Comparison of Assays for Measuring Sensitivity of *Phytophthora infestans* Isolates to Fungicides

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ABSTRACT

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Pathogenic strains of *Phytophthora infestans* insensitive to phenylamide have been reported from around the world and are responsible for a lack of late blight control in both potatoes and tomatoes. In vivo laboratory assay methods used to determine the sensitivity of *P. infestans* to fungicides include floating of leaflet disks on fungicide suspension and the use of potato tuber disks. In our study, these two methods were compared with a new detached-leaf assay. The fungicides chlorothalonil (protective), oxadixyl and metalaxyl (systemic), and cymoxanil (locally systemic) were utilized. Phenylamide-insensitive and -sensitive genotypes of *P. infestans* were used as inoculum. Significant differences between preventive and systemic fungicides were found, and all assay methods differentiated between phenylamide-sensitive and -insensitive genotypes of *P. infestans*. Differences among assays were significant when comparing mean inhibitory values. There was no significant difference between the detached leaf and the tuber disk assay, but both methods were significantly different from the floating-leaflet disk assay.

Late blight, caused by *Phytophthora infestans*, is an international disease problem that is mainly controlled by a program of appropriately scheduled fungicide applications (6). On a worldwide basis, fungicidal control of potato late blight accounts for one-fourth of the total annual expenditure for fungicides on all crops (9).

Metalaxyl resistance is mediated by a single gene (4). Cases of resistance have evolved where this fungicide was used as a single product in curative or eradicator treatments. Resistant isolates also have been detected in fields not treated with metalaxyl and on partially resistant cultivars of potato (4,5,7,19), indicating a high fitness of the resistant strains in the absence of selection pressure (16). Metalaxyl-resistant genotypes emerged rapidly worldwide, and cross-resistance was reported for this class of fungicides (3,5,16). A coordinated strategy of avoiding eradicator applications and using metalaxyl in mixtures with protectant multisite fungicides has been effective in slowing the development of resistance (2,5,10,16).

Testing for the sensitivity of *P. infestans* to fungicides is conducted either under greenhouse conditions or by using common

laboratory techniques such as media amended with different concentrations of fungicides, floating leaflet disks on fungicide suspensions, and use of tuber disks (8). This study was conducted to evaluate and compare these methods with a new detached-leaf assay.

MATERIALS AND METHODS

Isolates. Three isolates of *P. infestans* were used in this study: (i) US-8 acquired from the culture collection at Cornell University, (ii) isolate 1192, and (iii) isolate 1292. Isolates 1192 and 1292 were provided by E.I. DuPont de Nemours, AG Products, and were previously identified as sensitive (1192) and insensitive (1292) to metalaxyl. Isolates were maintained on V8 agar (20). To verify and maintain their pathogenicity, they were routinely inoculated into potato plants grown in the greenhouse and then reisolated on rye agar (1).

Fungicides and concentration. Three types of fungicides were used in this study: (i) the commercial formulation of chlorothalonil (Bravo 720); (ii) 96% technical product of cymoxanil provided by DuPont de Nemours; and (iii) two systemics: a 98% technical product of oxadixyl provided by CIBA Crop Protection and a commercial formulation of Ridomil 2E (25% a.i.). A range of fungicide concentrations (from 0.1 µg/ml to 150 µg/ml) was used in our study. However, based on prior studies (8) and our results, the concentration of 10 µg/ml solution is presented in this paper as the standard for all assay methods. The 10 µg/ml concen-

tration represents the critical dose that separates metalaxyl-sensitive and -insensitive isolates of *P. infestans*.

