Protection of Tobacco Against Phytophthora parasitica var. nicotianae by Cultivar-Nonpathogenic Races, Cell-Free Sonicates, and Pratylenchus penetrans

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ABSTRACT

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Tobacco cultivars L8 and 1071 were protected against cultivar-pathogenic races of *Phytophthora parasitica* var. *nicotianae* by cultivar-nonpathogenic races. Protection was localized within 1 cm of the site of injection. Sonicates of all races protected these cultivars and the susceptible cultivar Windsor Shade 117 (WS 117). Protection occurred only when tobacco cultivars were exposed first to the sonicate and then were challenged. Protection occurred within 12 hr of sonicate application, reached a maximum by 24 hr, and thereafter decreased through 96 hr. Sonicates did not inhibit germination of zoospores of the pathogen in vitro. Infection by the lesion nematode, *Pratylenchus penetrans,* also protected WS 117 against *P. parasitica* var. *nicotianae*. This protection was similar to the above except that it was longer-lived, presumably due to the continual irritation of the plant by the nematode. The results indicate that the protective response is nonspecific and that a host response is required for protection to occur.

Additional key words: induced resistance, *Nicotiana tabacum,* black shank.

Induced resistance has been demonstrated for several host-parasite systems when races of the pathogen and differential cultivars of the host are used (5, 24, 25, 28, 30, 31). Protection of tobacco, *Nicotiana tabacum* L., against several pathogens, including *Peronospora tabacina* (4, 14), *Erysiphe cichoracearum* (3), *Pseudomonas solanacearum* (13), *Pseudomonas tabaci* (11), and tobacco mosaic virus (26, 27) has been demonstrated. Wills and Moore (33) have reported that when tobacco cultivars were inoculated with *P. parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker and later inoculated with the same pathogen at a site near the first lesion there was a reduction in the size of the second lesion. This paper reports the protection of tobacco against cultivar-pathogenic races of *P. parasitica* var. *nicotianae* by cultivar-nonpathogenic races and also by cell-free sonicates of the pathogen and by *Pratylenchus penetrans*. Preliminary accounts have been published (15, 17).

MATERIALS AND METHODS

Isolates of *P. parasitica* var. *nicotianae*, races 0 and 1 (isolates 1587 and 1668-1, received from C. C. Litton, University of Kentucky, Lexington) and race 3 (Connecticut isolate M-15T) were used. Isolates were maintained and zoospore suspensions were prepared as described previously (23). Optimum results were obtained with an inoculum of 10^4 zoospores per ml, and this concentration was used in all studies.

The following cultivars of *N. tabacum* were used: L8 (resistant to race 0, moderately susceptible to race 3, and susceptible to race 1) (19, 32); 1071 (resistant to races 0 and 3, and susceptible to race 1) (19, 32); and Windsor Shade 117 (WS 117) (susceptible to races 0, 1, and 3) (19). Seeds were germinated on a sterile mixture of soil, peat, and sand (1:1:1, v/v), and seedlings at the four-leaf stage of development were transplanted to 240-ml Styrofoam® (Dow Chemical Co., Midland, MI 48640) cups containing a similar potting mixture. Plants were fertilized when necessary (Peters' fertilizer, R. B. Peters Co. Inc., Allentown, PA 18104) and maintained at 25 ± 2 C and under fluorescent light with a 12-hr photoperiod. Twelve- to 16-wk-old plants were used for all studies.

In initial studies, L8 or 1071 were stem-inoculated (7, 32) with mycelium of race 0 of the pathogen. Control plants were wounded but not inoculated. Plants were challenged at the same site with race 1 either immediately or 24 hr later. To standardize the inoculum, zoospore suspensions were used. Inoculations were made into the stem about 1 cm above the soil line by piercing through the stem with a syringe needle [1.6 cm long, 0.71 mm in diameter (22 gauge)]. As the needle was withdrawn, one drop of inoculum (containing about 1.7×10^2 zoospores) was deposited at both the entrance and exit sites. Tobacco lines L8 and 1071 were injected with water or race 0 zoospore suspensions and challenged immediately or 24 hr later by inoculation with a race 1 zoospore suspension. Plants were challenged at the site of initial inoculation, or at 0.5 or 1.0 cm above that site.

