Induction of Systemic Resistance in Tobacco Against Metalaxyl-Tolerant Strains of *Peronospora tabacina* and the Natural Occurrence of the Phenomenon in Mexico

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ABSTRACT

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Field experiments were conducted in San Andrés Tuxtla, Veracruz, Mexico, during 1986 and 1989 to test the effectiveness of induced systemic resistance (immunization) against metalaxyl-tolerant strains of *Peronospora tabacina,* causal agent of tobacco blue mold. Plants of *Nicotiana tabacum* cultivar Jaltepec were stem-injected with sporangiospores of metalaxyl-tolerant strains of *P. tabacina.* Half of the plants were treated with metalaxyl. The number and size of blue mold lesions that developed because of infections caused by ambient inoculum were significantly fewer and smaller on plants injected with *P. tabacina* as compared with noninjected stem controls, regardless of metalaxyl applications. Some vigorously growing, naturally infected plants with necrotic stem lesions but markedly reduced foliar lesions of blue mold were observed in commercial fields surrounding the experimental plots. Stem necrosis was consistently associated with these plants. Stem necrosis was not detected in plants infected heavily with foliar lesions of blue mold. Several attempts to isolate the causal agent of necrosis associated with acquired resistance to blue mold failed. Only live sporangiospores of *P. tabacina* produced necrosis typical of that found in the naturally protected plants. Natural immunization apparently occurs at scattered locations on the gulf coast of Mexico and is associated with vigorous growth. Furthermore, the endemic nature of the disease, presence of sexual stages of the fungus, long-term moisture in the field, and high constant disease pressure created conditions very favorable for the development of tolerance to any protective agent, such as metalaxyl, with a single site of action; however, development of tolerance to immunization has not yet been observed.

The downy mildew disease, blue mold, incited by *Peronospora tabacina* D. B. Adam, has caused severe economic losses in tobacco (*Nicotiana tabacum* L.) (12). Losses due to this disease were estimated to exceed $250 million in the United States and Canada during the 1979 epidemic (12). In recent years, losses in the United States due to the disease have declined because of unfavorable weather conditions and extensive use of the systemic fungicide, metalaxyl. Strains of *P. tabacina* tolerant to metalaxyl, however, have been found in the Caribbean region, in several Central American countries, and on the gulf coast of Mexico (8,12,18). Metalaxyl-tolerant strains have caused serious losses to crops in the gulf zone of Mexico during the past 5 yr. Currently, fungicides do not provide acceptable control, although weekly applications of fungicides containing mancozeb are used. The sporangiospores of *P. tabacina* can travel long distances by air currents (6), and the presence of metalaxyl-tolerant strains in Mexico poses a threat to tobacco in the United States.

Development of induced resistance of tobacco foliage to blue mold was first reported on systemically stem-infected and stunted field-grown tobacco in Australia (10). Further research demonstrated that tobacco plants were systemically protected against blue mold by stem injection with sporangiospores of *P. tabacina* (2) in the greenhouse and field as well as by application of sporangiospore suspensions to the potting media used to grow tobacco (1). Protection, expressed by development of fewer and smaller lesions, was greater than 95% compared with that of controls 3 wk after stem injection; however, in all cases, plants were stunted and showed growth abnormalities. High N fertilization partially overcame stunting (7). Modification of the technique for stem injection with *P. tabacina,* which introduces inoculum specifically into the stem tissue external to the xylem, overcame stunting and resulted in immunized plants with increased growth (5,13-15). Field tests with the modified technique were conducted over a 3-yr period in Kentucky and Puerto Rico with a metalaxyl-sensitive strain of the fungus. The tests indicated that immunized plants were as well protected as plants treated with metalaxyl. Furthermore, even in the absence of disease, immunized plants in the field grew more vigorously and increased in yield by 10-25% compared with that of the controls (16). The purpose of this study was to determine the effectiveness of immunization against metalaxyl-tolerant strains of *P. tabacina* in Mexico. Preliminary results have been reported (17).

