Evaluation of Systemic Resistance to Blue Mold Induced in Tobacco Leaves by Prior Stem Inoculation with *Peronospora hyoscyami* f. sp. *tabacina*

Yigal Cohen and Joseph Kuć

Department of Plant Pathology, University of Kentucky, Lexington 40546. The senior author was on leave from, and has returned to, the Department of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel.

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ABSTRACT

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Conidia of *Peronospora hyoscyami* f. sp. *tabacina* (which causes blue mold of tobacco) applied to the stem-root interface zone of potted burley tobacco plants induced systemic protection of the foliage against the blue mold disease caused by the same pathogen. Neither heat-killed or sonicated conidia of *P. hyoscyami* f. sp. *tabacina* nor untreated inocula of three other tobacco pathogens or three nonpathogens induced protection against blue mold. Protection was associated with the necrosis that developed in the external phloem and cambium of the stem subsequent to the initial inoculation with *P. hyoscyami* f. sp. *tabacina*. Approximately 2 wk was required for development of ~50% protection and 3 wk for ~95%. Resistance in the leaves of protected plants was not confined to the epidermis; conidia infiltrated into leaf panels failed to produce normal lesions.

Additional key words: Nicotiana tabacum.

Induced resistance of tobacco foliage to the blue mold disease was first reported by Cruickshank and Mandryk in 1960. In both greenhouse and field experiments, they observed (2) that stem infection with *Peronospora hyoscyami* f. sp. *tabacina* markedly reduced the severity of disease on foliage subsequently challenge inoculated with the fungus (2,6). Survival of protected plants in the greenhouse requires high nitrogen fertilization and incubation at a day temperature of 29 C with high relative humidity (RH) (7). In a later study, Shepherd and Mandryk (12) reported the extracting from protected plants of water soluble compounds that inhibited both germination and sporulation of *P. tabacina*.

The severe epidemic of blue mold in the USA in 1979 encouraged us to further explore the possibilities of protecting tobacco against this disease.

MATERIALS AND METHODS

Plants. Burley tobacco, *Nicotiana tabacum* L., 'Judy's Pride,' 'Ky 14,' 'Ky 16,' 'B 21,' and hybrid B21 × L8, were used. Plants were grown in the greenhouse (20–26 C in fall and winter, 20–33 C in spring and summer, 16 hr of light per day) in 2-L pots containing Pro-Mix BX, a commercial potting mixture. Pots were watered with a fertilizer solution (14.79 cc [1 tablespoon] of 20-20-20 [N-P-K] soluble fertilizer per 3.79 L [1 gallon]) twice a week.

Inducing inoculations. Unless stated otherwise, plants were inoculated at the five- to seven-leaf stage with *P. hyoscyami* f. sp. *tabacina* (collected in Georgetown, Kentucky, in 1979), *Thielaviopsis basicola*, or *Phytophthora parasitica* var. *nicotiana* race 0 (kindly supplied by J. Hendrix, Department of Plant Pathology, University of Kentucky, Lexington). Three nonpathogens of tobacco also were tested: *Phytophthora infestans* race 0, *Pseudomonas lachrymans*, and *Colletotrichum lagenarium*. All pathogens were maintained at 25 C on appropriate agar media except *P. hyoscyami* f. sp. *tabacina*, which was maintained on tobacco plants in growth chambers at 20 C. Inoculations were done by infiltrating 0.2–0.5 ml of H_2O suspension (5×10^5 cells per milliliter) into the base of the stem, infiltrating conidial or bacterial suspensions into leaf panels, or pouring conidial suspensions on the soil at the base of the stem of potted plants.