Tuber disk assay. The tuber disk method of Kadish and Cohen (15) was used. A susceptible potato cultivar, Bintje, grown from tissue culture and provided by E.I. DuPont de Nemours, was grown in the greenhouse without any fungicide treatment. Disks were cut from the Bintje tubers with a sterile cork borer (20 × 3 mm) and placed in 90-mm-diameter petri dishes on filter paper wetted with either 2.5 ml of sterile deionized water (control) or a fungicide suspension. Inoculum was produced by the following procedure: sporangia were washed from culture plates and adjusted to 10⁴ sporangia per ml using a hemacytometer. To release zoospores, the sporangial suspension was chilled for 2.5 h at 6°C and incubated for at least 30 min at 25°C before inoculation. Inoculum consisted of a 25-µl droplet of a suspension of sporangia and zoospores. The droplet was deposited in the center of three tuber disks per petri dish using a micropipette. There were three petri dishes per treatment. Inoculated tuber disks were incubated in a dual program illuminated incubator (818, SAS Scientific Apparatus Service) at 15°C in 12 h of light per day. The response to different fungicides was measured using a scale adopted from Deahl et al. (8): % lesion development = (average lesion diameter on tuber disk with fungicide)/(average lesion diameter on tuber disk without fungicide) × 100. This experiment was completed twice under the same conditions.

Floating leaflet disk assay. The technique described by Kadish et al. (16) was utilized in this experiment. Leaflet disks (20 mm diameter) cut with a sterile cork borer from 6- to 8-week-old greenhouse grown potato cv. Bintje were floated with the abaxial surface up in 90-mm petri dishes containing either sterile deionized water (control) or suspensions of different fungicide concentrations. Inoculum preparation and inoculation procedures were the same as for the tuber disk assay (three petri dishes with four leaflet disks each). Inoculated floating disks were then incubated in the light (16-h day, intensity 75,000 lx) at 15°C for 5 to 10 days. The percentage of each leaflet disk showing lesion development was rated using a scale adopted from Deahl et al. (8). Lesion development greater than 20% indicated fungicide sen-

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sitivity. The assay was conducted twice under the same conditions.

Detached-leaf assay. The technique published by Goth and Kean (13), developed to evaluate late blight resistance in potato and tomato, was modified for use in this study. Primary leaves from the third to the sixth node of potato plants cv. Bintje were excised at the stem. Petioles were inserted into 14 × 100 mm floral aqua tubes (from local florist) containing 9 ml of sterile distilled water. The exposed leaves, with petioles sealed in the aqua tubes, were placed with abaxial sides down on galva-

nized metal hardware cloth (12 × 12 mm mesh). The supporting hardware cloth was then placed 4 cm above distilled water (2 cm deep) in 30 × 12 × 8 cm plastic boxes with tight-fitting lids. Inoculum preparation was the same as for the tuber disk assay, and the inoculation procedure was as follows: a 12-mm-diameter filter paper disk was dipped for 3 min in an inoculum solution of 10⁴ sporangia per ml, then placed on each leaflet from each leaf. Each treatment included three inoculated leaflets per leaf (three leaves per fungicide). Controls were inoculated with sterile deionized

water. Inoculated leaflets were then incubated at 15°C in a 12-h daylight regime. Lesion development was usually evident 5 to 7 days after inoculation. The rating scale was the same as for the floating leaflet disk assay, i.e., it was based on the percentage of leaflet area showing lesion development. The experiment was repeated once.

Statistical analyses. Analyses were performed using three-way ANOVA with assay methods, fungicides, and isolates as factors. Mean values were separated by Fisher's least significant difference (LSD) using the GLM procedure of SAS (SAS Institute, Cary, NC). If *F* values were significant, means were separated by LSD.

Table 1. Summary of statistical analysis for *P* value using GLM procedure of SAS to compare different isolates

Source	df	Sum square	Mean square	<i>F</i> value	Pr > <i>F</i>
Isolate	3	10,425.611	2,606.27	125.73	0.0001 ^a
Chemical	3	1,144.85	381.60	23.17	0.0001
Isolate × chemical	9	10,288.84	857.40	52.06	0.0001
Error	159	2,618.55	16.46		
Total	319	121,769.48			

^a *P* = 0.05, significant at the 0.05 level.