MATERIALS AND METHODS

Plants, preparation of fields, and chemical treatments. Experiments were conducted in five different field locations near San Andrés Tuxtla, Veracruz, Mexico, where metalaxyl-tolerant strains of *P. tabacina* caused severe losses during 1986 and 1989 despite extensive treatment with metalaxyl plus mancozeb. Mexican cigar-type dark tobacco, cultivar Jaltepec, commonly grown in this region, was used in experiments. In addition, several commercial fields were surveyed during 4 yr of collaborative effort with Tabacos Mexicanos (TABAMEX) for the natural occurrence of protection. Natural protection data presented here were collected from two fields in which Jaltepec was grown during 1986. Seedlings were produced in seedbeds at the locations of the experiments according to standard procedures used by TABAMEX (unpublished TABAMEX procedures for growing cigar tobacco on the gulf coast of Mexico). Seedbeds were treated with methyl bromide (45 g/m^2) and fertilizer containing N, P, and K (10:17:17) before seeding. Granular metalaxyl (12.5 kg/ha, Ridomil 5G, Ciba-Geigy Mexicana, S. A. de C. V., Mexico, D. F.)

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was applied to the seedbeds before seeding at the commercial fields. Plants in the seedbeds were treated with mancozeb (1–2 g/L, Manzate 200, du Pont, S. A. de C. V., Mexico, D. F.) twice weekly, starting 1 day after germination, and with metalaxyl (5 g/L, Ridomil Mz58, Ciba-Geigy Mexicana) 22 days after germination. Metalaxyl application was repeated after every third application of mancozeb. Fertilization rates, N, P, and K (16:9:8 + 1% $K_2Mg(SO_4)_2$, 950 kg/ha) were based on soil tests, and the fertilizers were applied before seedlings were transplanted. Seedlings (about 64 per plot) were transplanted 45 cm apart in rows spaced 90 cm apart. Plants in test plots were sprayed with mancozeb (2.5 kg/ha, Manzate 200, du Pont) until 3 wk after stem injection. At the time of stem injection, metalaxyl (Ridomil 2E, Ciba-Geigy, Greensboro, NC) was applied to soil (15 ml/L) as a control (Tables 1 and 2). Plants in commercial fields in which natural protection data were collected were treated with Manzate 200 (5 g/L) once weekly and Ridomil Mz58 every 12 days. Diazinon (50 kg/ha, Basudin 4G, Ciba-Geigy Mexicana), Orthene 75 WP (0.5–1 kg/h, Insecticides Ortho, S. A. de C. V., Mexico, D. F.), and Folimate 1200 (1 L/ha, Bayer de Mexico, S. A. de C. V., Mexico, D. F.) were applied, alternately, once weekly to control aphids and other insects during the experiments. Weed control was maintained by cultivation. Plants were topped by removing the terminal bud 45 days after transplanting. Lateral bud growth was controlled by hand. Plots and fields were not irrigated. Treatments (Tables 1–3) were arranged in randomized complete block designs with three replications.

Fungus and inducing inoculations. Sporangiospores were collected from sporulating leaves of plants in nearby commercial fields treated with Ridomil Mz58 and Manzate 200 as described earlier (14,16). Within 3 h before stem injections, sporangiospores were brushed from leaves into distilled water, cooled to 5 C, and washed on 0.8-μm Millipore filters (Millipore Corp., Bedford, MA) to remove fungicides and self-inhibitors. Germination tests indicated >85% sporangiospore viability in these preparations. Tolerance of the fungus to metalaxyl was determined by using a detached leaf assay. The minimum inhibitory concentration of metalaxyl for the spore populations used for stem injections (isolate A) was found to be 10–15 μg/ml (18). This is about 200 times more tolerant to metalaxyl than is the wild-type *P. tabacina* from the United States (18). Sporangiospore suspensions were kept in an ice bath during the period that stems were being injected.

Plants were stem-injected when they were about 20 cm in height. One milliliter of inoculum (5×10^5 sporangiospores per milliliter) was injected into the stem external to the xylem using a 1-cm^3 tuberculin syringe with a 26 G3/8 intradermal bevel needle (Becton Dickinson and Co., Rutherford, NJ). In some experiments, a second stem injection (booster) followed 1 wk after the first injection. Controls were not injected with H_2O or liquid obtained from uninoculated leaves to save time because such treatments have not induced resistance in previous greenhouse and field experiments (13,14) and in Mexico during preliminary studies. None of the plants in commercial fields in which natural protection data were collected was stem-injected.

