Screening Tobacco Seedlings for Resistance to Phytophthora parasitica var. nicotianae

John L. McIntyre and G. S. Taylor

Department of Plant Pathology and Botany, The Connecticut Agricultural Experiment Station, New Haven, 06504, and Windsor, 06095.

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ABSTRACT

A technique was developed for screening 2- to 3-week-old tobacco seedlings for resistance to black shank, caused by *Phytophthora parasitica* var. *nicotianae*. Tobacco seedlings of different cultivars at the 4-leaf stage were placed on moistened filter paper disks in square petri plates and sprayed with approximately 3 ml of a zoospore suspension of race 0, 1, or a Connecticut (C) isolate of *P. parasitica* var. *nicotianae*. Black shank symptoms developed within 3 days after the seedlings were sprayed with a suspension of 10^4 zoospores/ml and within 5 days disease resistance ratings of the cultivars and breeding lines tested paralleled results obtained by others from field and greenhouse observations. The seedling data indicate that the C isolate was different from races 0 and 1. Stem inoculations of 8-week-old differential tobacco cultivars and lines L8, 1071, and mature *Nicotiana nesophila* with races 0 or 1 of *P. parasitica* var. *nicotianae* gave the expected black shank symptom responses. Stem inoculation of 1071 with the C isolate gave results comparable to race 0, while results obtained with *N. nesophila* were comparable to race 1. Inoculation of L8 with this isolate caused some necrosis of stem tissue. These results suggest that the C isolate of *P. parasitica* var. *nicotianae* may be more virulent than or different from races 0 and 1 of the pathogen.

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Evaluation of tobacco cultivars for resistance to black shank, caused by *Phytophthora parasitica* (Dast.) var. *nicotianae* (Breda de Haan) Tucker, has been difficult. Black shank resistance has been tested elsewhere in naturally infested field plots. The occurrence of different races of the pathogen, variations in disease intensity from location to location and season to season, and lack of control over inoculum potential and environmental conditions made this screening method difficult to utilize effectively. To overcome these problems, greenhouse techniques were developed to screen tobacco cultivars for whole-plant resistance to black shank (5, 10). These greenhouse methods use standardized conditions of temperature and light, and one (5) controls the initial inoculum concentration. The method of Troutman et al. (10) requires transplanting the tobacco seedlings, a time-consuming process if large numbers of cultivars are to be screened. The methods of Troutman et al. (10) and Litton et al. (5) require up to 6- and 7-weeks, respectively, to determine final resistance ratings. Resistance to black shank has also been evaluated by leaf tissue strips (14) and stem inoculations (4). Results of the leaf technique have shown that in some cases leaf resistance differs from whole plant resistance (13, 14), and the stem inoculation technique is time-consuming if large numbers of tobacco lines are screened.

The objectives of our experiments were to develop a rapid means of evaluating young tobacco seedlings for black shank resistance which utilized standardized conditions of inoculum concentration, temperature, and light. A comparison of the Connecticut (C) isolate of *P. parasitica* var. *nicotianae* (12) with races 0 and 1 of the pathogen was also made.

MATERIALS AND METHODS.—Race 0 (isolate 1587, obtained from C. C. Litton, University of Kentucky, Lexington, Ky; isolates 1189 A-2 and 1189 A-3, obtained from J. L. Apple and G. B. Lucas, respectively, North Carolina State Univ., Raleigh, N. C.), race 1 (isolates 1668-1, obtained from C. C. Litton, and 1452-1-2-2 from J. L. Apple) and the C isolate (isolates M-15S and M-15T) of *P. parasitica* var. *nicotianae* were grown on fresh lima bean agar for 1 week and transferred to water agar plates until abundant zoosporangia were present. Zoospores were released by a modification of the method of Dukes and Apple (2) and zoospores from isolates of the same race were combined. Zoospores from a sample of each suspension were inactivated by heating at 60 C for 15 seconds and counted with a hemacytometer. The zoospore suspensions were adjusted to 10^5, 10^4, or 10^3 zoospores/ml. The best separation of whole-plant black shank resistance of the cultivars was obtained with an inoculum suspension of 10^4 zoospores/ml, and this concentration was utilized in subsequent experiments.

