Virulence of an Endemic Isolate of *Peronospora tabacina* from Texas to *Nicotiana tabacum* and *N. repanda*

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ABSTRACT

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Isolates of *Peronospora tabacina*, causal agent of blue mold of tobacco, were obtained in 1983 and 1984 from naturally infected wild *Nicotiana repanda* plants near Uvalde, TX, and were increased on *N. repanda* in a growth chamber. Disease severity was similar on both *N. tabacum* 'Ky 14' and *N. repanda* plants following serial inoculations with sporangiospores and was positively correlated to inoculum density. The host plant (Ky 14 or *N. repanda*) used to maintain the isolates did not affect disease. *N. repanda* plants became systemically infected and supported sporulation for 25–60 days (30,000–240,000 sporangiospores/cm^2 of leaf tissue). These findings differ from those we obtained in earlier experiments with a Kentucky isolate (Ky 79). Virulence of the Ky 79 and Texas 84 isolates of *P. tabacina* was compared directly on leaf disks of Ky 14 plants of different ages under controlled conditions. The Texas isolate caused greater disease severity than the Kentucky isolate. This disease severity was temperature-dependent during the infection period. The data indicate that the Texas isolate is more virulent than the Kentucky isolate, and that virulence may be a useful marker for comparison of the two isolates. The epidemiological significance of these findings is discussed.

Wolf (11) reported in 1947 that *Nicotiana repanda* Willd., a noncultivated tobacco in Texas, was infected with a downy mildew fungus. During March and April of 1983 and 1984, Nesmith (4,5) observed that *N. repanda* L., growing naturally in Texas, was infected with a downy mildew fungus that sporulated profusely. Preliminary experiments showed that the fungus was pathogenic to cultivated tobacco and the disease was caused by a *Peronospora* sp. The potential for *N. repanda* to serve as a source of *P. tabacina* Adam for cultivated tobacco was hypothesized.

This study was undertaken to determine the pathogenicity of Texas isolates of *P. tabacina* to *N. tabacum*, burley, and *N. repanda* under controlled conditions. Comparisons are made to our previous study (7) that established the pathogenicity of a Kentucky isolate to these tobacco hosts. The virulence of a Texas isolate and a Kentucky isolate was also compared directly on burley tobacco in this study. A preliminary report on these findings has been presented (6).

MATERIALS AND METHODS

Host plants. Seedlings of burley tobacco (*N. tabacum*, 'Ky 14') and *N. repanda* (accession No. 46, *Nicotiana* species collection, Oxford, NC) were grown to 7–8 weeks of age in the greenhouse and then preconditioned in growth chambers as previously described (8).

Sources and maintenance of *P. tabacina* isolates. The following isolates of *P. tabacina* were used for this study: 1) Tx 83, sporangiospores were collected following overnight incubation under dark and moist (100% humidity) conditions in May 1983 from local chlorotic lesions on leaves of *N. repanda* plants growing wild near Uvalde, Tx; 2) Tx 84, sporangiospores were obtained under similar conditions in May 1984 from systemically colonized leaves of *N. repanda* plants growing in the wild on the Frio River near Knippa, TX; and 3) Ky 79, sporangiospores were collected in 1979 from burley tobacco plants in a field near Georgetown, KY. Contamination among the isolates was prevented by keeping each of the isolates in separate growth chambers. Both Texas isolates were maintained by weekly transfer using *N. repanda* plants, and the Kentucky isolate was maintained using Ky 14 plants. Sporangiospores of each isolate were gently brushed into a small quantity of distilled water, collected on a 3-μ filter, resuspended in distilled water, and sprayed on the appropriate host to produce inoculum. Germination of sporangiospores in the inoculum was monitored for each isolate by microscopic examination of 20 μl of inoculum suspension previously incubated 20 hr at 18 C in the dark on depression slides. The mean percentage of germination was recorded for eight replications.