Challenge inoculation. All challenge inoculations were done with *P. hyoscyami* f. sp. *tabacina* conidia. Freshly produced conidia at a concentration of 5×10^4 conidia per milliliter of H_2O were used in all experiments. Germinability of conidia varied from

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5 to 50% in various experiments. Suspensions of conidia were uniformly sprayed on the upper leaf surfaces, the plants were placed in a dark moist chamber at 20 C for about 20 hr, and then were transferred to a greenhouse bench or growth chamber (20 C illuminated 12 hr/day with cool-white fluorescent lights supplemented with incandescent light at ~180 μEinsteins · m^{-2} · s^{-1}). Lesion development was recorded 7 days after challenge. When separate lesions developed, the number and sizes of lesions were recorded. To describe disease severity when lesions became confluent, the percentage of leaf area infected was estimated (11). Plants were then returned to moist chambers at 20 C in the dark for 24 hr to induce sporulation. The number of conidia produced per unit of leaf surface was determined as described before (11). In some experiments a visual estimation of sporulation on a 0–4 scale was made to describe proportion of the leaf area covered with mildew (11). Experiments were repeated two or more times with either six five- to seven- leaf, or three 10- to 15-leaf replicate plants receiving each treatment.

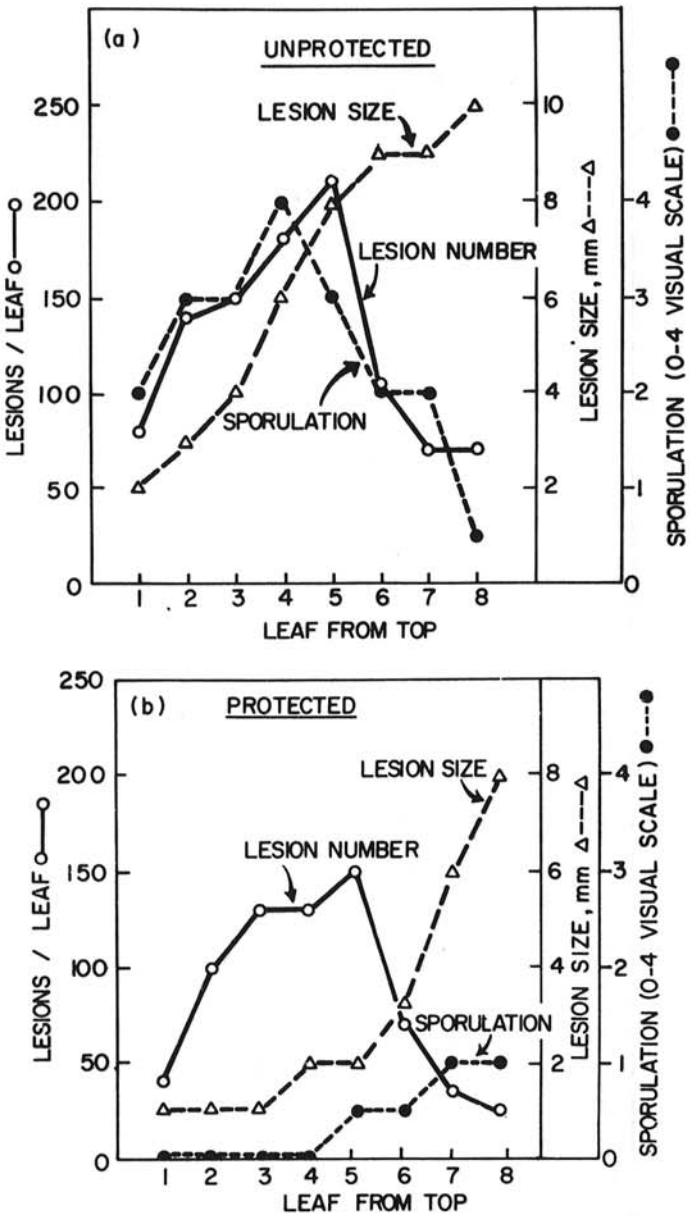

Fig. 1. Systemic protection against blue mold in tobacco cultivar Ky 16 plants against blue mold induced by prior inoculation at the root-stem interface with *Peronospora hyoscyami* f. sp. *tabacina*. **a,** Control, unprotected. **b,** Protection induced with 1 ml of a spore suspension containing 10^6 conidia per plant. Plants were challenged 16 days after induction by spraying conidia of *P. hyoscyami* f. sp. *tabacina* onto leaf surfaces.

