Accuracy of Methods to Monitor Sensitivity of *Phytophthora infestans* to Phenylamide Fungicides

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ABSTRACT

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Sensitivity of *Phytophthora infestans* to metalaxyl was monitored on potato leaf disks, detached leaves, or whole plants. Only minor differences between the various experimental systems were found. Values obtained on amended agar showed similar resistance factors. The less differentiating slopes of the dose response curves from agar tests, however, leave more space for interpretation of sensitivity of test isolates. The drawback of all methods is that they are not sufficiently quantitative. Mixed populations of sensitive and resistant sporangia may give either a completely sensitive or resistant reaction, depending on the ratio of the two types of sporangia in the mixture. Levels of resistance lower than 0.1%, which occur at an early phase in the development of resistance, remain undetected with these methods. Lower levels of resistance down to 0.001% were detected only when large numbers of sporangia were bulked and subjected to preselection of metalaxyl-treated potato plants. Sensitivity testing was carried out subsequently with sporangia recovered from lesions developing on the treated plants. The methods described, therefore, are well suited for monitoring programs aimed at detecting fungicide resistance at higher levels late in the overall selection process. Other methods have to be developed to detect early selection with resistance frequencies lower than 10^{-3}.

The introduction of site-specific fungicides has led to an increasing number of fungicide-resistant plant pathogens during the last 10 yr (6). A typical example is the use of the highly active phenylamides (13), which interfere specifically with RNA polymerases of fungi in the order of the Peronosporales (4). Cases of resistance were found soon after the introduction of these fungicides as single products on various crops (3,5,7,9,11), and cross-resistance was invariably confirmed among various representatives of this chemical group (2,8). A strategy of using these products in mixtures with protective multisite fungicides has been successful in slowing down the development of resistance (11,12). In this context, various methods are used to measure fungicide sensitivity and to detect resistance in target populations. Such methods have not only allowed detection of resistance but have contributed to designing and validating antiresistance strategies or helped to clarify suspected cases of resistance. The methods do not allow precise quantitative descriptions of target populations, because their power to detect low frequencies of resistant spores is not elucidated. The purpose of this paper is to review the strengths and limitations of these resistance detection methods.

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MATERIALS AND METHODS

Potato test material. Experiments on whole plants were carried out on 4-wk-old, single-stemmed potato plants (cultivar Bintje) grown in the greenhouse from eye cuttings on peat in 5-cm pots. For tests on detached leaves or leaf disks, fully expanded leaves of plants grown similarly from whole tubers in 5-L pots were used.

Fungicide. Metalaxyl was used in all trials either as active ingredient (a.i.) or formulated as Ridomil 25WP. Fresh fungicide suspensions were made with tap water for each experiment.

Pathogen. Three metalaxyl-sensitive (CH 004.80, IRL 233.80, and NL 293.80) and three metalaxyl-resistant isolates (CH 138.80, IRL 144.80, and NL 126.80) of *Phytophthora infestans* were recovered from commercial potato fields and maintained at 18 C on detached potato leaves in petri dishes containing moist filter paper. Pure cultures were kept on rye agar slants at 11 C. Rye agar was made by boiling 200 g of rye in 1 L of distilled water for 1 hr. The broth was then filtered through cheesecloth, amended with 20 g of agar (Difco) and 5 g of D-glucose, and adjusted up to 1 L.

Inoculum preparation. Detached potato leaves were inoculated with a sporangial suspension of *P. infestans* and incubated in petri dishes as described earlier. After 1 wk, freshly formed sporangia were collected from the leaf surface with a fine, sterilized paintbrush and suspended in distilled water. The concentration was adjusted to 25,000 sporangia per milliliter unless stated otherwise. Inoculum was chilled at 6 C for 2 hr before inoculation to induce zoospore release.

Fitness. Sporangial suspensions at various concentrations were used to inoculate untreated whole plants in three replicates (1.5 ml of inoculum per plant) or leaf disks in 10 replicates (10 μl of inoculum per leaf disk). After incubation at 18 C during a 14-hr light period (10,000 lux) and 15 C at at night in 100% relative humidity for 7 days, percentage of sporulation on leaf surfaces or on leaf disks was assessed and plotted against inoculum density.

Sensitivity tests. In vitro mycelial growth of *P. infestans* was measured on rye agar amended with different concentrations of metalaxyl previously dissolved in acetone. Final concentration of acetone did not exceed 0.5%. Mycelial plugs were cut from the margins of 7-day-old rye agar plate cultures and transferred to the test media in six replicates. Colony diameter was measured after a 14-day incubation period at 18 C in the dark.

