phytophthora infestans* did not produce many sporangia on potato slices, it was cultured on rye-decoction-agar; sporangia were washed from 7-day-old cultures. Sporangia of *Plasmopara viticola* were washed from infected leaves by shaking them with water in an Erlenmeyer flask. Suspensions of about 10^5/ml were used for both sensitive-(56) and phenylamide-resistant (103) strains. Both *Phytophthora* strains and both *Plasmopara* strains were about equally sensitive to either cymoxanil or mancozeb independent of their degree of resistance to oxadixyl (11); there is no cross resistance between the three molecules. The two pairs of *Phytophthora* and *Plasmopara* strains did not vary much in the fitness (sporulation intensity) or the pathogenicity to potato, tomato, or grape plants when inoculated singly and in absence of fungicide (data not shown).

Plant material. Tomato (*Lycopersicon esculentum*) plants, cultivar Baby, were produced from seeds grown at about 25 C for 5 wk; potted plants with seven leaves were used for all growth chamber experiments (Table 1). Grape (*Vitis vinifera*) plants, cultivar Riesling Sylvaner, were produced from 1-yr-old cuttings, which were incubated at 25 C for about 6 wk in perlite. The young potted plants were grown for another 3 wk until the 10-leaf stage was reached (growth chamber experiments, Table 2 and Fig. 1). Twenty plants per treatment (fungicide concentration) were used in all growth-chamber experiments. Potato plants, cultivar Bintje, were produced using two tubers per pot filled with 1 L of sandy loam. They were incubated in a plastic house at about 25 C. After 4 wk, 17–20 shoots, bearing 24–32 leaflets per shoot, developed in each pot; 30 pots per treatment were used in the plastic house experiments (Fig. 2), and 200 pots per treatment were used in the plastic house experiments (Table 3).

Epidemic experiments in plastic house. Plants were sprayed three times at intervals of 14 days with three concentrations of fungicide suspensions using a garden sprayer. About 24 hr after the first treatment plants were spray-inoculated with sporangium suspensions of the phenylamide-resistant strain (13) of *Phytophthora infestans.* Disease developed continuously on the growing plant parts and was under a continuous selection pressure; plants were in proximity to facilitate sporangium spreading. Disease development was recorded at 7-day intervals and expressed as percentage of infected leaflets per pot. Percent disease control was calculated according to Abbott (6,9), and dose response curves were computed for all components to quantify the interactions of the single components in the fungicide mixtures (synergy ratio SR) according to the Wadley approach (22,23). Synergistic interactions (on the 90% efficacy level) indicate an observed response to the fungicide mixture higher than the sum of the responses to the individual components. Further details on the basis of the analysis method are given by Gisi et al (9) and Samoucha and Gisi (16). Only the results of the highest of the three fungicide concentrations are shown in Figure 2.

Population cycling experiments. The change of resistant subpopulation in a mixture of strains was estimated by establishing a discontinuous selection pressure with fungicide mixtures on the developing disease over a period of up to eight generations; these cycling experiments were done either in growth chambers (Tables 1 and 2; Fig. 1) or in a plastic house (Table 3).

Growth chamber experiments. The sporangium suspensions of sensitive (S) and resistant (R) strains (each 10^5/ml) of

TABLE 1. Influence of repeated fungicide treatments (oxadixyl/mancozeb, 1 + 7; oxadixyl/mancozeb/cymoxanil, 1 + 7 + 0.4) on the proportion of a phenylamide resistant strain in mixed populations (original amount of R: 50 or 20%) over eight sporangium generations of *Phytophthora infestans* on tomato[a]

	Resistant strain ± SD (%)			
	Oxadixyl/mancozeb/cymoxanil treated plants		Oxadixyl/mancozeb treated plants	
Cycles	Original R = 50%	Original R = 20%	Original R = 50%	Original R = 20%
1	45 ± 9	18 ± 4	63 ± 11	33 ± 6
2	46 ± 6	19 ± 6	72 ± 9	54 ± 10
3	40 ± 7	16 ± 5	90 ± 5	80 ± 7
4	45 ± 11	19 ± 3	100	100
5	43 ± 8	18 ± 7	...	...
6	40 ± 6	15 ± 4	...	...
7	40 ± 9	15 ± 5	...	...
8	38 ± 5	13 ± 6	...	...