Microscopic examination. Germination of *P. hyoscyami* f. sp. *tabacina* in situ was determined on leaf disks inoculated on the upper surface and incubated in moist chambers at 20 C in the dark for 20 hr. Disks were then placed in lactophenol for a few hours, stained with cotton blue, and mounted in lactophenol.

In some experiments epidermal strips were removed from inoculated leaves and stained as above. This technique was used mainly when lower leaf surfaces were inoculated. Other epidermal strips were stained with 1% phloroglucinol-HCl reagent to determine whether the cells were lignified.

RESULTS

Resistance induced by prior inoculation of a leaf. Leaf 3 of plants in the five- to seven-leaf stage was infiltrated with conidia of three pathogens and three nonpathogens. After 24–48 hr, severe necrosis developed in leaves infiltrated with *P. parasitica* var. *nicotiana, P. infestans, T. basicola, C. lagenarium,* and *P. lachrymans,* whereas those infiltrated with *P. hyoscyami* f. sp. *tabacina* developed chlorosis within 3 days. The plants did not show systemic protection against blue mold after challenge inoculation of upper leaves 7 days after the initial inoculation. Water extracts and sonicates of conidia of *P. hyoscyami* f. sp. *tabacina* infiltrated into leaves produced chlorosis at 24–48 hr, but did not induce systemic protection. When leaf 3 was infected with *P. hyoscyami* f. sp. *tabacina*, browning in the petiole and stem developed in about 2 wk, and systemic protection developed after an additional 3 wk (see below).

Resistance induced by prior inoculation of the stem. When infiltrated into the stem, *P. infestans, C. lagenarium, P. lachrymans,* and conidial sonicates of *P. hyoscyami* f. sp. *tabacina*, all produced restricted necrotic lesions (1–2 cm) in pith tissue, but did not induce resistance to blue mold when the leaves of the plants were challenge inoculated 1–4 wk later. *T. basicola* produced a stem canker 5–6 cm long, but also did not induce resistance. *P. parasitica* var. *nicotiana* Race 0 produced a canker 5–6 cm long, which was followed by rapid wilting. Leaves of such plants were kept in moist chambers for 7 days after the challenge inoculation. Resistance to blue mold was not induced on either detached leaves or intact plants.

Systemic resistance to blue mold was induced 2–3 wk after stems were infiltrated with viable conidia of *P. hyoscyami* f. sp. *tabacina*. Stem inoculation in all cases caused necrosis in the cambium and external phloem. Pith, cortex, and xylem were mostly free of necrosis, although in the fall and winter greenhouse-grown plants temporarily wilted at midday 4–5 wk after inoculation. Usually a positive association was obtained between protection of foliage and area of necrosis in the stem. Stem injection with *P. hyoscyami* f. sp. *tabacina*, which was feasible in plants older than the seven-leaf stage, never resulted in the development of blue mold in leaves. By studying freehand sections, we showed that necrosis did not move from the stem into the leaves. On very rare occasions, however, stem-injected plants showed systemic symptoms of the disease in the apical bud.