Challenge inoculations. During the course of the experiments, abundant sporulating lesions occurred on tobacco plants at and around field locations. Therefore, blue mold developed naturally in the experimental plots and commercial fields. The source of inoculum presumably was from commercial tobacco plants treated with Ridomil Mz58 as described.

Isolation of microorganisms from tobacco stems. This test was conducted to investigate the source of the apparent natural immunization in the commercial fields. Tobacco stems with apparent systemic blue mold (naturally stem-infected and showing resistance to blue mold) and noninfected (susceptible to blue mold) tobacco stems were cut at the soil line and taken to a laboratory. Sections of stems around and including the necrotic areas were excised, surface-sterilized with 0.5% sodium hypochlorite (10% commercial bleach) for 10 min, washed with sterile distilled water twice, and plated on media. Two different media, potato-dextrose agar (39 g/L, Difco Laboratories, Detroit, MI) and nutrient agar (23 g/L, Difco) were used for culturing fungi and bacteria present in the stem. Plates (20 plates per medium) were incubated at 22 C for 7 days, and bacteria and fungal spores were collected by placing 20 ml of sterile distilled water into the plates. These microbial suspensions were diluted 1, 10, and 100×, and 1 ml of suspension was injected into stem tissue of healthy Jaltepec tobacco plants (10 plants per treatment) growing in the field as described above. Stem material obtained from naturally infected plants was ground in a mortar with 20 ml of sterile distilled water and allowed to settle. The fluid was injected into stem tissue. Plants injected with *P. tabacina* served as positive controls, and plants injected with sterile distilled water used for serial dilutions served as negative controls. Stems were examined for necrosis and evaluated 21 days after stem injections.

Evaluations. Severity of blue mold infection was determined. The number of blue mold lesions was counted, and the percentage of leaf area damaged by blue mold was estimated on all of the plants in experiments reported in Tables 1 and 2. For experiments reported in Table 3, a rating scale was used to estimate lesion

TABLE 1. Effects of stem injections with sporangial suspensions of *Peronospora tabacina* on development of blue mold in Jaltepec tobacco plants treated or not treated with metalaxyl during 1986

Treatment	Number of lesions per leaf per plant[w]	Percent leaf area with lesions per plant	N[x]
Stem-injected with *P. tabacina*[y]	1.7 b	0.8 b	164
Metalaxyl, stem-injected with *P. tabacina*[y]	1.6 b	1.1 b	162
Metalaxyl, not stem-injected[z]	5.8 a	4.2 a	181
Not stem-injected[z]	8.4 a	5.7 a	197

[w] Different letters indicate significant difference within the column according to Waller-Duncan k-ratio t test.
[x] The number of plants remaining in the experimental field. Although the number of plants at the start of the test was the same for each treatment, some plants were lost during the course of experiment.
[y] Data include plants without stem necrosis.
[z] Data include plants with stem necrosis possibly due to natural infection.

TABLE 2. Effects of stem necrosis developed either naturally or because of stem injections with *Peronospora tabacina* on the development of blue mold on Jaltepec tobacco during 1986

	Stem necrosis			No stem necrosis		
Treatment	N[y]	Mean number of lesions per leaf per plant[z]	Percent leaf area with lesions per plant	N	Mean number of lesions per leaf per plant	Percent leaf area with lesions per plant
Stem-injected with *P. tabacina*	159	1.1 bc	0.5 a	5	23.5 a	14.2 a
Metalaxyl, stem-injected with *P. tabacina*	151	0.7 ab	0.3 a	11	15.0 a	12.2 a
Metalaxyl, not stem-injected	137	0.5 a	0.2 a	44	22.1 a	16.5 a
Not stem-injected	143	1.7 c	0.1 a	54	26.1 a	17.0 a

[y] Number of plants remaining in the experimental field.
[z] Means within a column followed by the same letter are not significantly different ($P < 0.05$) according to a t test. For each treatment, the mean for each variable for plants with stem necrosis was significantly less ($P < 0.001$) than that for plants with no stem necrosis.

area and sporulation (Table 3, footnotes w and x). At the termination of the experiments, the stem of each plant was cut to determine presence or absence of necrosis similar to that obtained after stem injection with *P. tabacina*. Natural protection data from the two commercial fields were collected from 100 plants at the center of each field. In such commercial fields, adjacent plants within a row were rated for disease and then cut to determine presence or absence of necrosis in the stems. Data were subjected to analysis of variance and *t* tests. Waller-Duncan *k*-ratio *t* test was used for separation of means when appropriate.