Fig. 1. Sensitivity of different isolates of *Phytophthora infestans* to metalaxyl using different assay methods. Means within assay method with the same letter are not significantly different according to Fisher's test at *P* = 0.05.

Fig. 2. Sensitivity of different isolates of *Phytophthora infestans* to oxadixyl using different assay methods. Means within assay method with the same letter are not significantly different according to Fisher's test at *P* = 0.05.

RESULTS AND DISCUSSION

Results indicated there was a highly significant difference among the isolates in response to the fungicides as measured by percent lesion development (Table 1). The metalaxyl-insensitive isolates were also insensitive to oxadixyl, and the metalaxyl-sensitive isolates were also sensitive to oxadixyl. All assay methods differentiated among the phenylamide sensitivities of different isolates of *P. infestans*, and there was a significant difference between sensitive and insensitive isolates (Figs. 1 and 2). Based on the mean value of lesion development, results indicated that the reactions of different isolates of *P. infestans* to chlorothalonil and cymoxanil were generally similar (Figs. 3 and 4).

There was a highly significant difference among assay methods and between the fungicides and the isolates used in this study (Table 2). There were no significant differences between the detached leaf and tuber disk assays as measured by percent lesion development. Both of these assay methods were different from the floating leaflet disk method for the cymoxanil experiment (Fig. 4).

In all assays, *P. infestans* isolates were sensitive to chlorothalonil and cymoxanil; however, sensitivity and insensitivity responses to phenylamide were consistent. These results confirm other findings (9,12,18,21).

The metalaxyl-insensitive isolates also were insensitive to oxadixyl, which indicates the existence of cross-resistance between these two systemics since the US-8 genotype with no prior exposure to oxadixyl (not yet registered in the United States) was also insensitive to this fungicide. By contrast, chlorothalonil affects many cellular processes, and resistance to this fungicide in *P. infestans* has not been reported, although it has been used extensively around the world (17). Cymoxanil has been used intensively for more than 20 years in Europe and Africa, in mixtures with fungicides having different modes of action, and no resistance has been reported (11,14,22). Our study supports these findings. This is important, since Vanderplank (23) describes *P. infestans* as a complex

pathogen with high genetic variability and a high potential for resistance development.

The newly introduced modified detached leaf technique yielded the most reliable assay of *P. infestans* sensitivity to the fungicides tested. The environmental conditions associated with this technique were favorable for fungal growth (100% RH and 15°C). The tuber disk assay showed similar results, which supports those obtained using the modified detached leaf assay. It appears, based on this study, that any results obtained using the floating leaflet disk method (currently a commonly used laboratory assay) should be confirmed by other assay methods such as those

tested in this study. The results of the leaflet disk assay indicate that by itself it is not reliable for testing the sensitivity of *P. infestans* to cymoxanil.

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Assay method for chlorothalonil

Fig. 3. Sensitivity of different isolates of *Phytophthora infestans* to chlorothalonil using different assay methods. Means within assay method with the same letter are not significantly different according to Fisher's test at $P = 0.05$.

Assay method for cymoxanil

Fig. 4. Sensitivity of different isolates of *Phytophthora infestans* to cymoxanil using different assay methods. Means within assay method with the same letter are not significantly different according to Fisher's test at $P = 0.05$.

Table 2. Summary of statistical analysis for P value using the GLM procedure of SAS to compare assay methods

Source	df	Sum of square	Mean square	F value	Pr > F
Method	3	148.78	148.78	2.04	0.0001 ^a
Fungicide	3	3,086.65	1,028.88	14.13	0.0001
Isolate	2	5,176.40	1,725.55	84.94	0.0001
Method × isolate	5	5,790.91	482.57	29.30	0.0001
Error	63	538.98	72.20		

^a $P = 0.05$, significant at the 0.05 level.

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