Resistance induced by prior inoculation of the stem-root interface zone. Conidia of *P. hyoscyami* f. sp, *tabacina* poured on the soil around the base of the stems of potted five- to seven- leaf tobacco plants induced resistance in the foliage to *P. hyoscyami* f. sp. *tabacina* (Fig. 1). Pouring the conidia onto the soil at the base of the stems of three-leaf plants caused systemic infection with blue mold. Root wounding was unnecessary for protection. As little as 1 ml of conidial suspension (5×10^3 conidia) poured onto the soil surface close to the base of the stem induced resistance. One milliliter of suspension with 5×10^4 and 5×10^5 conidia was required to induce resistance in 10- and 15-leaf plants, respectively. Histological examinations revealed that the pathogen infected the cortical tissue at the stem-root interface and produced necrosis in the cambium and external phloem tissues. Necrosis progressed upward in the stems as in stem-inoculated plants. Resistance developed gradually and usually was associated with the area of necrosis in the stem. At 1, 2, 3, and 4 wk after initial inoculation (fall and winter) necrosis in Ky 16 reached a height of 0, 4, 7, and 13 cm

in stems, and resistance (based on the leaf area infected) was about 0, 50, 90, and 95%, respectively. Use of a more concentrated inoculum (up to 10^6 conidia per milliliter) did not result in an earlier expression of foliar resistance.

In a typical experiment (Table 1), seven-leaf plants were inoculated at the stem base and challenged after 3 wk, when they had about 15 leaves. Resistance was induced in all leaves, but was more pronounced in the top six leaves than in the bottom six leaves (the three lowest leaves senesced). Lesions on upper leaves of protected plants were circular and 1–2 mm in diameter, compared to the irregular, 4- to 8-mm-diameter lesions on control plants. On lower leaves, lesions were irregular and 3–7 mm in diameter, compared with 12–18 mm on those of the controls. Lesion number mostly was dependent on the percent germination of the conidia used (see Materials and Methods). With low germinability, 0–30 lesions per leaf developed on protected plants versus 30–60 on unprotected plants, whereas with high germinability, 20–150, and 50–200 lesions per leaf developed on protected and unprotected plants, respectively. When inoculum concentration was 5×10^5 conidia per milliliter with about 30–50% germination, small differences in numbers of lesions were recorded between protected and unprotected plants. Differences in lesion size, however, were still clearly noticeable.

Although it sporulated profusely on lesions in young leaves of unprotected plants (1,500–2,500 conidia per square millimeter), *P. hyoscyami* f. sp. *tabacina* either did not sporulate or only scarcely sporulated at all (about 20 conidia per square millimeter) on lesions in young leaves of protected plants (Table 1). Sporulation generally was lower in older leaves but it was higher in the older leaves (300–700 conidia/mm^2) than in the younger leaves of protected plants. Challenged leaves of unprotected plants became desiccated in about 2-wk (older leaves) or 3-wk (younger leaves), whereas those of protected plants remained alive for more than 8 wk with very little expansion and/or necrotization of lesions, especially in the younger leaves.

Protection persisted in detached challenged leaves; at 3 wk after infestation of the soil at the base of the stem leaves 1, 2, 3, and 4 of B21 developed 0, 5, 18, and 24 lesions ~5 mm in diameter per leaf as compared to 3, 11, 32, and 55 lesions ~15 mm in diameter per leaf in the control plants, respectively. As with Ky 16 at 4 wk, 0, 1, 9, and 8 lesions ~3.5 mm diameter per leaf developed in protected plants, compared to 8, 24, 55, and 66 lesions ~15 mm diameter per leaf on leaves 1, 2, 3, and 4 of unprotected plants, respectively.

To determine the effect of incubation temperature on the expression of resistance, plants were transferred at 6 wk after initial inoculation with *P. hyoscyami* f. sp. *tabacina* to growth chambers at either 15 or 25 C (12 hr of light per day). After 1 wk, they were challenge-inoculated, kept at 20 C for 24 hr, and returned to the original chambers for symptom production. Systemic resistance was induced both at 15 and 25 C (Table 2).

Prior inoculation of the stem-root interface zone was also done with either *P. parasitica* var. *nicotiana* Race 0, *T. basicola, C. lagenarium,* sonicates of conidia of *P. hyoscyami* f. sp. *tabacina*, and repeatedly freeze-thawed conidia of *P. hyoscyami* f. sp. *tabacina* . None of these treatments induced resistance except for the freeze-thawed conidia of *P. hyoscyami* f. sp. *tabacina* which were found to retain viability after this treatment.