RESULTS

Sporulating blue mold lesions were evident at all stages of tobacco development in surrounding fields. Weather conditions remained favorable for development of disease; therefore, no additional efforts were necessary to elicit and enhance disease. Results of detached leaf assays, as reported previously (18), indicated that the isolate present in this area was 200–300 times more tolerant to metalaxyl than was the wild type present in the United States.

Stem injections with *P. tabacina* resulted in significant protection in Jaltepec tobacco (Tables 1–3). Both the number of lesions and the percentage of infected leaf area were significantly less than that of the controls. Metalaxyl treatment did not affect disease development, regardless of whether the stems were injected or not injected (Tables 1–3). Longitudinal cutting of the stems of some of the experimental plants that had not been stem-injected revealed necrosis, which resembled that associated with *P. tabacina* stem injections. A few plants stem-injected with *P. tabacina* did not exhibit necrosis. Stems of each plant in the experimental plot were cut and examined for necrosis. Plants with necrosis on the stem were significantly protected compared with plants that did not have any evident necrosis, regardless of any other treatment (Tables 1–3). Small, chlorotic, sparsely sporulating lesions developed on plants with visible stem necrosis.

Most plants in commercial fields in 1986 were covered with abundantly sporulating lesions, but some plants grew vigorously and had only small, chlorotic, sparsely sporulating lesions. The plants protected against blue mold had stem necrosis, whereas the heavily diseased plants did not (Table 4, Fig. 1). The necrosis resembled that caused by stem injection with sporangiospores of *P. tabacina*. Plants with stem necrosis consistently appeared taller and more vigorous than those without necrosis. Usually protection associated with stem necrosis occurred on plants scattered throughout the tobacco-growing areas of San Andrés Tuxtla during the period 1986–1989, but occasionally extensive protection was observed in some fields in which over 90% of the plants had stem necrosis. These were fields that usually had extensive blue mold damage in previous seasons. Blue mold activity in such fields was restricted, and plants were protected similarly to plants having stem necrosis in Table 4 (data not presented). Micro- and macroscopic investigations to detect the cause of natural stem necrosis indicated that only stem injection with live sporangiospores of *P. tabacina* produced necrosis similar to that observed in stems of naturally protected plants in the field. Because *P. tabacina* is an obligate parasite and the fungus was not detected in the stem, we were unable to prove that the natural stem necrosis was caused by *P. tabacina*. However, in separate experiments, the injection of stems with *P. parasitica* and other tobacco pathogens did not induce systemic resistance to blue mold or produce stem necrosis characteristic of that produced by injection with *P. tabacina*.

TABLE 3. Effects of stem injection with sporangiospores of *Peronospora tabacina* on the development of blue mold on Jaltepec tobacco during 1989[v]

Field	Treatment	Disease rating[w]	Sporulation[x]
1	Metalaxyl	2.5 a[y]	3.1 a
	Control[z]	3.0 a	2.9 a
	Stem-injected with *P. tabacina*	1.0 b	0.6 b
2	Metalaxyl	2.8 a	2.9 a
	Control	2.7 a	2.9 a
	Stem-injected with *P. tabacina*	0.3 b	0.2 b
	Stem-injected and metalaxyl-treated	0.7 b	0.2 b

[v] Data were obtained from approximately 120 plants per treatment per field.

[w] Disease was rated according to a 0–10 scale, where 0 = no visible lesions and 10 = 100% of leaf surface covered by chlorotic and necrotic lesions.

[x] Sporulation was rated according to a 0–4 scale, where 0 = no evident sporulation and 4 = lesions heavily sporulating.

[y] Data include some plants injected with sporangiospores that did not develop stem necrosis and some plants not injected that developed stem necrosis. Means within a column followed by the same letter are not significantly different according to a Waller-Duncan *k*-ratio *t* test.

[z] Control plants were not stem-injected or treated with metalaxyl.

