Resistance of *Peronospora tabacina* to Metalaxyl in Texas and Mexico

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ABSTRACT

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Isolates of *Peronospora tabacina* were collected and tested for metalaxyl resistance in Kentucky during 1979–1987, southwestern Texas during 1983–1987, and Veracruz, Mexico during 1985–1987, using a detached leaf assay. The minimum inhibitory concentration (MIC) that prevented sporulation of all Kentucky isolates was 0.066 μg/ml. The Texas 1985 isolate was three times more sensitive to metalaxyl than Ky 1979. Reisolation of the Tx 85 isolate from leaves treated with the highest concentration of metalaxyl that allowed sporulation indicated an increase in the level of resistance. However, because of the approaching tobacco-growing season, it was necessary to freeze the isolate. When further experiments were conducted after the growing season, the isolate recovered from storage was as sensitive as the Ky 79 isolate. Isolates collected in all years from the gulf zone of Veracruz, Mexico were determined to be highly resistant. The MIC ranged from 12.5 to 27 μg/ml, and chlorotic lesions developed at concentrations as high as 66.6 μg/ml. With this level of sensitivity, metalaxyl is no longer an effective control. The proximity of metalaxyl-resistant *P. tabacina* isolates to the U.S. and Canadian tobacco production areas merits the continual monitoring of isolates in Texas and Mexico, as well as development of effective strategies to control such isolates should they arrive.

Metalaxyl is a systemic fungicide active against pathogenic fungi in the order Peronosporales (14). Its effectiveness against blue mold of tobacco, caused by *Peronospora tabacina* Adam, has made metalaxyl the primary fungicide for the control of this fungus (1). However, metalaxyl-resistant isolates among the Peronosporales have been reported from various parts of the world (2,3,5,11). Moreover, the resistance to metalaxyl in some of these isolates also makes them more tolerant to other *N*-phenylamide systemic fungicides, thus narrowing the choice of effective fungicidal strategies for control (3).

Resistance of *P. tabacina* to metalaxyl was first noted on cigar tobacco in Central America in the early 1980s (15). More recently, epidemics caused by resistant isolates in Mexico (*personal communication*) and Cuba (8) have caused significant economic losses in these tobacco-producing areas. Furthermore, a wild species of tobacco (*Nicotiana repanda*) present in southern Texas has been shown to be an ideal host for *P. tabacina* (9,10). The proximity of Mexican and Cuban metalaxyl-resistant isolates to areas of tobacco production, and the presence of a native species of *Nicotiana* in Texas, may pose a threat to the United States tobacco crop. Consequently, a knowledge of the location and level of resistance in these isolates is very important to the tobacco industries of the United States, Mexico, and Canada. The objective of this study was to determine the sensitivity of *P. tabacina* isolates to metalaxyl from southwestern Texas and Veracruz, Mexico. A preliminary report was presented earlier (12,16).

MATERIALS AND METHODS

The bioassay used to determine the sensitivity of *Peronospora* isolates to metalaxyl was developed at our laboratory and was described previously (13). In summary, seedlings of *Nicotiana tabacum* L. 'Ky 14' were grown in the greenhouses of the University of Kentucky (Lexington). Leaves were preconditioned, detached, and allowed to take up various concentrations of metalaxyl prior to inoculation with isolates of *P. tabacina* (13). Isolates from wild tobacco in Texas and cultivated tobacco in Mexico and Kentucky were compared with isolate Ky 79, which is metalaxyl sensitive at a minimum inhibitory concentration (MIC) for sporulation of 0.066 μg/ml (13). Due to quarantine restrictions, separate tests were conducted at Lexington, KY and at San Andres Tuxtla, Veracruz, Mexico. Initial tests also were conducted in Texas.

Texas isolates were collected from 1982 to 1987 on *N. repanda* growing wild along the Frio and Sabanal river basins in Uvalde county in Texas (W. Nesmith, *unpublished*). Plants with chlorotic or systemic lesions were collected and sporulation was induced under dark and moist conditions in plastic bags at 20 C. Approximately 25 sporulating leaves then were placed in 9-cm plastic petri dishes with wet filter paper and shipped to Lexington, KY via overnight mail. Upon arrival, isolates were immediately inoculated onto *N. repanda* plants (four to six leaves, grown in the greenhouse). A portion of the original sporangiospores were stored in liquid nitrogen (5), as were some from the first generation produced in the laboratory. For each year of collection, a composite of sporangiospores from lesions was then used for all further experiments; they were designated Tx 82, Tx 83, etc. The isolates were maintained on *N. repanda* in separate growth chambers (12-hr photoperiod, 100 $\mu E \cdot m^{-2} \cdot s^{-1}$, at 21 C) and transferred weekly. Periodically, fresh sporulating lesions were placed between layers of filter paper in petri dishes and frozen at approximately −20 C for up to 6 mo.

