A Detached-Leaf Technique for Detecting Resistance to *Phytophthora parasitica* var. *nicotianae* in Tobacco

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ABSTRACT

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We developed a nondestructive technique for evaluating tobacco (*Nicotiana tabacum*) germ plasm for resistance to *Phytophthora parasitica* var. *nicotianae*. Leaves (7–9 cm long) from healthy tobacco plants were excised, surface-sterilized for 30 sec in a 0.05% $NaOCl_3$ solution, and inoculated with four mycelial plugs (1 cm in diameter) of 2-day-old *P. p.* var. *nicotianae* cultures grown on oatmeal agar. Inoculated leaves were placed in petri dishes containing one sterile filter paper and 10 ml of sterile distilled water. Petri dishes were sealed with Parafilm and incubated in a growth chamber at 27 C with constant fluorescent light (65 $\mu E \cdot m^{-2} \cdot s^{-1}$). Number of lesions per leaf and percentage of leaf area infected were recorded daily for 6 days after inoculation. Genotype rankings for lesion development and percentage of infected leaf area were similar to known rankings of the same genotypes for field resistance. The technique was not effective in detecting race-specific resistance derived from *N. longiflora*. The technique is quick, repeatable, and nondestructive, and it may be used in combination with detached-leaf screening techniques for other pathogens.

Black shank, caused by *Phytophthora parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker, is a destructive root and stem disease of tobacco (*Nicotiana tabacum* L.). Use of resistant cultivars is the best way to control the disease on infested land; however, even the most resistant cultivars of burley tobacco have only moderate levels of resistance.

Techniques used to screen tobacco germ plasm for black shank resistance have typically involved inoculation of stems or roots. Hendrix and Apple (3) developed an effective stem inoculation technique for detecting monogenic resistance to race 0 of *P. p.* var. *nicotianae;* however, the method was inadequate for differentiating levels of horizontal resistance. Wills and Moore (12) modified this stem inoculation technique, but their methods were also too variable to detect horizontal resistance.

Root inoculation techniques for evaluating tobacco for black shank resistance have been developed and improved by many researchers (1,2,4, 7,8). In all cases, inoculation of young seedlings with either mycelial suspensions or zoospores results in symptom expression on and rapid mortality of susceptible cultivars. In one frequently used procedure (4), young seedlings are grown in plastic tubes filled with vermiculite. The tubes, which have holes in the bottom, are dipped into a zoospore suspension to inoculate the plants. This method of screening is fairly reliable but destructive, as even cultivars with moderately high levels of resistance die before reaching reproductive maturity. The method is therefore ineffective for the advancement of early-generation lines.

We attempted to develop a screening technique for the evaluation of black shank resistance that would be quick, nondestructive, and effective in demonstrating resistance at an early growth stage.

MATERIALS AND METHODS

The following cultivars were used in all experiments: Burley 21 (B 21) and Kentucky 14 (Ky 14), burley tobacco cultivars susceptible to races 0 and 1 of *P. p.* var. *nicotianae;* Virginia 509 (Va 509), Burley 37 (B 37), and Kentucky 17 (Ky 17), burley cultivars moderately resistant to both races; Kentucky 14 × L8 (Ky 14 × L8), a burley cultivar with high resistance to race 0 but susceptible to race 1; and Beinhart 1000-1 (Beinhart), a highly resistant cultivar of a cigar-type tobacco. Seeds of the seven cultivars were germinated in Jiffy-7 peat pellets (Jiffy Products [N.B.] Ltd., Shippegan, Canada) and maintained in a growth chamber at 28 C with a 12-hr photoperiod of combined fluorescent and incandescent light (50 $\mu E \cdot m^{-2} \cdot s^{-1}$) for about 30 days. Plants were then moved to the greenhouse (18–27 C), where they were kept in metal pans and watered daily. Plants were fertilized weekly with Peters 20:20:20 (N:P:K) fertilizer (W. R. Grace & Co., Fogelsville, PA) to maintain vigorous growth.

About 45 days after seeding, one young apical leaf 7–9 cm long was excised at the base of the petiole from each plant to be tested. Leaves were soaked for 30 sec in a 0.05% $NaOCl_3$ solution for surface-sterilization, rinsed in sterile distilled water, and blotted dry on sterilized paper towels. Leaves were placed abaxial side down in plastic petri dishes measuring 100 × 15 mm, each containing one layer of sterile Whatman No. 1 filter paper and 10 ml of sterile distilled water.