Induced protection in suckers. Suckers, stimulated by both the stem and stem-root interface inoculation, usually emerged from the lower part of the stem and developed five, seven, and nine leaves at 5, 6, and 7 wk, respectively, after the preliminary inoculation. Intact suckers were fully susceptible to *P. hyoscyami* f. sp. *tabacina*, whereas the leaves of the attached main shoot were resistant. Longitudinal sections revealed that necrosis did not develop in cambial and phloem tissues of the suckers.

To test whether resistance could be developed in suckers, sucker production was stimulated in normal tobacco by ringing seven-leaf plants 5 cm above the soil level. At 2 wk, when suckers had developed, a conidial suspension of *P. hyoscyami* f. sp. *tabacina* was applied to the soil at the base of the stem. All suckers developed

TABLE 1. Systemic resistance against blue mold induced in tobacco cultivar Ky 16 by prior inoculation with *Peronospora hyoscyami* f. sp. *tabacina*

Treatment[a]	Leaf position from top	Lesions per leaf	Lesion (mm^2) size[b]	Average area/leaf infected (cm^2)	Spore production per unit (mm^2) of lesion area
Unprotected	1–5	124 ± 52	6 ± 2	35	2,100 ± 500
Protected	1–5	43 ± 27	1.3 ± 0.6	0.6	20 ± 20
% protection		65	78	98	99
Unprotected	6–10	75 ± 41	15 ± 3	132	1,300 ± 300
Protected	6–10	20 ± 20	5 ± 2	3.9	500 ± 200
% protection		74	67	97	62

[a] Plants were inoculated at the seven-leaf stage and challenged 5 wk later. After challenge, plants were grown in controlled environment chambers at 20 C.
[b] Data are the diameters for round lesions or the longest axes for irregular lesions. Protected plants mostly produce round lesions, especially on upper leaves.

TABLE 2. Expression of systemic induced resistance in tobacco (cv. B21) against blue mold at two temperatures of incubation

	Number and sizes of lesions in plants maintained at:							
	15 C[b]				25 C[b]			
	Protected		Unprotected		Protected		Unprotected	
Leaf position[a]	Number	Size[c]	Number	Size[c]	Number	Size[c]	Number	Size[c]
1	10 ± 9	1 ± 0.5	42 ± 10	12 ± 3	32 ± 9	2 ± 0	30 ± 15	15 ± 3
2	4 ± 4	1 ± 0.5	67 ± 21	8 ± 6	3 ± 2	2 ± 1	50 ± 10	17 ± 3
3	2 ± 3	1 ± 0.5	51 ± 10	15 ± 5	9 ± 3	2 ± 0	50 ± 0	17 ± 4
4	1 ± 1	1 ± 0.5	50 ± 17	17 ± 3	7 ± 6	7 ± 3	75 ± 15	20 ± 5
5	0	...	62 ± 18	17 ± 2	2 ±2	2 ± 1	75 ±25	27 ± 6
6	0	...	90 ± 10	17 ± 3	4 ± 3	3 ± 2	85 ± 22	32 ± 5
7	1 ± 2	2 ± 1	52 ± 28	22 ± 3	0	...	85 ± 23	35 ± 7
8	0	...	50 ± 0	25 ± 5	3 ± 3	5 ± 0	100 ± 10	37 ± 5
9	0	...	52 ± 2	25 ± 6	9 ± 8	17 ± 3	100 ± 7	37 ± 6
Average	1.8	1.1	57.3	17.6	7.1	5.0	72.2	26.3

[a] Beginning at the top of the plant.
[b] Tobacco plants, were inoculated at the seven-leaf stage and challenged 7 wk later. Leaves 10–23 senesced. Records were taken on the 7th and 9th day after challenge at 25 C and 15 C, respectively.
[c] Data are the diameters for round lesions or the longest axes for irregular lesions.

necrosis in cambium and phloem and their foliage was resistant to *P. hyoscyami* f. sp. *tabacina*.