Sterile cell-free sonicates of race 0, 1, or 3 mycelium were prepared to determine if they could protect plants of the three lines against cultivar-pathogenic races of *P. parasitica* var. *nicotianae*. Mycelia were harvested from 5-day-old cultures grown on fresh lima bean agar by

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freezing the plates on dry ice and aseptically removing the aerial mycelium. Mycelia from 7-day-old cultures growing in P-1 broth (8) were harvested by filtration and washed with sterile distilled water prior to sonication. Mycelia (2 to 3 g wet weight) were placed in 10 ml sterile 0.05 M phosphate buffer, pH 6.8, and sonicated in an ice bath at maximum power for two 1-min intervals (100 W output, Model 350 sonicator, Heat Systems - Ultrasonics, Inc., Plainview, NY 11803). The sonicate was centrifuged at 4 C for 10 min at 12,000 *g*. The supernatant liquid was decanted and filter-sterilized (0.22 μm Millipore filter), the protein concentration was determined by the Lowry method (12), and the sterile cell-free sonicate was adjusted with phosphate buffer to about 7 mg protein per ml. Sonicates were tested for sterility on potato-dextrose agar (Difco) or in nutrient broth (Difco).

Plant stems were injected with sonicates at three sites: 1, 2, and 3 cm above the soil line, by the method described for the zoospore inoculations. In different experiments, L8 or 1071 were injected with sterile phosphate buffer or sonicates of races 0, 1, or 3 and challenged immediately or 24 hr later with zoospores from race 1. The zoospores were injected only into the center site (2 cm above the soil line) of sonicate application. The susceptible cultivar WS 117 was injected with phosphate buffer or sonicates from races 0, 1, or 3 and challenged immediately or 24 hr later with race 0, 1, or 3 zoospore suspensions. All combinations of sonicate source and challenge were tested.

The protection was further characterized with the susceptible cultivar WS 117 as host, race 3 sonicates as initial treatments, and race 3 zoospores as challenge inoculum. To determine the onset and duration of protection, plants were injected with phosphate buffer or sonicate and challenged immediately or 12, 24, 48, 72, 96, or 120 hr later. To determine the effect of sonicate concentration on protection, plants were injected with 1:1 and 1:9 dilutions of sonicate and challenged 24 hr later. To determine the heat-stability of the protectant, plants were injected with autoclaved sonicate (15 min) and

TABLE 1. Protection of tobacco lines L8 or 1071 against race 1 of *Phytophthora parasitica* var. *nicotianae* by race 0

Tobacco cultivar	Inoculum[a]	Treatment and time of inoculation (hr)		Plants with symptoms at days after inoculation (%)[b]				t[c]
		0	24	3	4	5	8	
L8	Mycelium	Race 0		0	0	0	0	
		Plant injured[a]	Race 1	100	100	100	100	
		Race 0 + Race 1		100	100	100	100	
		Race 0	Race 1	0	0	20	30	15.12**
	Zoospores	Race 0		0	0	0	0	
		Water[a]	Race 1	100	100	100	100	
		Race 0 + Race 1		100	100	100	100	
		Race 0	Race 1	0	0	0	0	∞**
		Race 0	Race 1 (0.5 cm)[d]	0	0	70	100	2.82**
		Race 0	Race 1 (1.0 cm)[d]	0	70	100	100	1.34
1071	Mycelium	Race 0		0	0	0	0	
		Plant injured[a]	Race 1	100	100	100	100	
		Race 0 + Race 1		100	100	100	100	
		Race 0	Race 1	0	0	0	0	∞**
	Zoospores	Race 0		0	0	0	0	
		Water[a]	Race 1	100	100	100	100	
		Race 0 + Race 1		100	100	100	100	
		Race 0	Race 1	0	0	0	0	∞**
		Race 0	Race 1 (0.5 cm)[d]	0	0	0	0	∞**
		Race 0	Race 1 (1.0 cm)[d]	0	0	0	30	15.12**