Isolates ATCC-66208 (American Type Culture Collection, Rockville, MD; isolate from Kentucky) (race 0) and ATCC-66209 (Kentucky isolate) (race 1) of *P. p.* var. *nicotianae* were maintained on oatmeal agar slants incubated at 27 C with no light and were tested frequently for pathogenicity. Isolates were grown on freshly prepared oatmeal agar for 2 days at 27 C in the dark. Plugs of mycelium and agar (1 cm in diameter) were taken from the outside edge of mycelial mats with a sterile cork borer and were used as inoculum.

One plug from each of four separate culture dishes was inverted and placed on each leaf so that two plugs were distributed uniformly on each side of the midvein. Petri dish lids were replaced and sealed with Parafilm "M" (American Can Company, Greenwich, CT) to maintain high relative humidity. Dishes were incubated in a growth chamber at 27 C with constant fluorescent light (65 $\mu E \cdot m^{-2} \cdot s^{-1}$). The number of lesions per leaf (0–4) and the percentage of leaf area infected (0–100%) were recorded every 24 hr for 6 days after inoculation.

In the first experiment, leaves of the seven cultivars were evaluated for resistance to race 0 of *P. p.* var. *nicotianae* using isolate ATCC-66208. The experimental design was a randomized complete block with five replications of one leaf per replication. The experiment was done twice, and data from both experiments were pooled after a test for homogeneity of variance.

A second experiment tested the effect of pathogen race. Detached leaves of the seven cultivars were inoculated with either isolate ATCC-66208 (race 0) or isolate ATCC-66209 (race 1). The exper-

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imental design was a split plot with five replications. Cultivars were assigned to main plots, and pathogen races were assigned to subplots. The experiment was done twice, and data from both experiments were pooled after a test for homogeneity of variance.

To compare our detached-leaf assay with an established greenhouse evaluation procedure, we evaluated the seven cultivars for resistance to both isolates of *P. p.* var. *nicotianae* in a timed experiment using the seedling root inoculation technique described by Litton et al (4). A split-plot design with five replications was used. Cultivars were assigned to main plots, and pathogen races were assigned to subplots. Resistance was expressed as the percentage of living plants 4 days after inoculation with 1,000 zoospores of the appropriate isolate per milliliter.

RESULTS

In the first experiment, symptoms developed between 24 and 48 hr after inoculation on detached leaves of susceptible plants but not on leaves of resistant plants, with the exception of Ky 14 × L8 (Fig. 1). Lesion development on leaves of Ky 14 × L8 inoculated with the race 0 isolate of the pathogen was similar to that on leaves of the susceptible cultivars Ky 14 and B 21. Lesion development on all three of these cultivars was noticeable much earlier than on the four resistant cultivars (Fig. 1). Lesion ratings were analyzed separately for each observation date. The three cultivars differed significantly in number of lesions per leaf 2 and 3 days after inoculation, but not at later dates (Fig. 1). Disease ratings of the resistant cultivars were clearly lower than those of the susceptible cultivars beginning 2 days after inoculation. Leaves of Beinhart had lower disease ratings than those of the three moderately resistant cultivars, but differences were not significant except 4 days after inoculation, and even then Beinhart was significantly different only from Ky 17 (Fig. 1).