Growth retardant effects of ethrel. In fall and winter, plants inoculated at the stem-root interface exhibited growth retardation (stunting) and yellowing. In summer, on the other hand, such plants grew taller and produced inflorescences earlier than did the untreated control plants. To determine whether growth retardation may have induced resistance to blue mold, healthy tobacco plants were treated with an aqueous solution of ethrel (2-chloroethylphosphonic acid). Ethrel (10^2–5×10^3 mg/L in 0.05 M phosphate buffer, pH 6.4) was sprayed, injected into stems, or poured on the soil. Plants were challenged 7–15 days after treatment. Resistance was not induced even though stunting, yellowing, and accelerated senescence responses were elicited by the chemical.

Spore germination in protected leaves. Conidial germination of *P. hyoscyami* f. sp. *tabacina* was studied in situ on leaf disks of unprotected and protected plants (3 wk after either stem-injection or stem-root interface inoculation). Conidial germination on unprotected plants was very poor (1–2%), confirming previous reports (3,13). Similar poor germination was observed on protected leaves. Phloroglucinol-HCl staining of epidermal strips did not indicate lignification in the vicinity of germinating spores in either protected or unprotected leaves.

To determine whether resistance was restricted to the epidermal layer, leaves of Ky 16 were infiltrated with 5×10^4 conidia per milliliter, thus bypassing the epidermis. Both lesion size and fungal sporulation in protected leaves were reduced, especially in younger leaves, compared with those parameters for unprotected leaves (Fig. 2).

DISCUSSION

Results of these experiments confirmed earlier observations by Pont (9) of the field resistance of infected tobacco to reinfection, and by Cruickshank and Mandryk (2) and Mandryk (6–8) on the protection of tobacco against blue mold by stem injection with *Peronospora tabacina*. This study also demonstrates that protection can be achieved by applying conidia of *P. hyoscyami* f. sp. *tabacina* to the soil surface around the stem of potted tobacco plants. With such preliminary inoculation, a remarkably high level of resistance developed. Systemic protection in leaves increased gradually, reaching about 95% about 3 wk after introducing conidia of *P. hyoscyami* f. sp. *tabacina* to the soil at the base of the stem. Bottom leaves were always less protected than were the upper leaves. Once developed, however, resistance persisted for the lifetime of detached leaves. Protected plants developed minute restricted lesions on which the pathogen produced very few or no spores. These lesions were more frequent on younger leaves, especially when a high concentration of challenge inoculum was used. About 4–5 wk after induction, these tiny lesions were so rare that the amount of protection based on infected leaf area was 99%.