[a]Plants were stem inoculated with mycelium or with zoospores (10^4 per ml). See text for the method of zoospore injection. Control plants were wounded but mycelium was not placed into the wound, or they were injected with sterile distilled water.

[b]Ten plants per treatment. Experiments performed a minimum of two times, and the average number of plants with symptoms (%) was calculated from all observations.

[c]Calculated t-value obtained with a paired t-test. Values significant at $P = 0.01$ (**).

[d]Zoospores of race 1 were injected into the plant stem either 0.5 or 1.0 cm above the site of race 0 zoospore injection.

challenged 24 hr later.

To determine if sonicate inhibited zoospore germination, 1 ml of a race 3 zoospore suspension was added to 1, 5, or 10 ml of phosphate buffer or race 3 sonicate. These preparations were incubated at 25 C in the dark and observed daily for zoospore germination.

Inoculation with *Pratylenchus penetrans,* the lesion nematode, was also used to protect 12- to 16-wk-old WS 117 plants against the black shank pathogen. Plants were transplanted into 240-ml Styrofoam cups containing the following: soil which was naturally infested with 47 *P. penetrans* adults and larvae per 100 g of soil; the same soil but previously frozen at −12 C for 48 hr to kill the nematodes (22); or the sterile soil mixture described previously. Plants were challenged 4 days later by pipetting 1 ml of the race 3 zoospore suspension (10^4 zoospores per ml) around the base of the plant.

To determine the effect of nematode concentration, adults and larvae of *P. penetrans* were extracted from infested soil by a combination of the centrifugation (21) and tissue (20) methods and adjusted in water to 0, 25, 50, 100, or 200 living nematodes per milliliter. Plants were inoculated with nematodes as described previously (18) and challenged 4 days later with a race 3 zoospore suspension as described above. Nine days after the plants were challenged the number of nematodes in individual root systems was determined (18). Roots also were exposed to 200 nematodes and challenged either immediately, 4, or 9 days later.

All experiments contained a minimum of 10 plants per treatment and were repeated at least once. Data were analyzed by the paired *t* - test (29).

RESULTS

Black shank symptoms appeared as a necrosis of the stem tissue around the inoculation site 2 days after stem-inoculating plants with mycelium or zoospores of cultivar-pathogenic races. When zoospore suspensions were pipetted around the bases of the plant stems, stem necrosis developed 3 days after inoculation. Three days after the initial observation of stem necrosis the entire stem had become necrotic and the tissues were collapsed. Symptoms were not observed on many of the plants which were protected against cultivar-pathogenic races by cultivar-nonpathogenic races, cell-free sonicates of the pathogen, or *P. penetrans*. When symptoms did occur on "protected" plants, they often were delayed by several days compared to plants inoculated only with cultivar-pathogenic races. However, once symptoms began to develop in these "protected" plants they often progressed as rapidly as in nonprotected plants.

Mycelia or zoospores of race 0 protected L8 against race 1 when they preceded race 1 by 24 hr, but not if both races were used simultaneously (Table 1). The cultivar 1071 did not develop black shank symptoms when challenge was applied at or 0.5 cm above the site of race 0 injection. Moderate protection occurred when initial and challenge inoculation sites were 1.0 cm apart. There was only a delay in symptom expression when L8 plants were challenged 0.5 or 1.0 cm above the race 0 injection site.