Testing virulence of the Texas isolates. After one generation on *N. repanda*, to produce inoculum for the experiment, the virulence of the Texas isolates was evaluated during serial inoculations of Ky 14 and *N. repanda* (Fig. 1). Sporangiospores were sprayed uniformly over the upper leaf surface of 7- to 8-wk-old plants of Ky 14 and *N. repanda* at concentrations of 0, 4×10^1, 4×10^2, 4×10^3, and 4×10^4 sporangiospores/ml. Each inoculum concentration was sprayed on three to four leaves per plant using a glass chromatography sprayer. Six plants of each species were used per concentration. After inoculation, plants were covered with plastic bags (sprayed on the inside with water) and kept at 18 C for 20 hr in the dark. Plants were then uncovered and incubated in a growth chamber (23 C, 70 $\mu E \cdot s^{-1} \cdot m^{-2}$, 12 hr light per day supplied by cool-white fluorescent light) for a minimum of 7 days. The chlorotic area of each inoculated leaf was estimated visually starting on the seventh day after inoculation using a 0–4 scale: 0

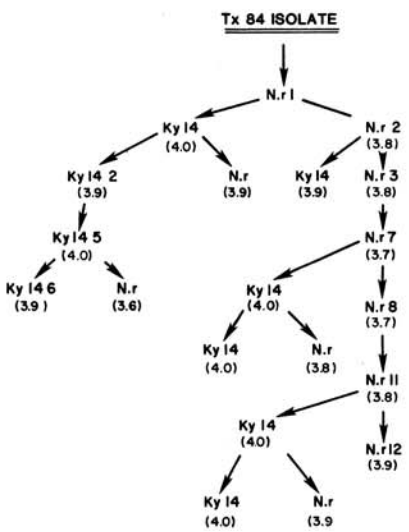

Fig. 1. Scheme for the study of the virulence of *Peronospora tabacina* (Tx 84 isolate) during serial passage through *Nicotiana tabacum* (Ky 14) and *N. repanda* (N.r). Numbers following plant represent the generation of *P. tabacina*. Numbers in parentheses are the disease severity rating (0–4 scale) 7 days after inoculation with 4×10^4 sporangiospores per ml. There were no significant differences ($P = 0.05$) in disease severity of the isolate on the different hosts.

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= no lesions; 1 = <25% of the leaf area chlorotic; 2 = 25–50% chlorotic; 3 = 50–75% chlorotic; and 4 = chlorosis on >75% of the leaf (7).

The above procedure was followed through 11 and 5 generations on *N. repanda* and Ky 14, respectively, using inoculum generated from the immediately preceding generation. Periodically, the inoculum produced on one host was used to challenge both hosts separately through serial passage to test for possible changes in virulence that might have occurred during successive generations (Fig. 1). The complete serial passage was performed twice for both Texas isolates with some interim steps performed two or more additional times.

Comparison of virulence of Texas and Kentucky isolates. An assay using leaf disks of Ky 14 plants (9) was used to compare the virulence of Texas 84 with the Ky 79 isolate of *P. tabacina*. Two experiments were designed to evaluate virulence of the two isolates, at six inoculum concentrations, two plant ages, and five temperatures.

In the first experiment, the virulence of Tx 84 and Ky 79 was compared at six inoculum concentrations on leaf disks of 9- or 19-wk-old plants. One hundred leaf disks of each age plant (18 mm diameter, excised with a cork borer No. 13) were obtained from two fully expanded leaves from the same plant. Ten disks were placed in a petri plate on filter paper wetted previously with 4 ml of a 1-μg/ml aqueous solution of kinetin. Sporangiospore suspensions of each isolate were applied uniformly to the disk using an airbrush sprayer (9) at concentrations of 0, 1.5, 7.5, 15, 45, and 75 sporangiospores/cm^2 of leaf tissue. The top surface of each plate was sprayed lightly with distilled water. Following inoculation, the plates were maintained at 18 C for 20 hr in the dark, then transferred to a growth chamber (23 C, 70 $\mu E \cdot s^{-1} \cdot m^{-2}$, 12 hr light per day) for disease development. Each treatment was replicated three times in a completely random design and the experiment was conducted twice. The data was statistically analyzed using Duncan's multivariate analysis.

In the second experiment, the effect of five temperatures (13, 18, 23, 28, and 33 C) during the infection period on the virulence of the isolates was evaluated. The conditions were similar to those of the first experiment, except that only 9-wk-old plants and three inoculum concentrations (0, 7.5, and 75 sporangiospores/cm^2 leaf disks) were used. The chlorotic area on each disk was rated visually using the scale of 0–4 described above, beginning 7 days after inoculation.

Sporulation. Differences in sporangiospore production of the three isolates (inoculation with 4×10^4 sporangiospores/ml) were determined on detached leaves of both *N. repanda* and Ky 14. A leaf from each treatment was detached from the plant at various times after inoculation and incubated on wet filter paper in petri dishes at 18 C for 20–24 hr in the dark to induce sporulation (7). Sporangiospores of each isolate from each host were harvested and collected as described above for inoculum preparation. The sporangiospores were resuspended in distilled water and spore concentrations were determined with a hemacytometer.