DISCUSSION

This study indicated that tobacco plants can be protected against strains of *P. tabacina* tolerant to metalaxyl by stem injections with the sporangiospores of the same isolate of the fungus from the same field. The protection was very effective, appeared to occur naturally, and was naturally distributed among the tobacco-growing areas on the gulf coast of Mexico.

Because of Mexican quarantine regulations, we were not able to use the inducer isolates used in previous experiments in the United States; however, results indicated that metalaxyl-tolerant *P. tabacina* effectively induced resistance. Immunized plants were taller than the control plants; however, this might have been due to the presence of blue mold on controls. Stem injection experiments with burley tobacco cultivars were unsuccessful under the growing conditions in San Andrés Tuxtla. The burley tobacco cultivar we used (Ky 17) was severely damaged by tobacco etch virus. The effectiveness of immunization against metalaxyl-tolerant strains of *P. tabacina* on burley tobacco cultivars more resistant to tobacco etch virus is being tested currently.

The wide distribution of stem necrosis and protection in research plots led to investigation of several nearby commercial fields for evidence of the same phenomenon. In all cases examined, protection against blue mold was associated with stem necrosis. Development of natural protection in infected plants growing in commercial fields was observed in Australia (10); however, such plants were severely stunted. Although growth measurements were not collected in San Andrés Tuxtla, plants with stem necrosis were apparently the most vigorous plants at all locations. Although induced systemic resistance has been reported for many other host-parasite interactions (3–5), reports of natural occurrence in fields are rare. In addition, occurrence of natural protection in the fields with the most blue mold activity in previous seasons may indicate that this mechanism of natural protection evolved during the years of interaction between the plant and the pathogen.

TABLE 4. Effect of naturally occurring stem necrosis on blue mold in Jaltepec tobacco plants grown in commercial fields during 1986

	Plants with stem necrosis		Plants with no evidence of stem necrosis	
Field	Number of plants[y]	Percent leaf area with lesions per plant[z]	Number of plants[y]	Percent leaf area with lesions per plant[z]
1	57	3.1	43	34.8
2	55	2.8	45	27.8

[y] One hundred plants in a row in each field were rated first for disease. Plant stems then were cut to detect necrosis.

[z] Analysis of variance indicated a significant ($P < 0.001$) difference in the percentage of leaf area with lesions for plants with stem necrosis compared with that of plants without stem necrosis. Significant difference between the two fields was not observed.

Investigations of stem necrosis indicated that none of the organisms isolated from naturally infected stems produced the same type of necrosis produced by *P. tabacina*. Previous research indicated that plants stem-infected by application of sporangiospore suspensions to soil were protected in the greenhouse (1) and field (9); however, such plants were severely stunted. Sporangiospores present in the soil may penetrate stems and cause necrosis similar to stem-injected plants. However, the disease also may develop on the leaves at early stages of plant growth and enter the stems through systemic development as reported for *N. repanda* (11). The occurrence of necrosis external to xylem of stems is apparently important for the development of systemic resistance without stunting (14).

No tolerance of the fungus to immunization was detected under the growing conditions in San Andrés Tuxtla. At this location, tobacco and blue mold are present in the field throughout the year. Oospores are abundantly present on the sporulating leaves, and weather conditions are favorable for most of the year for the development of disease. These factors increase pressure for the development of resistance in pathogens to systemic fungicides with a single site of action such as metalaxyl. Although natural immunization was probably widely distributed on the gulf coast of Mexico, a first occurrence in 1986 is unlikely. Natural immunization has been active at least during the 4-yr period of collaborative studies with TABAMEX, and possibly present for many years before 1986, so the very high degree of protection achieved by plant immunization is stabile under the most disease-conducive conditions, and the fungus is not readily able to overcome the natural defense mechanism(s) activated by immunization.

Fig. 1. Occurrence of blue mold and stem necrosis in Jaltepec tobacco grown in a commercial field in Mexico. Plants were grown within the same row in close proximity to one another. The plants were treated by the farmer with Ridomil MZ58 and other chemicals as described in Materials and Methods. Plants were not stem-injected with *Peronospora tabacina*. **A,** Two adjacent plants, one of which appears to be immunized (right) and the other nonimmunized (left). **B,** A plant without stem necrosis that is heavily infected. **C,** Stems of the plants shown in **A** with presence (right) and absence (left) of necrosis. **D,** A plant with stem necrosis that is well protected.

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