Isolates from Mexico, denoted A, B, C, and D, were collected in 1985–1987 from cigar tobacco, cultivated for commercial or research purposes at the Tabacos Mexicanos (TABAMEX) Experimental Station, San Andres Tuxtla, Veracruz, Mexico. The isolates were collected from chlorotic lesions on plants growing in areas where metalaxyl was not controlling blue mold. Due to quarantine restrictions, these isolates were tested at the TABAMEX site. Due to time constraints, inoculations of experimental treatments were made with sporangiospores harvested from sporulating lesions occurring in the field, greenhouse, or seedbeds rather than from isolates increased in the laboratory.

Prior to conducting the detached leaf assay to determine metalaxyl sensitivity, preliminary experiments were conducted in Texas to obtain information on the sensitivity of *P. tabacina* collected from *N. repanda*. These tests were performed in growth chambers (12-hr photoperiod at 20 C) at the Texas A&M Research and Extension Center in Uvalde. The foliage of Ky 14 seedlings, which had been grown and preconditioned at Lexington, KY as per the bioassay procedure, but without detachment, was sprayed with metalaxyl

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(Ridomil 2E) concentrations of 0, 0.066, 1, 10, or 100 μg a.i./ml to runoff 24 hr prior to inoculation. Each treatment was applied to six to eight plants and was replicated three times in a completely randomized design. *P. tabacina* was inoculated to treated plants at a concentration of 5×10^4 sporangiospores per milliliter. The inoculated plants were incubated as previously described (13). Disease was evaluated by counting the number of plants with sporulating lesions 7–10 days after inoculation. In Mexico, preliminary evaluations were conducted on untreated field plots or on plots treated with Ridomil 2E at 2.2 kg/ha preplant incorporated at transplant time. Each plot consisted of three rows of 20 plants each replicated four times in a randomized complete block. The number of naturally infected chlorotic lesions per plot was counted.

Tests of the Texas isolates in Lexington followed the protocol of Reuveni et al (13). However, because the Mexican isolates were all tested on site at the TABAMEX laboratory, some modifications were made. Leaf material for the Mexican assays was treated with metalaxyl in Lexington, immediately packaged in plastic petri plates with filter paper inserted, and flown directly to TABAMEX. The time from fungicide treatment to fungal inoculation of treated leaves was less than 48 hr. Upon arrival at the TABAMEX laboratories, the leaves were exposed for 3 hr to flourescent light at approximately 125 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 22–25 C. Inoculum was collected from the field and prepared as previously described (13). Six 3-μl drops of a 50,000 sporangiospore/ml suspension were applied to each detached leaf. Following inoculations, the leaves were misted with distilled water and placed in the dark for 20 hr at 18–25 C. The leaves were then transferred to petri dishes (with paper filter disks saturated in 1 μg/ml kinetin solution) and incubated at 18–25 C, 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ in a 12-hr photoperiod. Seven days after inoculation, disease was evaluated by counting the number of chlorotic and sporulating lesions on each leaf. Fourteen metalaxyl concentrations (treatments), ranging from 0 to 100 μg/ml, were tested. Each laboratory sensitivity assay was replicated at least four times and most were repeated two or more times. Additionally, germination of sporangiospores was monitered for each experiment by examining 10 μl of an aqueous suspension of sporangiospores incubated for 20 hr at 18 C in the dark.

Table 1. Mean number of Ky 14 seedlings with blue mold lesions following treatment with metalaxyl and inoculation with *Peronospora tabacina* isolate Tx 85[a]

Metalaxyl concentration (μg/ml)	Days after inoculation	
	7	10
0	8.0[b]	8.0[b]
0.066	3.3[b]	8.0[b]
1	0	7.0
10	0	2.3
100	0	0

[a] Foliar application of Ridomil 2E to 6-wk-old plants, 24 hr prior to inoculation, eight per concentration.
[b] Sporulation was present.

RESULTS

With the exception of the isolate collected in Texas in 1985, all Texas and Kentucky isolates were sensitive to metalaxyl at an MIC that prevented sporulation of 0.066 μg/ml. However, in preliminary tests conducted in Texas with the 1985 isolate, chlorotic lesions were produced on approximately 25% of the plants sprayed with 10 μg/ml of metalaxyl and on most of the plants sprayed with lower concentrations (Table 1). Sporulation was not evident above the level for the previous years. Lesion development was slower on the treated leaves, and symptoms did not become evident until the 10th day after inoculation. In the more sensitive detached leaf assay, the isolate from 1985 sporulated on leaves treated with metalaxyl at a concentration of 0.1 μg/ml. Further tests using the sporangiospores collected from leaves treated with the highest concentration of fungicide that allowed sporulation in the previous test, indicated that sporulation now occurred at levels as high as 3.33 μg/ml (Table 2). Due to local regulations involving the possible release of resistant propagules during the growing season, the experiments were delayed for several months until the commercial crop had been harvested and the isolate was frozen in liquid nitrogen. When the isolate was recovered after 5 mo, the level of sensitivity had decreased to 0.066 μg/ml.