RESULTS

Symptoms. Inoculation of Ky 14 plants with 4×10^4 sporangiospores/ml of Tx 83 or Tx 84, obtained from *N. repanda*, resulted in distinct chlorotic lesions within 4 or 5 days. Seven days after inoculation of the plants, extensive chlorosis and wilting occurred. Extensive necrosis developed 7–9 days after inoculation and plant death followed 12 or 13 days after inoculation. On *N. repanda* inoculated with the Texas isolates, symptoms developed more slowly with distinct, chlorotic lesions not visible until the seventh day after inoculation. At that time, inoculated leaves were also curled, but leaves of *N. repanda* did not wilt as they did with Ky 14. By the 10th day, general chlorosis was extensive, but no necrosis was present. Within 14–21 days after inoculation, chlorosis and leaf curling developed on adjacent newly emerged, unsprayed leaves, starting at the petioles and progressing toward the tip of leaves. Necrosis of inoculated leaves was not evident until 26–30 days after inoculation. Necrosis was initially limited to the oldest inoculated leaves, and progressed up the plant to noninoculated leaves. Noninoculated control plants of Ky 14 and *N. repanda* (sprayed with water) were free of symptoms of blue mold.

Symptom development on both hosts after inoculation with the Ky 79 isolate (4×10^4 sporangiospores/ml) was similar to that reported in a previous study (7), but different from those observed with the Texas isolates. The Ky 79 isolate produced symptoms on Ky 14 similar to those observed with the Texas isolates. However, on *N. repanda*, symptom development and systemic colonization were much slower than when inoculated with Texas isolates. Plant death did not occur until about 60 days after

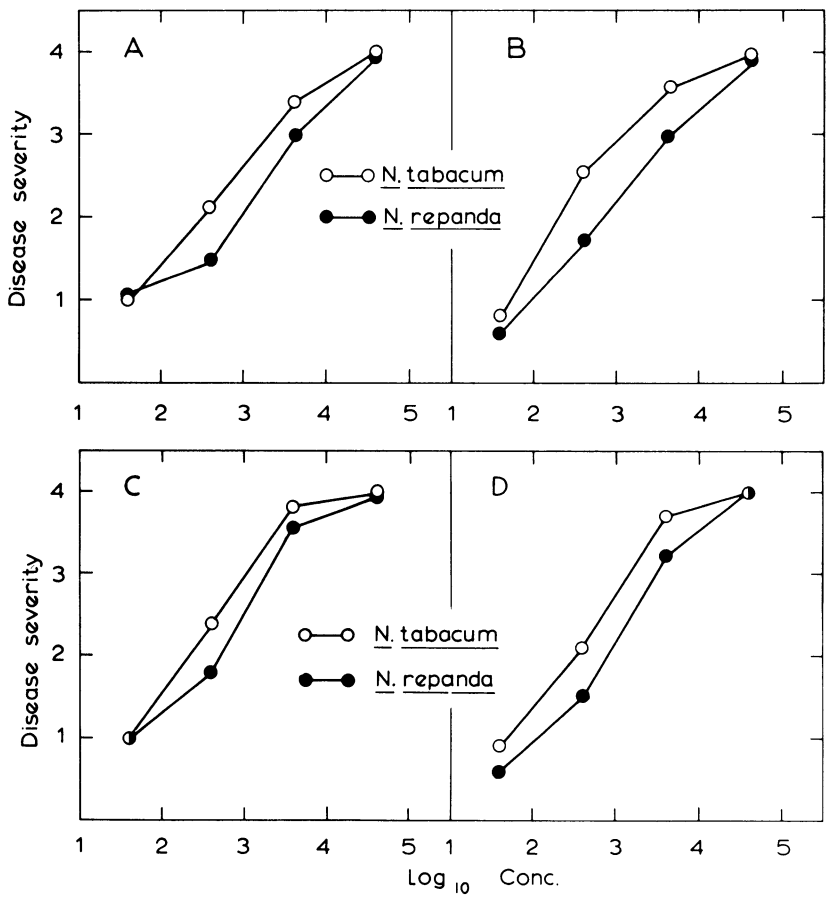

Fig. 2. The severity of blue mold on *Nicotiana tabacum* (Ky 14) and *N. repanda* inoculated with various sporangiospore concentrations of *Peronospora tabacina* (Tx 83 or Tx 84 isolates). Each point represents the mean of disease ratings on 30 disks. There were no significant differences ($P = 0.05$) between isolates at any concentration of inoculum. **(A)** Tx 83 sporangiospores were obtained from *N. repanda* plants, **(B)** Tx 83 sporangiospores were obtained from Ky 14 plants, **(C)** Tx 84 sporangiospores were obtained from *N. repanda* plants, and **(D)** Tx 84 sporangiospores were obtained from Ky 14 plants.

inoculation.