[a] For every cycle (7 days) new plants were used for inoculation and fungicide application.

TABLE 2. Influence of repeated fungicide treatments (oxadixyl/mancozeb, 1 + 7; oxadixyl/mancozeb/cymoxanil, 1 + 7 + 0.4) on the proportion of a phenylamide-resistant strain in mixed populations (original amount of R: 50 or 20%) over eight sporangium generations of *Plasmopara viticola* on grape[a]

	Resistant strain ± SD (%)			
	Oxadixyl/mancozeb/cymoxanil treated plants		Oxadixyl/mancozeb treated plants	
Cycles	Original R = 50%	Original R = 20%	Original R = 50%	Original R = 20%
1	49 ± 11	20 ± 6	55 ± 4	35 ± 7
2	49 ± 8	19 ± 5	68 ± 9	54 ± 5
3	47 ± 10	18 ± 6	94 ± 3	86 ± 3
4	45 ± 6	18 ± 4	100	95 ± 1
5	43 ± 11	17 ± 5	...	100
6	42 ± 8	16 ± 7	...	...
7	40 ± 5	15 ± 3	...	...
8	40 ± 7	15 ± 4	...	...

[a] For every cycle (7 days) new plants were used for inoculation and fungicide application.

Phytophthora infestans or *Plasmopara viticola* were prepared separately as described and mixed by volume to create the following proportions of R in the final suspension: 50, 20, 10, 1, 0.1, and 0.01%. The sporangium suspension was used immediately after mixing for inoculation. Four days after inoculation, the infected plants were sprayed (curative treatment) with the two- or three-way mixture (oxadixyl/mancozeb, 50 + 350 mg/L; oxadixyl/mancozeb/cymoxanil, 50 + 350 + 20 mg/L). Seven days after inoculation abundant sporangia were produced on the infected leaves and were harvested from the leaves of each treatment separately for inoculation of new plants for the next cycle. Because all treatments were curative (4 days after inoculation), there was no preselection during penetration but a discontinuous selection pressure during sporangium formation of each new generation. The same sporangium suspension was used to estimate the percentage of the resistant subpopulation (see below). Eight 7-day cycles, one after the other on new plants, were used. Every new sporangium generation was treated with the fungicide mixtures resulting in a very high selection pressure (curative treatment, short cycles, high sporangium concentrations). Statistical analyses of the amount of resistance in the population were made with Duncan's multiple range test (Tables 1–3).

Plastic house experiments. The S and R strains of *Phytophthora infestans* were mixed at a ratio (S:R) of 9:1. Four days after artificial inoculation of potato plants with the mixed sporangium suspension, plants in the first plastic house were treated with the two-way mixture (oxadixyl/mancozeb = 100 + 700 mg/L) and plants in the second house with the three-way mixture (oxadixyl/mancozeb/cymoxanil = 100 + 700 + 40 mg/L) using a garden sprayer. The fungicide applications were repeated three more times at intervals of 14 days. Every 7 days sporangia were harvested from newly infected leaves of each plastic house and used to estimate the percentage of the resistant subpopulation (see below). Because of the high disease pressure and high humidity in the plastic houses, the new growth of the potato plants was continuously infected by the next generation of sporangia (about every 7 days); thus, the developing disease was under a continuous selection pressure of the fungicides.