Observations and preliminary tests in Mexico during 1985 and 1986 indicated no difference in lesion numbers between metalaxyl-treated and nontreated test plots. All Mexican isolates tested exhibited extremely high levels of resistance to metalaxyl (Table 3). Isolate A had an MIC of 10–15 μg/ml. At all concentrations below this level, the fungus sporulated. Even though sporulation was completely inhibited above 10 μg/ml, small chlorotic lesions were present up to a concentration of 66.6 μg/ml of metalaxyl. Isolate B had a slightly higher level of resistance, with an MIC of 15.0 μg/ml. However, chlorotic lesions were visible only up to a concentration of 20 μg/ml. Isolate C, which was collected later in the year, had an MIC of 20.0 μg/ml (Table 3). At the highest concentration of metalaxyl tested in this case, 25 μg/ml, chlorotic lesions were present. Isolate D had an MIC between 15 and 27 μg/ml. Leaves inoculated with these isolates and treated with concentrations up to 66 μg/ml of metalaxyl exhibited chlorotic lesions. Germination of freshly harvested sporangiospores from Texas and Kentucky isolates were all high, 70–81% on depression slides. Isolates from Mexico varied greatly in their germination ranging from 5 to 80%.

DISCUSSION

This study is the first to determine the level of metalaxyl resistance present from isolates of *P. tabacina* collected from wild tobacco in Texas (1983–1987) and the Gulf zone region of Mexico (1986–1987). Levels of sensitivity were determined by the detached leaf assay

Table 2. Severity of blue mold on detached tobacco leaves treated with metalaxyl and inoculated with sporangiospores of *Peronospora tabacina* isolates Ky 79 and Tx 85[a]

Metalaxyl concentration μg/ml	Isolate					
	Ky 79		Tx 85[b]			
	Original		Original		Increased[c]	
	Disease[d]	Sporulation[d]	Disease	Sporulation	Disease	Sporulation
0	3.00	4.00	3.00	4.00	3.00	4.00
0.033	1.25	1.00	1.50	2.00	...	...
0.066	0	0	1.00	1.00	1.50	0.50
0.10	0	0	1.00	1.00	0.50	Tr[e]
0.33	...	...	...	...	0.50	Tr
0.66	...	...	...	...	0.75	Tr
1.00	...	...	0	0	0.50	Tr
3.33	...	...	...	...	0.50	Tr

[a] Four leaves per concentration per isolate.
[b] Tx 85 was a composite of isolates collected from six locations in Uvalde county in Texas.
[c] Sporangiospores used in this assay were collected from the original Tx 85 assay at metalaxyl levels above 0.033 μg/ml.
[d] Severity rating based on a 0–4 scale: 0 = 0% infection or sporulation; 1 = 1–25% of leaf area chlorotic or sporulating; 2 = 26–50% of leaf area chlorotic or sporulating; 3 = 51–75% of leaf area chlorotic or sporulating; and 4 = 76–100% of leaf area chlorotic or sporulating.
[e] Tr = trace of sporulation.

developed by Reuveni et al (13).

Preliminary tests in Texas in 1985 suggested the Texas isolate had a greater tolerance to metalaxyl than samples from previous years. When originally tested, the MIC for this composite isolate was only slightly higher than that of Ky 79. As with other reports of resistance (2,3), the MIC level increased with each test in which metalaxyl-exposed sporangiospores were used. This procedure allowed us to increase the level of resistance of Tx 85 up to 100 times the level present in Ky 79 (Table 2). It should be noted that the Ky 79 isolate has never decreased in sensitivity using this assay (13). It was not possible to determine if greater decreases in sensitivity to metalaxyl of the 1985 isolate would have occurred, for fear of contaminating the commercial crop. As the level of metalaxyl resistance was high, it was decided to postpone further testing until all burley tobacco had been harvested. Therefore, the composite isolate was frozen at −180 C (liquid nitrogen) and −20 C for 6 mo. When the isolate was recovered from both storage temperatures, resistance was not found in isolates from Texas in that year. However, the level of germination of this inoculum was extremely low, 0.5–12%, and the resistant portion of the inoculum may have been destroyed with freezing. A similar phenomenon was observed for resistant isolates discovered in North Carolina in 1982 (1). In unpublished data from experiments in Mexico with the resistant isolates from San Andres, short-term freezing (1 wk) had no effect on the level of resistance present. Studies using spores frozen for longer times did not yield conclusive results. Clearly, the effect of long-term freezing on resistant propagules needs to be examined further. The effect could be significant because airborne spores are probably frozen during movement along the boundary layer (7).