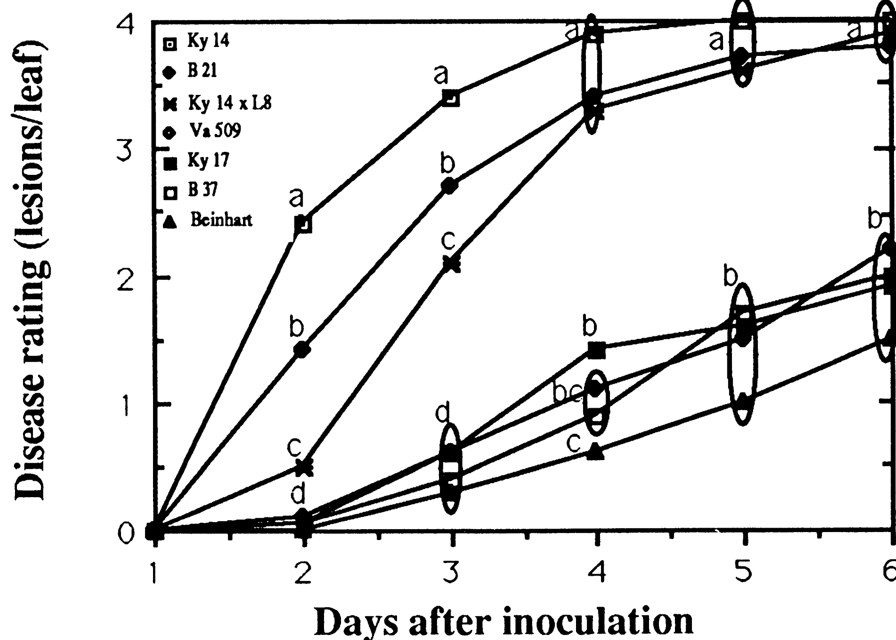

Fig. 1. Mean disease ratings (lesions per leaf) of seven tobacco cultivars after inoculation of detached leaves with four mycelial plugs of isolate ATCC-66208 (race 0) of *Phytophthora parasitica* var. *nicotianae* per leaf. Disease ratings on a given day labeled with different letters differ significantly ($P = 0.05$) according to Duncan's multiple range test.

Lesions developed faster on the susceptible cultivars (Fig. 2) and were visually distinct from those on the resistant cultivars. Lesions on leaves of resistant cultivars were usually very small and were often accompanied by a chlorotic halo. Lesions on the susceptible cultivars and on Ky 14 × L8 expanded rapidly and appeared water-soaked. A higher percentage of leaf area of Ky 14 leaves than of B 21 or Ky 14 × L8 leaves was infected at all times until 6 days after inoculation (Fig. 2). The other susceptible cultivar, B 21, and the moderately resistant cultivar Ky 14 × L8 were similar in the percentage of infected leaf area at all observation times beyond the first 3 days after inoculation. Excluding Ky 14 × L8, all resistant cultivars had significantly less infected leaf area than did susceptible cultivars beginning 2 days after inoculation (Fig. 2). Beinhart and Va 509 did not differ significantly in lesion expansion until 5 days after inoculation, and even then there were no significant differences between Beinhart and the other moderately resistant burley cultivars.

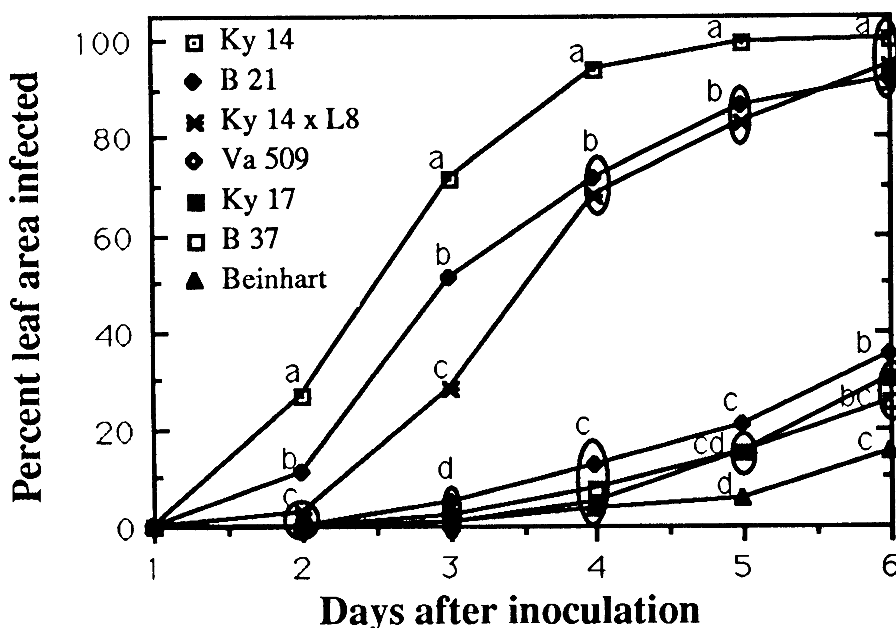

Fig. 2. Mean percentage of leaf area infected for seven tobacco cultivars after inoculation of detached leaves with four mycelial plugs of isolate ATCC-66208 (race 0) of *Phytophthora parasitica* var. *nicotianae* per leaf. Values on a given day labeled with different letters differ significantly ($P = 0.05$) according to Duncan's multiple range test.