Estimation of the percentage of resistant subpopulation. Sporangia from leaves of each fungicide treatment were harvested at the end of each cycle and used to inoculate new plants, which were either untreated or sprayed with 250 mg/L of oxadixyl. The percentage of leaf area of the treated and untreated plants that became diseased after 7 days as a result of this treatment was used to estimate the percent of resistant subpopulation in the sporangium suspension as follows:

$$\% \text{ resistant strain} = \% \text{ disease of plants } \frac{\text{treated} \times 100}{\text{untreated}} .$$

RESULTS

The influence of three spray applications of oxadixyl, mancozeb, cymoxanil, and the three-way mixture (1 + 7 + 0.4) was tested on the progress of the disease development caused by a phenylamide-resistant strain (13) of *Phytophthora infestans* on growing potato plants (Fig. 2). When the fungicides were tested singly, none of them provided sufficient disease control shortly after inoculation, whereas the three-way mixture resulted in almost full disease control over the whole test period. The excellent efficacy of the mixture is a result of significant synergistic interactions (synergy ratio) between oxadixyl, mancozeb, cymoxanil, which gradually increased during the disease development from a value of 12 at the beginning to 67 after 42 days.

A sporangium suspension of *Phytophthora infestans* (Table 1) and *Plasmopara viticola* (Table 2), with initial proportions of S:R = 4:1 or 1:1, was used to inoculate plants before fungicide

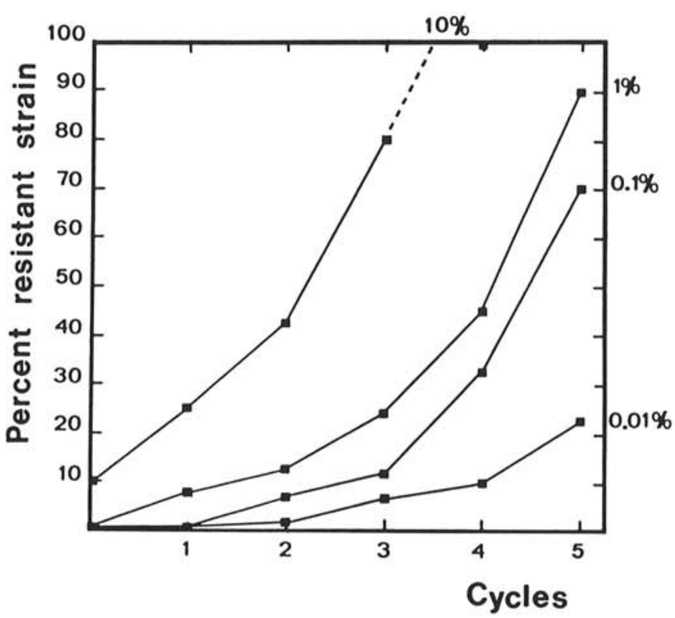

Fig. 1. Influence of repeated fungicide treatments (oxadixyl/mancozeb, 1 + 7) on the proportion of phenylamide-resistant strain in mixed populations (original amounts of R in sporangium suspension used for inoculation were 10, 1, 0.1, and 0.01%) over five sporangium generations of *Phytophthora infestans* on tomato. For every cycle (7 days) new plants were used for inoculation and application.