Tobacco cultivars and breeding lines were grown in disposable loaf pans ($15 \times 15 \times 5$ cm) containing a sterile potting mixture of soil:peat:sand (1:1:1, v/v). Seedlings were maintained at 30 C with a 12-hour photoperiod until the four-leaf stage (2-3 weeks for most cultivars), at which time they were gently removed from the soil, washed free of soil debris with tap water, and rinsed with sterile distilled water. Ten seedlings of each cultivar were placed in sterilized square plastic petri plates ($100 \times 100 \times 15$ mm) which contained a 9-cm diameter filter paper disk moistened with 4 ml of sterile distilled water. The zoospore suspensions were applied with a Sprayon Jet-Pak Sprayer (Sprayon Products, Inc., Cleveland, Ohio) from a distance of 50 cm by evenly passing the zoospore-containing spray over a row of plates until approximately 3 ml of inoculum had been sprayed at each plate. Because of dispersion of the zoospore-containing spray, about 20% (0.6 ml) of inoculum landed on each plate. The lids were placed on the plates and the seedlings maintained at

30 C and 12-hour photoperiod for the duration of the experiment. Additional sterile distilled water was added to the filter paper disks as required.

All cultivars were screened three times and a minimum of 50 seedlings of each cultivar were tested. Seedlings sprayed with sterile distilled water served as controls. The number of seedlings with black shank symptoms was determined at 3, 5, and 7 days after inoculation. The percent of infected seedlings for each cultivar was divided by ten to make an index scale of 0 (no plants with black shank symptoms) to 10 (all plants with black shank symptoms). Variation in the black shank indices for each cultivar was ± 1 for the three screenings. Therefore the indices are presented as the average for all observations. These data were subjected to an analysis of variance and Duncan's multiple range test of significance.

To determine the number of viable zoospores which landed on or near each sprayed tobacco seedling, five square petri plates (100 × 100 × 15 mm) containing water agar were sprayed as previously described with approximately 3 ml of a suspension containing 10^5 or 10^4 zoospores/ml. The plates were incubated in the dark at 25 C for 48 hours and the total numbers of zoospores and germinated zoospores per plate were determined by counting 10 areas 1 mm in diameter on each plate. By assuming that each tobacco seedling was a rectangle with a width equal to the distance across the primary leaves and a length equal to seedling height (9 × 15 mm), the number of viable zoospores landing on or near each plant was determined.

To compare races 0, 1, and the C isolate of *P. parasitica* var. *nicotianae*, 8-week-old tobacco breeding lines and cultivars L8, 1071, WS 117, and mature *Nicotiana nesophila* were stem inoculated (4, 15) with race 0, race 1, or the C isolate. The plants were observed 4 days later for stem necrosis.

RESULTS.—Within 24 hours after inoculating tobacco seedlings with a concentration of 10^4 zoospores/ml, we observed the production of zoosporangia from infected tissues and the release of zoospores, providing a continual source of secondary inoculum. Black shank symptoms usually appeared on the seedlings as a necrosis at the base of the stem within 3 days after inoculation. Root necrosis was also commonly observed as an initial black shank symptom, and less commonly leaf lesions were the first symptoms. Several days after the initial appearance of symptoms, infected seedlings were entirely necrotic. Control seedlings developed chlorotic symptoms after one week. In these tests the symptom development that best separated resistant from susceptible cultivars was achieved by the inoculum containing 10^4 zoospores/ml. A concentration

TABLE 1. Black shank indices of tobacco cultivars 5 days after spraying tobacco seedlings with a zoospore suspension of race 0 or race 1 of *Phytophthora parasitica* var. *nicotianae*[v,w]

Tobacco cultivar	Reported reaction to *P. parasitica* var. *nicotianae*[x]		Black shank indices 5 days after inoculation		Mean black shank index[y]
	Race 0	Race 1	Race 0	Race 1	
1071	R	S	0.6	2.6[z]	1.6 a
TI 1583	R	R	1.4	3.0	2.2 a
Burley 37	R	R	2.0	3.4	2.7 ab
Fla 15	R	R	3.8	1.6	2.7 ab
NC 95	R	R	3.2	3.0	3.1 ab
Beinhart 1000-1	R	R	3.8	3.2	3.5 abc
Md 609	R	R	5.0	2.2	3.6 abc
Fla 301	R	R	4.4	3.2	3.8 abc
L 8	R	S	3.0	4.6	3.8 abc
Con. L	R	R	3.8	5.0	4.4 abc
Coker 298	R	R	3.2	5.6	4.4 abc
Coker 347	R	R	6.8	3.0	4.9 abcd
Speight G 140	R	R	5.6	5.6	5.6 abcd
Burley 21	S	S	6.0	5.6	5.8 abcd
Brown 2238			10.0	3.2	6.6 bcd
Coker 187	R	R	10.0	3.2	6.6 bcd
Nicotiana nesophila	S	R	9.0	4.4	6.7 bcd
WS 918			10.0	3.6	6.8 bcd
Culbro 396			10.0	5.0	7.5 cde
WS 715			10.0	7.4	8.7 de
WS 117			9.6	9.4	9.5 de

[x]Percentage of infected seedlings/10 = black shank index. A minimum of 50 seedlings was observed for each tobacco cultivar or line.