In vivo experiments were carried out in three ways (all experiments done at least twice):

1. Plants grown from eye cuttings were sprayed to runoff with various concentrations of metalaxyl formulated as wettable powder containing 25% a.i. After drying, they were spray-inoculated in three replicates with 1.5 ml of a sporangial suspension and incubated in a growth chamber at 18 C during a 14-hr light period (10,000 lux) and 15 C at night. High humidity was maintained by intermittent misting. Percentage of sporulation on leaf surfaces was assessed after 7 days.

2. Plants grown from tubers were sprayed with metalaxyl as indicated earlier. After drying, leaves (three terminal leaflets) were detached, placed in 9-cm petri dishes on moist filter paper, and inoculated in two replicates with four 10-μl droplets of a sporangial suspension per leaflet. Subsequent incubation and assessment were done as described for method 1.

3. Leaf disks (15 mm in diameter) were cut with a cork borer from fully expanded leaves of potato plants grown from tubers. Five disks were floated, upper surface down, on water (12 ml) in 5-cm petri dishes containing various concentrations of metalaxyl (a.i.) previously dissolved in acetone. Final concentration of acetone never exceeded 0.5%. Leaf disks were inoculated in 10 replicates with one 10-μl droplet each of a suspension containing sporangia of a single isolate or mixtures of sporangia from a sensitive and a resistant isolate at different ratios. They were incubated as described under method 1. Percentage of sporulation on leaf disks was assessed 7 days later.

RESULTS

Fitness. On potato plants, pathogen fitness expressed as the relationship between inoculum density and disease incidence was independent of fungicide sensitivity (Fig. 1A). Equivalent results were found in leaf disk tests (Fig. 1B), but in this system, lower inoculum concentrations were needed to produce disease. In each experiment, one strain showed a slightly superior fitness; in the plant experiment, it was the resistant IRL 144.80, and in the leaf disk test, it was the sensitive CH 004.80.