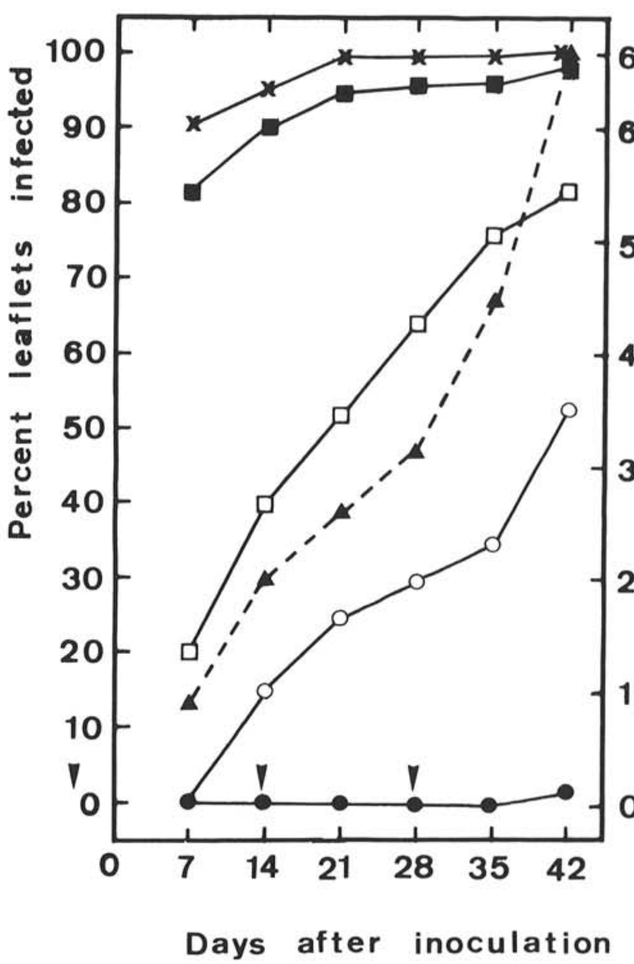

Fig. 2. Progressing disease development (×) on growing potato plants infected with a phenylamide-resistant strain (13) of *Phytophthora infestans* at day 0 and sprayed (▲) three times at interval of 14 days with oxadixyl (80 mg/L, ■), mancozeb (560 mg/L, o), cymoxanil (32 mg/L, □), or with a three-way mixture oxadixyl/mancozeb/cymoxanil (1 + 7 + 0.4, 336 mg/L, ●). Synergy ratio (▲) of the components in the mixture was calculated according to Wadley.

treatment; a discontinuous fungicide selection pressure was imposed over eight sporangium generations. The population treated with the two-way mixture oxadixyl/mancozeb, gradually shifted after each cycle and was resistant after four cycles for S:R = 1:1 and after five cycles for S:R = 4:1 (Table 1). In contrast there was no increase of the resistant subpopulation, even after eight cycles, when the three-way mixture oxadixyl/mancozeb/cymoxanil was used as selection agent. The proportion of the resistant strains even decreased slightly for both 4:1 and 1:1 sporangium mixtures. Similar results were observed with *Plasmopara viticola* populations (Table 2). With Duncan's analysis, significance was obtained for both two- and three-way mixtures at a level of $P < 0.005$. There was no significance at a level of $P < 0.05$ for "percentage of resistant strain" with three-way mixtures, but significance in all cases of two-way mixtures.

Growing potato plants were inoculated with a 9:1 (S:R) mixture of a sporangium suspension of *Phytophthora infestans* and incubated at 100% RH under plastic house conditions for 56 days (Table 3). During this time, a continuous epidemic developed corresponding to about eight generations of sporangia, which continuously infected the new growth; four fungicide applications were made at intervals of 14 days providing a continuous selection pressure for phenylamide resistant strains. When 800 mg/L of the two-way mixture oxadixyl/mancozeb (1 + 7) was used, the population was resistant after five sporangium generations and after three fungicide applications. In contrast, the S:R proportion of the mixed population remained fairly stable during at least eight sporangium generations when 840 mg/L of the three-way mixture oxadixyl/mancozeb/cymoxanil (1 + 7 + 0.4) were used four times at 14-day intervals for disease control.

A considerable delay in the buildup of resistance by the two-way mixture oxadixyl/mancozeb could be shown (Fig. 1). When the initial proportion of the resistant strain in the mixed population was less than 1%, the two-way mixture did not select much for resistance at the beginning of the experiment; resistant subpopulations did not increase to more than 15% after four, three, and two cycles, when initial R proportions were 0.01, 0.1, and 1%, respectively. In the case of 0.01% of initial resistance, the percentage of resistant strain reached only 23% at the end of the experiment (five cycles).