[w]Ten seedlings were placed in square petri plates containing a moistened filter paper disk and inoculated by spraying from a distance of 50 cm with approximately 3 ml of a zoospore suspension containing 10^4 zoospores/ml of race 0 or 1. Seedlings were maintained at 30 C and a 12-hour photoperiod.

[v]Whole-plant resistance (R) or susceptibility (S) to races 0 or 1 for the tobacco cultivars and lines were obtained from reports in the literature (1, 4, 6, 7, 8, 9, 11, 13).

[y]Numbers followed by same letter are not statistically different ($P = 0.05$) by Duncan's multiple range test.

[z]Seven days after inoculation the tobacco line 1071 exhibited type 0 resistance with a black shank index of 1.3 and 9.0 for races 0 and 1, respectively.

of 10^5 zoospores/ml caused black shank symptoms to appear within 3 days on highly resistant (Beinhart 1000-1) (7) and moderately resistant (Fla 301) (8) cultivars, while 10^3 zoospores/ml produced a slow response (7 days) on even the most susceptible cultivars (WS 117).

Approximately 20,400 zoospores, of which 50 ± 3% germinated, landed on water agar plates sprayed with a concentration of 10^5 zoospores/ml, or about 80 viable zoospores on or near each seedling. About eight viable zoospores landed on or near a seedling sprayed with a suspension containing 10^4 zoospores/ml. An index of 5.0 or less indicates tobacco cultivars or lines highly resistant to *P. parasitica* var. *nicotianae*, and an index of 5.1 to 7.5 represents cultivars or lines moderately resistant to the pathogen. A value > 7.5 indicates little if any resistance to *P. parasitica* var. *nicotianae* (1, 4, 6, 7, 8, 9, 11, 13). The black shank index taken 5 days after seedling inoculation (Table 1) is most comparable to indices obtained by others from field and greenhouse observations (1, 4, 6, 7, 8, 9, 11, 13).

The black shank indices obtained with the C isolate were not always the same as those obtained with races 0 or 1. Significant differences were found in the mean black shank indices for races 0 and 1 and the C isolate for several of the cultivars screened (Table 2).

Stem inoculations of 8-week-old WS 117, L8, 1071, and mature *N. nesophila* with races 0 and 1 gave typical symptom responses (Table 3). Symptom responses of these cultivars to the C isolate were not always comparable to either race 0 or race 1.

DISCUSSION.—The technique described for screening tobacco seedlings for black shank resistance separated resistant from susceptible cultivars. A concentration of 10^4 zoospores/ml gave a resistance index for all cultivars most comparable to published resistance indices (1, 4, 6, 7, 8, 9, 11, 13) and places approximately eight viable zoospores on or near each seedling. Gooding and Lucas (3) have shown that as the inoculum level is increased, even resistant cultivars elicit a susceptible response to *P. parasitica* var. *nicotianae*. This explains why black shank symptoms developed on resistant cultivars sprayed with a concentration of 10^5 zoospores/ml, which places about 80 zoospores on or near each seedling.

Differentiation of resistant and susceptible cultivars was most successful when seedlings were observed 5 days after inoculation with zoospores. Observation three days after inoculation did not permit adequate separation of the cultivars. Seven days after inoculation, a high percentage of some moderately resistant cultivars showed black shank symptoms, making it difficult to separate them from more susceptible cultivars. This was probably not due to the initial inoculum level, but rather to the production of new zoosporangia and zoospores within 24 hours so that the inoculum level within each plate was continually increasing, and eventually symptoms appeared even on moderately resistant cultivars. Since cultivars with high levels of resistance do not show black shank symptoms within 7 days, it is suggested that seedlings be maintained for several days after the 5-day indexing in order to detect the most resistant cultivars.