It has been postulated that the inoculum for blue mold epidemics in this hemisphere may share a common source (17). Once resistance of *P. tabacina* to metalaxyl had been discovered in Texas, we felt it necessary to determine the level of resistance present in commercial production areas where metalaxyl had previously not been effective as a fungicide. The San Andres Tuxtla region of Veracruz, Mexico was chosen because treatments with metalaxyl had recently failed to control blue mold in this area. Severe damage from blue mold of metalaxyl-treated fields of cigar tobacco occured in 1984 and 1985, amounting to a 50% reduction in yield, even though in previous years metalaxyl (Ridomil Mz58) had controlled the disease (*personal communication*).

Testing of the isolate present in January of 1986 confirmed that a highly resistant strain of *P. tabacina* existed in the San Andres area of Veracruz, in agreement with preliminary tests and field observations. A direct comparision of fungicide sensitivity of the Mexican isolates to the Ky 79 isolate was not possible under the conditions present in Mexico due to quarantine restrictions imposed by both U.S. and Mexican authorities. However, the same treatments were performed under similar conditions at both locations.

With resistance present in San Andres Tuxtla, Mexico the benefit of continual use of metalaxyl at that location is questionable. Experiments conducted at San Andres Tuxtla in 1985–1987 by University of Kentucky and TABAMEX reavealed that protection was similar for both the Ridomil Mz58 (metalaxyl plus mancozeb) and Manzate 200 (mancozeb). In those experiments, plots treated with metalaxyl alone (Ridomil 2E) had blue mold equal to that in untreated checks, but much more disease than those plots receiving Ridomil Mz58 or Manzate 200 (*unpublished*). On most commercial farms in the San Andres area, Ridomil Mz58 is sprayed biweekly and mancozeb alone is sprayed weekly. Therefore, protection of the tobacco crop by fungicides in San Andres is due solely to the mancozeb in the weekly application and Ridomil Mz58 and not by metalaxyl in Ridomil Mz58.

The proximity of metalaxyl-resistant Mexican isolates to the native species of *N. repanda* present in both southern Texas and the northern Mexico region should be cause for concern of all tobacco growers in the United States and Canada. The discovery in 1985 of a metalaxyl-resistant isolate in Texas raises a question as to how it arrived there. Metalaxyl-resistant strains of blue mold have been known to exist in areas of Honduras and Guatemala since the early 1980s (15). The northward movement and establishment of a resistant isolate in Mexico and Cuba in the mid 1980s could explain its arrival into Texas. Since epidemics of blue mold in Kentucky in 1983–1986 have a possible connection to the Texas inoculum source (6,7), the possibility exists that a link between San Andres and Kentucky, as well as the tobacco production areas of the United States, may one day be established. For this reason, the further testing of metalaxyl sensitivity in isolates collected from the same year in Veracruz, Texas, and Kentucky is crucial for understanding future epidemics as well as prevention of fungicide resistance.

Table 3. The average number of chlorotic and sporulating lesions caused by *Peronospora tabacina* on detached tobacco leaves treated with metalaxyl and inoculated with Mexican isolates[a]

Concentration $\mu g/ml$	Isolate A[b]		Isolate B[c]		Isolate C[d]	
	Lesions[e]	Sporulation[f]	Lesions	Sporulation	Lesions	Sporulation
0	5.2	5.2	6	6	5.7	4.3
0.066	6	6	···	···	4.0	3.5
1.0	5.3	5.3	···	···	4.0	4.0
3.33	5.5	5.5	···	···	···	···
5.0	4.0	3.0	···	···	4.1	3.9
6.66	3.3	3.3	···	···	···	···
7.5	3.5	2.8	···	···	···	···
10.0	3.8	2.4	5	5	4.8	4.3
15.0	3.8	0	3	1.7	3.0	1.4
20.0	2.5	0	1.0	0	1.7	1.3
25.0	···	···	0	0	0.6	0
33.3	1.3	0	···	···	···	···
66.6	0.7	0	···	···	···	···
100.0	0	0	···	···	···	···

[a] Four leaves per concentration per isolate.
[b] Isolate collected on 14 January 1986 from metalaxyl-treated cigar tobacco at the TABAMEX research plantation, San Andres Tuxtla, Mexico.
[c] Isolate collected on 28 January 1986 from TABAMEX experimental plots.
[d] Isolate collected 20 March 1986 from TABAMEX experimental plots.
[e] Average number of chlorotic lesions per leaf present from four replications.
[f] Average number of sporulating lesions per leaf present from four replications.

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