In the second experiment, lesions developed and expanded on detached leaves of the two susceptible cultivars and of Ky 14 × L8 much faster than on leaves of the resistant cultivars. Distinct differences between these cultivar groups were evident 4 days after inoculation regardless of pathogen race (Fig. 3). Race × cultivar interactions were not significant. Excised leaves of Ky 14 × L8 had a susceptible reaction to both races of the pathogen. Beinhart did not differ significantly from the other resistant cultivars (excluding Ky 14 × L8) 4 days after inoculation (Fig. 3).

In the third experiment, inoculation with zoospores resulted in rapid mortality of the susceptible cultivars (Ky 14 and B 21) (Fig. 4). Survival of the seven tobacco cultivars 4 days after young

seedlings were inoculated with zoospores of *P. p.* var. *nicotianae* correlated well with known field resistance. There was a significant cultivar × race interaction for this experiment (Fig. 4). All plants of Ky 14 × L8 infected by race 0 survived, whereas more than 40% of those infected with race 1 died. A significantly lower percentage of plants of the two susceptible cultivars (Ky 14 and B 21) than of the resistant cultivars survived following inoculation with the race 0 isolate (Fig. 4). All plants of Beinhart root-inoculated with either race of the pathogen survived. The race 0 isolate appeared to be slightly more pathogenic than the race 1 isolate in these tests, and this difference was especially evident for Ky 17.

DISCUSSION

Most of the laboratory methods for screening tobacco germ plasm for resistance to black shank (1,2,4,7,8) are laborious, not effective for detecting horizontal resistance, and often destructive. Resistance detected by stem-inoculation techniques has shown good correlation with whole-plant resistance (12); however, results have been too variable for these techniques to be used in tobacco breeding programs (3,12). The root-inoculation method of Litton et al (4) is effective for screening progeny of advanced breeding lines, but eventual death of even moderately resistant plants limits its use as a selection tool.

Our method of evaluating detached leaves inoculated with plugs of *P. p.* var. *nicotianae* permitted early identification of susceptible plants. Daily observations of lesion development and expansion on detached tobacco leaves inoculated with either race 0 or race 1 of the pathogen demonstrated that partial resistance may be expressed in leaves of cultivars that have the Florida 301 parental source of resistance. The resistance of all of the moderately resistant cultivars of burley tobacco evaluated in this study can be traced to the cultivar Florida 301. The resistance of Beinhart is similar to that of Florida 301 (6).

The detached-leaf method did not detect the type of resistance in the Ky 14 × L8 hybrid, which is derived from *N. longiflora* (9). Hendrix and Apple (3) suggested that the expression of resistance from *N. longiflora* is not entirely root-specific and that plants with black shank resistance from *N. longiflora* would survive stem inoculations, whereas those with the Florida 301 source of resistance would die. The present study and work done by Wills (10) confirm that detached leaves and intact plants may differ in resistance to *P. p.* var. *nicotianae*. In another study (11), the expression of resistance in leaf tissue also depended on the location of the leaf on the stalk: leaves from the upper and middle stalk positions were the most resistant, and tissue from the youngest apical leaf was extremely susceptible. We used young leaves (7–9 cm long) from near the apical meristem of young plants and found that resistance was expressed in the form of suppressed lesion development in leaves removed from cultivars possessing moderate levels of resistance.

Ranking tobacco cultivars for resistance to *P. p.* var. *nicotianae* on the basis of number of lesions per leaf and percentage of infected leaf area gave results that were consistent with known field resistance rankings in all cases except for Ky 14 × L8. Leaf tissue from resistant plants should not be scored inaccurately if the test is continued for at least 3 days. Leaf tissue of susceptible cultivars was almost completely water-soaked 4 days after inoculation, whereas leaves from resistant plants maintained their turgor throughout the experiment.

Levels of black shank resistance determined with the detached-leaf technique correlated well with results from Litton et al's root-inoculation technique except for the *N. longiflora* resistance of Ky 14 × L8. Selection of young seedlings of even the most resistant burley cultivars inoculated with either race of *P. p.* var. *nicotianae* resulted in approximately 95% mortality (*data not shown*). Making selections based on the lesion responses of detached leaves would provide a way