Sensitivity. Typical dose responses of different isolates of *P. infestans* to

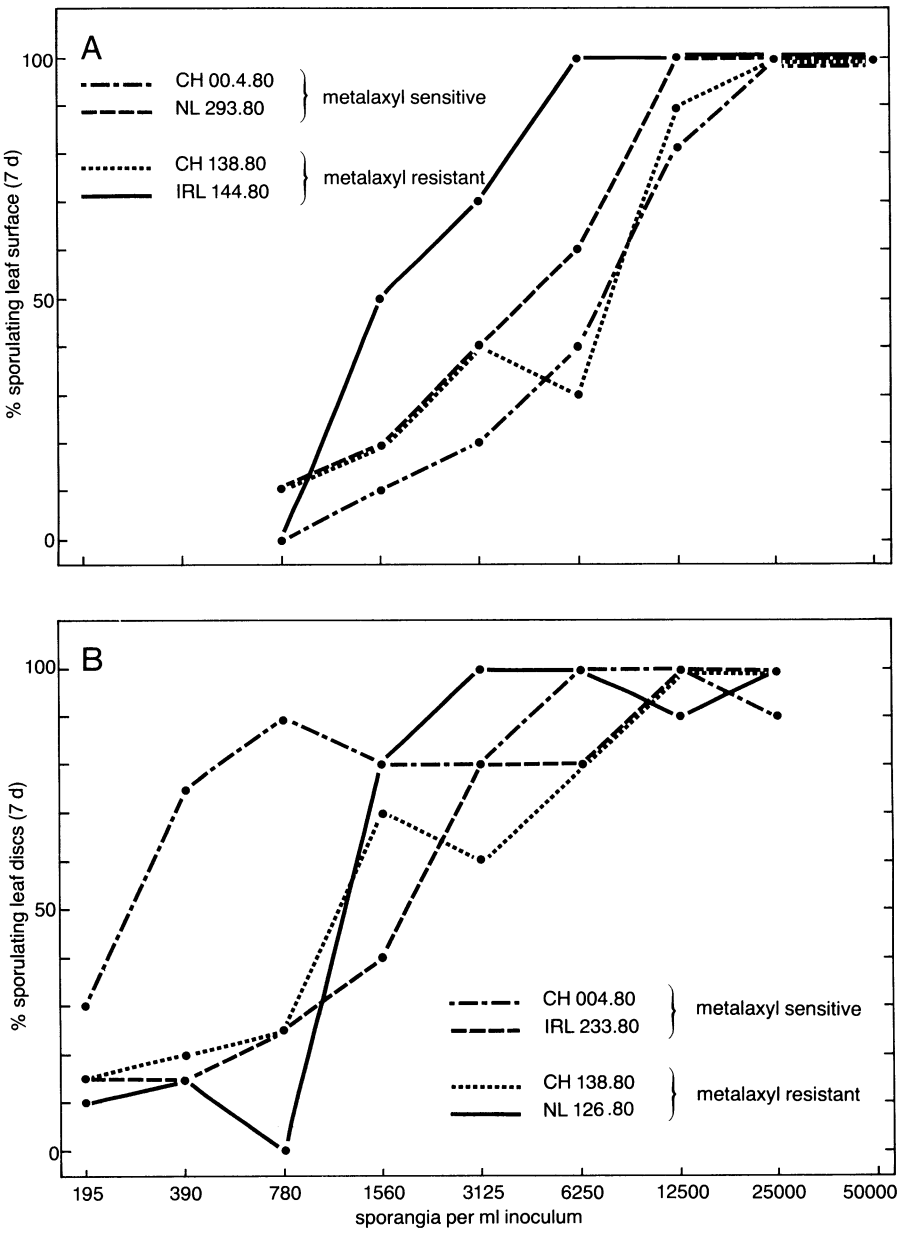

Fig. 1. Ability of phenylamide-sensitive and phenylamide-resistant *Phytophthora infestans* isolates to infect untreated **(A)** potato plants or **(B)** potato leaf disks at different concentrations of sporangia.

metalaxyl are given in Figure 2A–D. Responses of sensitive isolates on whole potato plants were marked, indicated by the steep slope of the curves (Fig. 2A). EC_{50} values of these isolates were <0.5 ppm, whereas resistant isolates were not or only slightly inhibited up to 200 ppm (Fig. 2A). Similar results were found with the detached leaves (Fig. 2B) and on leaf disks (Fig. 2C). However, on leaf disks, EC_{50} values of sensitive isolates were about 10 times lower, whereas resistant isolates again were not inhibited at all concentrations apart from a slightly delayed sporulation at 10 and 100 ppm (Fig. 2C). Also, the ranking of the sensitive isolates was inverted between the detached leaf and the leaf disk test, whereas on whole plants, the same reaction was observed for both isolates. In vitro results from agar tests (Fig. 2D) show a somewhat different picture. Although EC_{50} values of sensitive isolates were comparable to the leaf disk test results, the dose response was less marked, leading a less steep slope of the curves with higher minimal inhibitory concentrations (MIC). On the other hand, mycelial growth of resistant isolates was partly inhibited at concentrations as low as 0.1 ppm. Respective EC_{50} values and resistance factors are given in Table 1.

Sensitivity of mixed populations. Sensitivity of populations mixed at various ratios of sensitive and resistant sporangia in a leaf disk test is shown in Figure 3. Diluting the inoculum to 10% resistant sporangia did not significantly alter the reaction. At a ratio of 1% resistant sporangia, 30% of the disks still showed sporulation, whereas at a dilution to 0.1% resistant sporangia, there was no differentiation from the totally sensitive isolate. Similar observations were made with other pairs of isolates.

Detecting resistance levels of 0.1% or lower with an acceptable likelihood was possible only if large quantities of inoculum were submitted to preselection on potato leaves previously treated with 200 ppm a.i. metalaxyl. From a mainly sensitive inoculum of 10^6 sporangia containing 10 resistant sporangia, it was possible to reisolate 14 colonies resistant to metalaxyl after preselection of the population.

DISCUSSION

A criterion frequently used to demonstrate fungicide sensitivity is the linear growth of mycelial mass transfers on agar amended with fungicide (e.g., Fig. 2D). EC_{50} values and resistance factors from this test compare well with the respective values found in in vivo experiments (Table 1). In contrast to in vivo tests, however, the dose response curves of sensitive isolates on agar had a more gentle slope and a higher MIC. Resistant isolates, on the other hand, were already inhibited to some extent by low fungicide rates. On the basis of these in vitro EC_{50} values, the sensitivity of CH 138.80, for example, is ambiguous between 1 and 100 ppm. This may mislead interpretation of monitoring data, especially in the absence of reference isolates or baseline sensitivity data. Besides, obligate parasites (e.g., Peronosporaceae) cannot be evaluated on agar. For other fungi (e.g., Phythiaceae), isolation into pure culture is necessary with the risk of genetic changes by undesired laboratory selection. In addition, isolates showing resistance in vitro may still be sensitive in vivo (1,10).