Virulence of the Texas isolates. There were no statistical differences in the levels of disease developed on Ky 14 and *N. repanda* plants when inoculated with similar concentrations of either the Tx 83 or Tx 84 sporangiospores (Fig. 2). The host plant from which inoculum was obtained did not significantly affect the severity of disease. These observations were consistent during 11 serial passages of Tx 83 and Tx 84 isolates through *N. repanda* (Fig. 1). Following the 2nd, 7th, and 11th generations on *N. repanda* and the first and fifth generations on Ky 14, inoculum from each host was sprayed separately on both hosts for direct comparison. Disease severity on Ky 14 and *N. repanda* was similar, regardless of the source of inoculum.

Virulence of the Tx 84 and Ky 79 isolates. The Texas isolate caused greater disease than the Ky 79 isolate, despite the inoculum concentration, age of plant, or temperatures during the infection period (Figs. 3 and 4). However, at 33 C, both isolates failed to produce lesions (Fig. 4). Varying inoculum concentration, age of plant, and temperatures during infection influenced disease development, and allowed us to distinguish statistical differences in virulence between the isolates (Figs. 3 and 4). Disks of leaves from 9-wk-old plants were more susceptible to blue mold than were leaves of 19-wk-old plants, regardless of the isolates used for challenge (Fig. 3). The difference in virulence between the isolates was more easily distinguished with older leaves at higher inoculum concentrations. Effects of temperature changes on disease development during the infection period were determined on leaves of 9-wk-old plants (Fig. 4). Increased virulence of Tx 84 was greater than Ky 79 when leaves were subjected to low inoculum concentration (7.5 sporangiospores/ml) and low temperatures (13–23 C). When higher levels of inoculum or temperatures were used, no differences in virulence between the isolates were noted.

Sporulation. Morphology of the sporangiospores and sporangiophores of the Kentucky and the Texas isolates were indistinguishable. Irrespective of the isolate or host, infected live tobacco tissue could be induced to sporulate whenever placed under moist (100% humidity), dark conditions. Sporulation commenced 4–5 days following inoculation and continued until death of the host tissue. Infected Ky 14 plants were short-lived, supporting sporulation from 5 to 12 days after inoculation. In contrast, sporulation occurred on *N. repanda* plants for up to 60 days after inoculation with the Ky 79 isolate, but only 30–40 days with Texas isolates. This difference appeared to be related to the slower rates of necroses of initially infected leaves and the development of new infections through systemic colonization. Sporulation could be induced on initially inoculated leaves 5 days after inoculation (before symptoms were obvious) and continued until 20–25 days after inoculation. However, by that time, numerous other leaves had been colonized systemically and supported sporulation. This sporulation continued through maturity of the plants, permitting approximately 50 days total sporulation time.

Spore production varied from 30,000 (5 days postinoculation) to 240,000 (10 days postinoculation) sporangiospores per cm^2 of leaf tissue. The peak was reached about 10 days after inoculation, followed by a decline. The level of sporulation was not affected by isolate, although the *N. repanda* host supported high levels for about 20–30 days longer than did the Ky 14 host.

Germination of sporangiospores of Texas isolates obtained from either *N. repanda* or Ky 14 plants during the serial passage study was 66–78% and 70–81%, respectively. Sporangiospores produced on both hosts by all Texas isolates were of similar shape (lemon) and size (24 ± 2.0 μm long). Oospores were not observed during these laboratory studies.

DISCUSSION

This study compared the factors of virulence, symptomology, and sporulation of endemic Texas isolates of *P. tabacina* collected from noncultivated tobacco with an isolate (Ky 79) from a cultivated crop during the 1979 epidemic in Kentucky. Previously, we studied these factors with the Kentucky isolate on *N. repanda* and burley tobacco (7). Data from these studies indicate that the Ky 79 isolate is different from the Texas isolates obtained in 1983 and 1984.