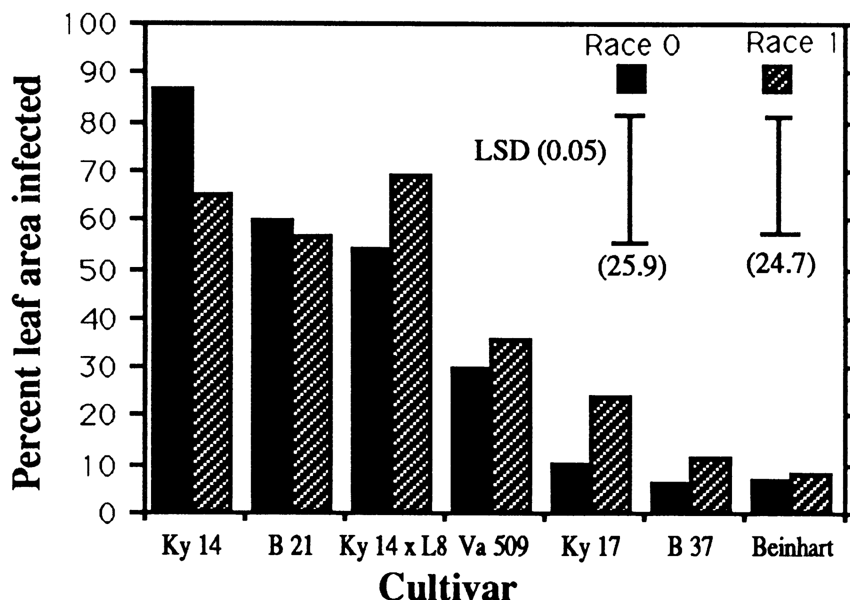

Fig. 3. Mean percentage of leaf area infected for seven tobacco cultivars 4 days after inoculation of detached leaves with four mycelial plugs of either isolate ATCC-66208 (race 0) or isolate ATCC-66209 (race 1) of *Phytophthora parasitica* var. *nicotianae* per leaf. The least significant difference (LSD) indicates the significance of differences among cultivars.

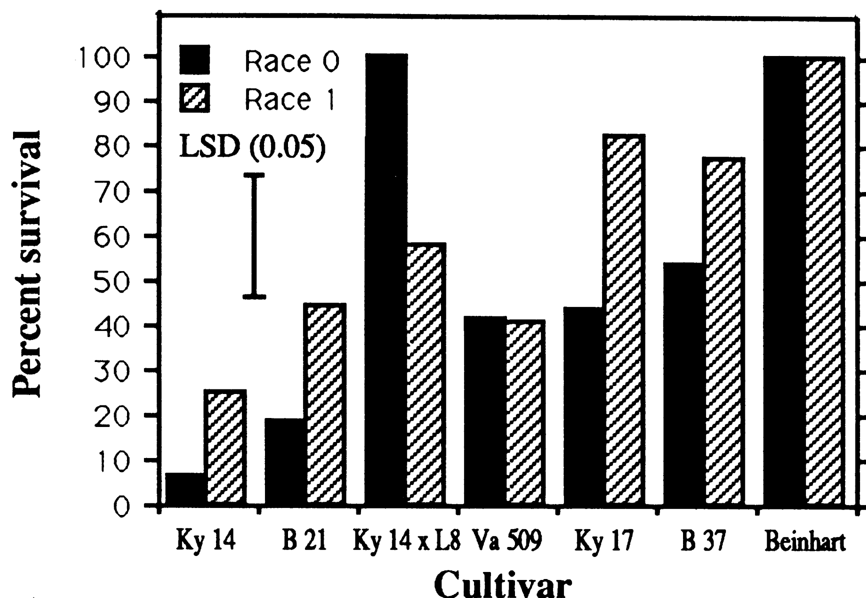

Fig. 4. Mean percentage survival of seedlings of seven tobacco cultivars 4 days after inoculation with 1,000 zoospores of isolate ATCC-66208 (race 0) or ATCC-66209 (race 1) of *Phytophthora parasitica* var. *nicotianae* per milliliter. The least significant difference (LSD) indicates the significance of differences between races.

to perpetuate resistant plants with the Florida 301 source of resistance.

The detached-leaf technique is not destructive and allows for the selection and continued propagation of resistant plants. This technique could be useful in early-generation tests and would help eliminate susceptible genotypes from segregating populations, thereby saving resources allocated to large-scale field testing for resistance to *P. p.* var. *nicotianae*. Because this method of screening uses peat pellets, it requires less soil and greenhouse space, and the potential for greenhouse contamination is greatly reduced. Only plants with high levels of resistance need be transferred into potting soil for generation advancement. The technique is also rapid, requiring only 7 days from inoculum transfer to completion. And because the technique is not destructive, plants may be screened for resistance to other diseases (5).

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