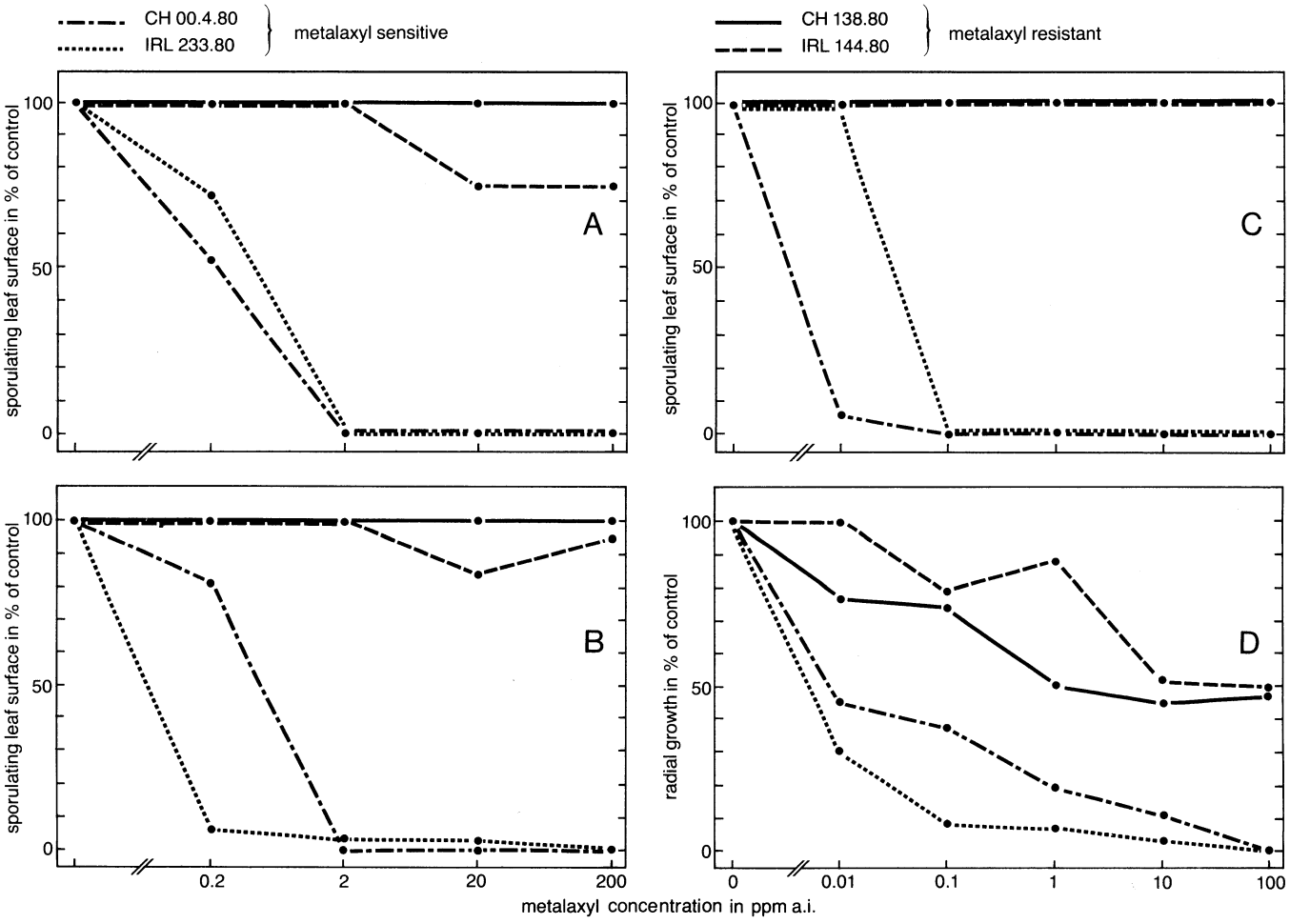

Fig. 2. Dose responses of phenylamide-sensitive and phenylamide-resistant *Phytophthora infestans* isolates to metalaxyl **(A–C)** 7 days and **(D)** 14 days after inoculation on **(A)** potato plants, **(B)** detached potato leaves, **(C)** potato leaf disks, and **(D)** rye decoct agar.

In vivo test systems using whole plants, detached leaves, or leaf disks give more precise indications of phenylamide sensitivity. The responses of isolates of *P. infestans* with different degrees of sensitivity of phenylamides were similar in the three test systems: no marked effect on resistant isolates even at the highest concentrations tested and complete control of sensitive isolates at low concentrations (MIC <2 ppm a.i.). Inversion of the ranking among sensitive isolates is within variations usually observed between test repetitions.

These in vivo results allow a clear definition of isolate sensitivity. Differing fitness of resistant and sensitive isolates is unlikely to influence in vivo test results, as can be derived from Figure 1, showing similar fitness of both isolate types on whole plants as well as leaf disks. High levels of attack on leaf disks obtained with lower inoculum concentrations may be attributed to the more directed inoculation compared with inoculation of whole plants.