DISCUSSION

The development of late blight on tomato or potato caused by *Phytophthora infestans* and downy mildew on grape caused by *Plasmopara viticola* was most successfully controlled with fungicide mixtures. The three fungicides oxadixyl, mancozeb, and cymoxanil, when used alone, provided poor or no control of a phenylamide resistant strain of *P. infestans*, whereas the three-way mixture, applied at intervals of 14 days, gave complete disease control. This result is due to the strong synergistic interactions between the three components of the mixture (10,11,16), which are based on phenomena like different modes and times of action of the three molecules (9,16) and to the systemicity of both oxadixyl and cymoxanil (17). Mixtures with equal or higher synergistic interactions against the resistant subpopulation are claimed (14) to have a much lower selection pressure than mixtures with lower synergism when resistant and sensitive subpopulations are compared. Grabski and Gisi (10,11) found that mixtures between oxadixyl and cymoxanil (with or without mancozeb) consistently showed higher synergy ratios for the control of phenylamide-resistant strains, whereas mixtures without cymoxanil showed decreasing synergy ratios against resistant strains of *Phytophthora infestans* and *Plasmopara viticola*. Thus, in situations where significant problems of resistance are anticipated or already exist, mixtures with favorable synergy properties for sensitive and resistant subpopulations should be preferred. On the other hand, in situations where the likelihood of selection for resistance is not very high, two-way mixtures offer a valid concept for delaying resistance buildup.

In all cycling experiments, either in growth chamber or plastic house using mixed populations with more than 10% of resistant strains in the initial population, a predominance of resistant strains occurred within four to five disease cycles of both *Phytophthora* and *Plasmopara* when two-way mixtures were sprayed (three to five applications) for disease control. When the initial percentage of resistant strains in the sporangium suspension used for inoculation was not higher than 0.01, the mixed population remained rather stable over at least three sporangium generations, and the percentage of the resistant strain increased only to 23% after four applications of the two-way mixture. Our results fully support previous reports (18,20) showing that two-way mixtures have much lower selection pressure than single fungicides when the level of resistance is low. Proportions of resistance within a field can be analyzed only by leaf sampling and testing the sensitivity of the population using standard methods under laboratory conditions (15,20). The method we used to estimate the final amounts of resistance at the end of each cycle is precise enough for mixtures of defined R and S strains containing the used initial R proportions; furthermore, no alternative analysis methods are available without consumption of much more time and space. Our results may reflect an over- rather than an underestimation of R proportions in the population. Depending on the initial level of resistance, one can justify either four applications (at 0.01% resistance), or three applications (at 0.1%), or two applications (at 1%) of two-way mixtures per season without getting into serious resistance problems. Thus, the FRAC recommendations (21) to restrict fungicide applications with acylalanines to two to four per season, are supported by these findings. In contrast, mixed populations with initial amounts of resistance up to 50% can be treated up to eight times with the three-way mixture oxadixyl/mancozeb/cymoxanil without any increase of resistance over at least eight sporangium generations. This observation may be related to a differential behavior in selection pressure of the three-way mixture. Three-way mixtures are therefore more flexible in their use under difficult disease situations; they not only minimize the buildup of resistance, as shown in this paper, but also contribute to control resistant populations (10).

TABLE 3. Influence of four fungicide treatments (oxadixyl/mancozeb 1 + 7; oxadixyl/mancozeb/cymoxanil 1 + 7 + 0.4) on the proportion of a phenylamide-resistant strain in mixed populations (original amount of R: 10%) over eight sporangium generations (7-day cycles) of *Phytophthora infestans* on growing potato plants under plastic house conditions

	Resistant strain ± SD (%)	
Cycles	Oxadixyl/mancozeb/cymoxanil treated plants	Oxadixyl/mancozeb treated plants
1	8 ± 4	12 ± 5
2	9 ± 3	20 ± 7
3	8 ± 5	40 ± 4
4	6 ± 2	60 ± 11
5	8 ± 3	100
6	9 ± 4	...
7	7 ± 3	...
8	6 ± 2	...

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