Sonicates of races 0, 1, and 3 protected L8, 1071, and the susceptible cultivar, WS 117, from all cultivar-pathogenic races if sonicate preceded challenge by 24 hr. Since protection was independent of host genotype, sonicate source, and cultivar-pathogenic race used as challenge, data are presented only for WS 117 with race 3 as the source of both sonicate and challenge. Significant protection was observed when sonicate preceded challenge by 12 to 72 hr (Table 2). Protection was highest after a 24-hr delay before challenge and decreased progressively with time. Protection became less effective as sonicate was diluted, and was ineffective with

TABLE 2. Protection of tobacco cultivar WS 117 against race 3 of *Phytophthora parasitica* var. *nicotianae* by cell-free sonicates of race 3 mycelium

Initial treatment	Time of inoculation with R3[a] (hr)	Plants with symptoms at days after inoculation (%)[b] 3	5	7	9	t[c]
Buffer (P)[a]		0	0	0	0	
Sonicate (S)[a]		0	0	0	0	
Challenge controls[d]		90	100	100	100	
S+R3		60	100	100	100	1.00
Sonicate	12	10	35	50	50	12.20**
Sonicate	24	15	25	25	25	106.64**
Sonicate	48	35	40	50	50	24.23**
Sonicate	72	30	70	70	70	7.84**
Sonicate	96	60	80	100	100	1.51
Sonicate	120	60	100	100	100	1.00
Sonicate (1:1)[e]	24	20	30	50	60	11.21**
Sonicate (1:9)[e]	24	60	90	100	100	1.87
Sonicate (Auto)[f]	24	100	100	100	100	

[a]Abbreviations: R3 = zoospores (10^4 per ml) from race 3; P = phosphate buffer; S = sonicate of race 3 mycelium.

[b]Ten plants per treatment. Experiments performed a minimum of two times, and the average number of plants with symptoms (%) was calculated from all observations.

[c]Calculated *t*-value from a paired *t*-test. Values significant at $P = 0.01$ (**).

[d]Challenge controls were injected with phosphate buffer and either simultaneously or 12, 24, 48, ..., 120 hr later with race 3 zoospores.

[e]Sonicate was diluted 1:1 or 1:9 in phosphate buffer.

[f]Sonicate was autoclaved for 15 min.

autoclaved sonicate.

Sonicate did not inhibit zoospore germination and 94% of the zoospores germinated within 24 hr. Sonicate, but not phosphate buffer, supported some growth of the fungus after zoospore germination.

Seedlings of WS 117 were partially protected against black shank when challenged 4 days after exposure to *P. penetrans*. All plants growing in the sterile soil mixture developed black shank symptoms by 4 days after zoospore challenge, but only 40% of the plants growing in the nematode-infested soil developed symptoms 12 days after challenge. Plants were not protected when grown in the *P. penetrans*-infested soil that had been frozen previously.

Protection of WS 117 by *P. penetrans* was dependent upon the interval between the initial and challenge inoculation. Plants were not protected if nematodes and challenge were applied simultaneously, but they were protected if challenge followed nematode application by 4 or 9 days (Table 3).

Protection also was dependent on nematode concentration and on the number of nematodes in the tobacco roots (Table 3). Partial protection occurred when plant roots were exposed to 200 nematodes but 100 nematodes afforded little protection and fewer nematodes afforded no protection. The average number of *P. penetrans* in the entire root system 9 days after challenging was 2, 4, 12, and 16 nematodes per plant when plants were exposed to 25, 50, 100, and 200 nematodes/ml, respectively.

DISCUSSION

For protection of tobacco against *P. parasitica* var. *nicotianae* a delay is required between exposure of the plant to the cultivar-nonpathogenic race, sonicate, or nematode, and application of challenge. This indicates that protection requires a host response. This conclusion is supported by the observation that sonicates did not inhibit zoospore germination or subsequent mycelial growth in vitro.