This 5-day black shank index for the cultivars tested differentiates the degree of resistance to race 0 or race 1. Tobacco cultivars possessing both stem and leaf resistance against *P. parasitica* var. *nicotianae*, such as Fla 301 and Beinhart 1000-1 (13), were also highly resistant in our screening procedure. The tobacco line 1071, which has both leaf and stem resistance to race 0 of *P. parasitica* var. *nicotianae* but is susceptible to race 1 (13), exhibited type 0 resistance which was not evident until seven days after seedling inoculation, when the black shank indices were 1.5 and 9.0 for races 0 and 1, respectively. Since our method requires spraying entire seedlings with zoospores of *P. parasitica* var. *nicotianae*, cultivars which possess stem resistance but leaf susceptibility would be expected to exhibit both stem and leaf reactions to the pathogen, and therefore perform less

TABLE 2. Black shank indices of several tobacco cultivars and lines 5 days after spraying tobacco seedlings with a zoospore suspension of races 0, 1, or a Connecticut (C) isolate of *Phytophthora parasitica* var. *nicotianae*[a,b]

Tobacco cultivar or line	Black shank indices five days after inoculation[c]		
	Race 0	Race 1	C isolate
NC 95	3.2	3.0	8.8
Beinhart 1000-1	3.8	3.2	5.0
MD 609	5.0	2.2	9.2
Speight G 140	5.6	5.6	8.6
WS 117	9.6	9.4	9.8
1071	0.6	2.6	0.4
L8	3.0	4.6	7.4
Nicotiana nesophila	9.0	4.4	5.0

[a]Percentage of infected seedlings/10 = black shank index. A minimum of 50 seedlings were observed for each tobacco cultivar or line.

[b]Ten seedlings were placed in square petri plates containing a moistened filter paper disk and inoculated by spraying from a distance of 50 cm with approximately 3 ml of a zoospore suspension containing 10^4 zoospores/ml of race 0, 1, or the C isolate. Seedlings were maintained at 30 C and a 12-hour photoperiod.

[c]Numbers underlined are not statistically different ($P = 0.01$) by Duncan's multiple range test.

TABLE 3. Black shank symptoms 3 days after stem-inoculating tobacco cultivars and lines with mycelium from race 0, 1, or the Connecticut (C) isolate of *Phytophthora parasitica* var. *nicotianae*[a]

Tobacco cultivar or line[b]	Number of plants that developed stem necrosis after stem inoculation (%)		
	Race 0	Race 1	C isolate
L 8	0	100	60
1071	0	100	0
Nicotiana nesophila	80	20	20
WS 117	100	100	100

[a]Five plants of each tobacco cultivar or line were stem inoculated as described by Hendrix and Apple (4) and Wills and Moore (15).

[b]Eight-week-old L8, 1071, WS 117, and mature *Nicotiana nesophila* were used in this study.

well than if only the stem were inoculated. This was true with Coker 187, which has excellent stem resistance but only moderate leaf resistance to *P. parasitica* var. *nicotianae* (13). Whole-plant susceptibility of Coker 187 to some race 0 isolates was found (13), which is supported by our data. The tobacco line L8, which is susceptible to race 1, has excellent stem resistance but its leaves are susceptible to race 0 (13). Our results indicate that L8 was resistant to both race 0 and race 1, with resistance to race 0 being only slightly better than resistance to race 1. Wills (13) has reported that whole-plant tests sometimes indicate L8 to have resistance to both races 0 and 1.

Since our technique does not differentiate stem from leaf resistance, it is not an absolute method for evaluating how a tobacco cultivar will perform in the field, but it does eliminate many highly susceptible cultivars. By placing succulent plants in conditions of optimum temperature, with free water, and high relative humidity, we provide optimum conditions for detecting seedlings which are resistant to *P. parasitica* var. *nicotianae*. The use of standardized environmental conditions and initial inoculum concentration, and the use of seedlings at the same stage of development provides conditions which can be duplicated each time tobacco seedlings are screened for resistance to black shank. This technique requires no more than 4 weeks from the time the seed is sown to determine the final resistance indices of the tobacco seedlings, uses a minimum of space and equipment, and progeny from several hundred crosses can be screened within one day.

The black shank indices obtained by our screening technique indicated that the C isolate was different from races 0 and 1. Cultivars NC 95, Md 609, and Speight G 140, which are resistant to races 0 and 1, were susceptible to the C isolate. In some cases the C isolate was most comparable with race 0, as with 1071, while in other cases it was most comparable to race 1, as with *N. nesophila*. Stem inoculations of L8, 1071, WS 117, and mature *N. nesophila* with race 0 or 1 of the pathogen exhibit the expected resistant or susceptible interactions. The C isolate is not comparable to race 0 or race 1 when these same tobacco cultivars and lines are stem inoculated with this isolate. These results suggest that the C isolate of *P. parasitica* var. *nicotianae* may be either more virulent than or different from race 0 and race 1 of *P. parasitica* var. *nicotianae*.

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