In considering the sensitivity test response of a particular field isolate, one should always bear in mind, however, that it is the response of a small population rather than a single-spore isolate. This means that the population tested may actually consist of a mixture of resistant and sensitive sporangia, even though the reaction is distinctly resistant or sensitive. Thus, the results are more qualitative than quantitative. This aspect is illustrated by the sensitivity ratings on leaf disks of a selected pair of resistant and sensitive isolates tested either alone or mixed at different ratios. Dilution of the resistant sporangia down to 10% of the inoculum still gave a completely resistant reaction and thus may be interpreted as a totally resistant population. Even at 1% resistant sporangia, a partially resistant reaction is found and may lead to an overestimation of resistance present in the field. This has important implications, for it is not the same to treat a completely resistant population of *P. infestans* with, for example, a mixture of metalaxyl and mancozeb or to do the same treatment on a mainly sensitive population containing 1% resistant sporangia. Both the basis for resistance selection and the performance of metalaxyl in the mixture are vastly different under these two extreme situations.

The results in Figure 3 also indicate that under the described test conditions, detection of 0.1% resistant sporangia in a population, as it occurs in an early phase of resistance development or in crops treated with mixtures of phenylamides with a residual compound, was not possible. Obviously, the limiting factor in the leaf disk test is the number of sporangia tested. Other experimental evidence showed that the method was accurate enough, however, to detect resistant *Plasmopara viticola* in vineyards treated with a mixture of metalaxyl and folpet or *P. infestans* treated with metalaxyl/mancozeb where no apparent breakdown of control had occurred (11). An attempt to detect lower frequencies of resistance by submitting large numbers of sporangia to selection on potato leaves treated with metalaxyl resulted in an improved resistance detection limit of 0.001%.

The data of this study show that tests carried out on potato leaf disks, detached leaves, or whole plants are reliable to monitor phenylamide sensitivity and thus are useful tools to assess aspects of the development of resistance in the field. Clear limitations to detect low levels of resistance were found, however, and more work in this direction is needed.

Table 1. EC_{50} values (in ppm a.i. metalaxyl) and resistance factors of *Phytophthora infestans* isolates under various test conditions

Isolate	Sensitivity	EC_{50} (ppm a.i.) Plant	Leaf	Disk	Agar
CH 004.80	S	0.22	0.38	<0.01	<0.01
IRL 233.80	S	0.33	<0.20	0.03	<0.01
CH 138.80	R	>200	>200	>100	100
IRL 144.80	R	>200	>200	>100	100
Resistance factor		>1,000×	>1,000×	>10,000×	>10,000×

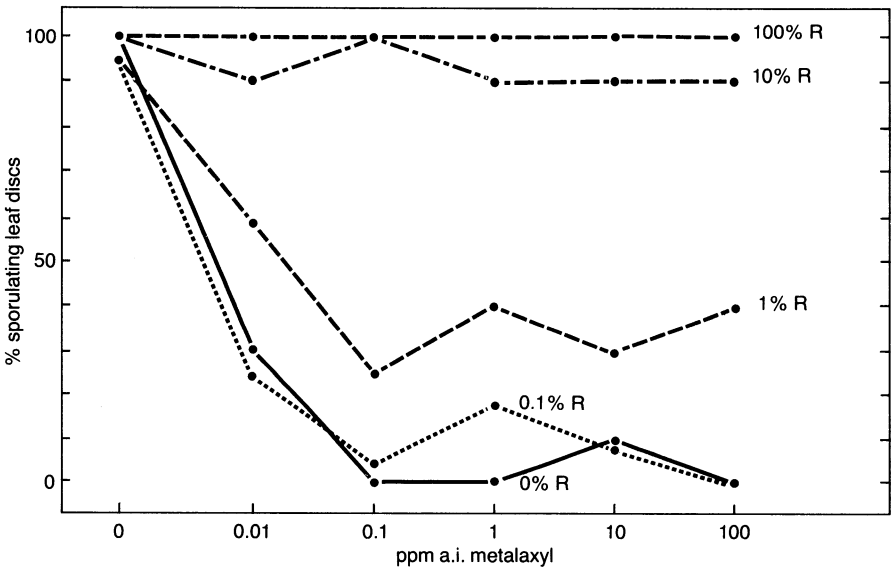

Fig. 3. Sensitivity reaction in a potato leaf disk test of a metalaxyl-sensitive or metalaxyl-resistant isolate of *Phytophthora infestans* or mixtures of both at various ratios (indicated as percent resistant [R] sporangia in the inoculum).

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