Protection induced by cultivar-nonpathogenic races was localized. It did not extend beyond 1.0 cm above the site treated with the cultivar-nonpathogenic race. Localization of protection also was evident in "protected" plants that developed black shank symptoms. Apparently healthy tissue often extended 1 to 2 cm above the site challenged. This also was observed when sonicates were used to protect plants. Control plants always exhibited initial symptoms at the site of challenge inoculation. These results are similar to those obtained with other host-parasite systems (5, 16, 24), although systemic protection also has been reported (4, 6, 10, 13, 14).

Protection afforded by sonicated mycelium was short-lived. It occurred when challenge followed sonicate by 12 hr, was at a maximum by 24 hr, and declined through 96 hr. These results also are in agreement with those obtained with certain other host-parasite systems (5, 16).

The protection of plants against cultivar-pathogenic races by cultivar-nonpathogenic races and sonicates has been reported for many host-parasite systems (5, 24, 25, 28, 30, 31). Glucans from several *Phytophthora* spp. also have been shown to stimulate the accumulation of phytoalexins in host tissues or to protect the plant against cultivar-pathogenic races of the pathogen (1, 2). In the tobacco-black shank system, sonicates of both cultivar-pathogenic and -nonpathogenic races induce protection. This indicates, as do results from other host-parasite systems for which sonicates or glucans are used to induce resistance (1, 2, 16, 28), that the protective response is probably nonspecific. These results also indicate that susceptible cultivars have the potential to respond in a resistant manner to the pathogen.

Protection of WS 117 by *P. penetrans* also required a delay before challenge, but it was more permanent. Plants were protected even when challenge followed initial exposure to the nematode by 9 days. This probably results from the continual irritation of the plant tissues by the nematodes. This protection appeared to be dependent upon the number of nematodes in or on the plant roots, and the protection was not localized since the roots were exposed to the nematode and the stem was protected

TABLE 3. Protection of tobacco cultivar WS 117 against race 3 (R3) of *Phytophthora parasitica* var. *nicotianae* by *Pratylenchus penetrans*

P. penetrans[a] (no./ml)	Time of inoculation with R3[b] (days)	Plants with symptoms at days after inoculation (%)[c] 4	5	7	9	t[d]	*P. penetrans* (avg. no. per root system)[e]
0	4	100	100	100	100		0
25	4	50	70	100	100	1.67	2
50	4	50	50	100	100	2.14	4
100	4	30	45	60	80	6.01**	12
200	4	15	30	35	35	22.15**	16
200	0	100	100	100	100		ND[f]
200	9	15	25	35	40	19.18**	ND

[a]Adult and larval nematodes were isolated from infested soil and added in suspension around the roots of the plant.

[b]Plants were inoculated by pipetting 1 ml of a R3 zoospore suspension (10^4 zoospores per ml) around the base of each plant.

[c]Ten plants per treatment. Experiments were performed a minimum of two times and the average number of plants with symptoms (%) was calculated from all observations.

[d]Calculated t-value from a paired t-test. Values significant at $P = 0.01$ (**).

[e]Nine days after plants were challenged with *P. parasitica* var. *nicotianae* the roots were ground in a Waring Blendor and the average number of *P. penetrans* per root system was determined.

[f]Abbreviation: ND = not determined.

against the black shank pathogen.

In contrast to our studies, Inagaki and Powell (9) reported that *Pratylenchus brachyurus* increased the susceptibility of tobacco to *P. parasitica* var. *nicotianae* when plants were exposed simultaneously to both pathogens or to the nematode and then to the fungus 1 or 2 wk later. However, when *P. parasitica* var. *nicotianae* followed exposure to *P. brachyurus* by 3 wk, black shank symptom development was delayed. They also observed that when plants with mechanically wounded roots were exposed to the fungus, black shank symptoms were more severe than when plants were exposed to the nematode and the fungus either simultaneously or sequentially. Inagaki and Powell used a different *Pratylenchus* species, different and more mature tobacco cultivars, more nematodes, and higher fungal inoculum, which was applied directly to the roots of the plants.

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