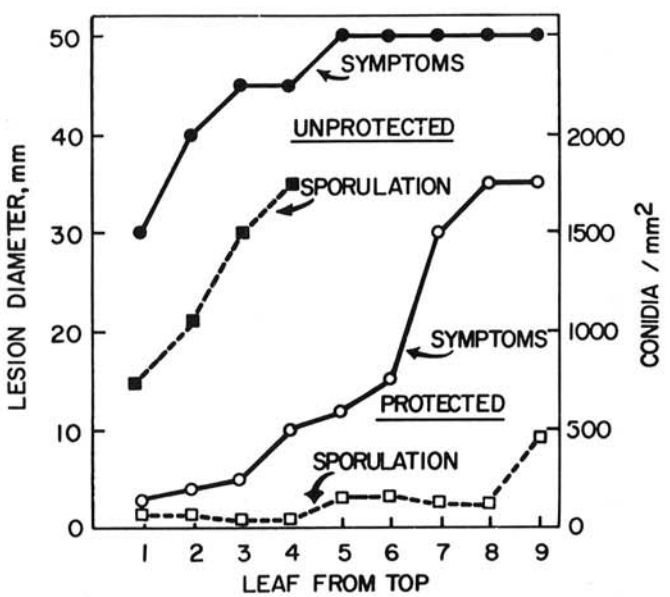

Fig. 2. Systemic protection against blue mold in tobacco cultivar Ky 16 plants induced by prior inoculation with *Peronospora hyoscyami* f. sp. *tabacina*. One milliliter of spore suspension containing 5×10^4 spores was added to the soil around the base of the stem, and 33 days after induction, plants were challenged by infiltrating conidia of *P. hyoscyami* f. sp. *tabacina* into leaf panels. Sporulation on leaves 5–9 of unprotected plants was negligible due to necrosis.

The appearance of the minute lesions clearly indicated penetration of the pathogen into the challenged protected leaves. Direct microscopic observations revealed no differences in the germination of conidia on protected or unprotected leaves.

That protection did not result from inhibition of penetration of the pathogen into the leaf was also deducted from leaf infiltration studies. These studies showed that protected leaves were highly resistant even when infiltrated with conidia of *P. hyoscyami* f. sp. *tabacina*. The resistance of *Nicotiana debneyi* to the same pathogen also does not lie in the epidermal layer (4,13). Infection of that host also was not obtained by removing epidermal strips and directly inoculating the mesophyll.

It seems that the resistance in protected plants lies in the inner leaf tissues. Although the pathogen penetrates and produces minute lesions in these tissues, its further growth is greatly restricted; both lesion expansion and sporulation were markedly inhibited. Possibly the resistance factors in inner tissues eventually reach a sufficiently high level to block germination and/or penetration of the pathogen on the surface of protected leaves. This may explain the decline in number of lesions on protected plants after the fourth week following induction onward.

Stunting and suckering were associated with resistance. These two features are indicative of an hormonal imbalance in protected plants. Stunting may account for 13–30% reduction in leaf area of protected plants depending upon the growing season and the cultivar. Mandryk (7) found that plants supplied with adequate nitrogen show no morphological changes associated with acquired resistance induced by stem injection. Growth inhibition alone is not responsible for the resistance of protected plants; neither Ethrel, which caused a similar setback in growth, nor the black shank pathogen, which caused a stunting and wilting, induced resistance. This implies that enhanced senescence or irritation as such are not the sole mechanisms responsible for induced resistance. That hypothesis is strengthened by observations that older leaves of both normal and protected tobacco plants are more susceptible to the disease than are younger ones.

Stem cankers induced by either *P. parasitica* var. *nicotiana* or *T. basicola* did not produce systemic resistance against blue mold. This indicates that *P. hyoscyami* f. sp. *tabacina* triggers a specific mechanism. Whether this mechanism is inhibitory to other pathogens in addition to TMV (8) is as yet unknown, and needs further study.

The mechanism for induced resistance in tobacco seems to differ in some characteristics from that reported in cucurbits by Kuć and coworkers (1,5,10). In cucurbits, infecting leaf 1 with fungi, bacteria, and viruses elicited within 4 days a systemic resistance that lasted until flowering (4–6 wk) against both the inducer and other fungal, bacterial, and viral pathogens. A booster inoculation 2–4 wk after induction protected cucurbits through the period of fruiting. In tobacco, leaf infection produced no measurable protection against blue mold in 3 wk, but stem or stem-root interface inoculation induced resistance in 2–3 wk that lasted through flowering and seed set. In cucurbits, but not in tobacco, one mechanism for protection was localized in the epidermis (10). In both cucurbits and tobacco, however, resistance was evident in leaves challenged by inoculum infiltration, and was not counteracted by high concentration of the challenge inoculation.

Induced resistance may play a role under natural conditions because: conidia washed to the soil surface may cause stem infection which will result in protection; and leaf infection may spread into the stem, thus stimulating the development of protection.

Such resistance will operate better in relatively older plants since they are less prone to systemic infection and also suffer almost no setback in growth.

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