Differential responses were observed between the hosts from inoculations with the various isolates as in our earlier experiments (7). Ky 14 plants were more susceptible than were *N. repanda* plants when inoculated with similar inoculum concentrations, regardless of the isolate used (Fig. 3). Symptom development was similar on the Ky 14 plants with all isolates. The Texas isolates killed plants of *N. repanda* in about half the time required for the Ky 79 isolate. Ky 14 plants died within 12–14 days after inoculation, while *N. repanda* plants survived from 45 to 60 days after inoculation despite systemic disease development. Leaves infected with *P. tabacina* were capable of sporulation while still alive. Consequently, *N. repanda* supported sporulation for up to 60 days after inoculation. These data suggest that the Texas isolates, should they become established in cultivated tobacco, could be more damaging than that of the 1979 epidemic (3).

The Texas isolates could not be distinguished from each other by any of the variables measured. They produced similar disease levels when inoculated at similar concentrations (Fig. 2). Morphologically, both isolates produced indistinguishable sporangiospores. Oospores were not observed in these controlled studies. This is a significant limitation, since oospore morphology is most valuable in identification of the Peronosporas. However, Nesmith and Keeny (5) had observed oospores on field-grown *N. repanda* plants. Although oospore size (18–58 μ) and shape (some uniformly ridged and others irregularly ridged) varied greatly from plant to plant, it was concluded that they generally fit the description for *P. tabacina*. Oospores of similar size and shape were observed on other plants from this population while collecting the isolates used in this study.

The host plant used to produce the

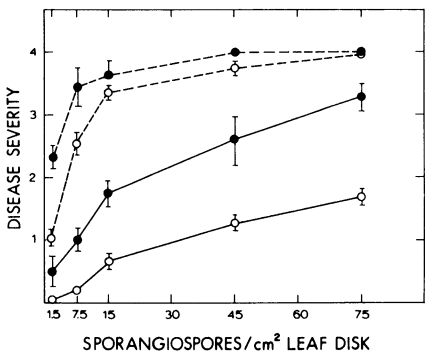

Fig. 3. Disease severity ratings on leaf disks of Ky 14 (dashed line = 9-wk-old, solid line = 19-wk-old) plants, challenged with various concentrations of sporangiospores (per cm^2) of *Peronospora tabacina* (open circle = Ky 79 isolate, closed circle = Tx 84 isolate). Each point represents the mean (± SD) of disease ratings on 30 disks. Germination of sporangiospores obtained from Ky 14 (Ky 79 isolate) and *N. repanda* (Tx 84 isolate) was 77.0 ± 3.0% and 65.0 ± 8.0%, respectively.

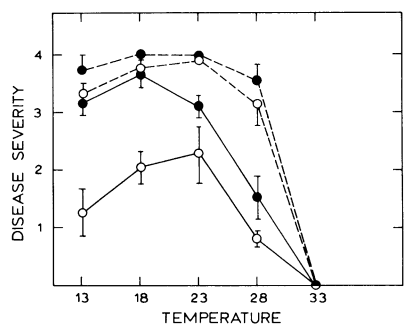

Fig. 4. The effect of temperature on disease severity ratings on leaf disks of 9-wk-old Ky 14 plants, challenged with 7.5 (solid line), or 75 (dashed line) sporangiospores (per cm^2 leaf disk) of *Peronospora tabacina* (open circle = Ky 79 isolate, closed circle = Tx 84 isolate). Germination of sporangiospores obtained trom Ky 14 (Ky 79 isolate) and *N. repanda* (Tx 84 isolate) was 78.0 ± 4.0% and 72.0 ± 5.0%, respectively. Each point represents the mean (± SD) of disease ratings on 30 disks.

inoculum affected the virulence of the Ky 79 isolate, but not when using either of the Texas isolates (7). Sporangiospores of the Ky 79 isolate produced on Ky 14 plants were more virulent than those produced on *N. repanda* plants. Also, sporangiospore size and the germinability of the Ky 79 isolate were altered during serial passage through Ky 14 and *N. repanda* plants (7). However, in this study, consistent results were obtained through 11 generations with the Texas isolates, regardless of host used (Fig 1). In contrast, with the Ky 79 isolate, virulence was affected by the host plant from which inoculum was harvested (7). The leaf disk assay was better at distinguishing the virulence of these isolates than were the whole plant assays. The leaf disk assay gave better control of more variables while directly comparing the Tx 84 and Ky 79 isolate. Overall, the Texas isolate caused more severe disease than did the Kentucky isolate, especially on older plants at all inoculum concentrations (Fig. 3). The difference in isolates was also observed with leaf disks from young plants, especially at low inoculum concentrations. Failure to observe differences at high inoculum levels on young leaves was due to higher susceptibility of these leaves and overloading at the higher levels of inoculum, as others have reported (9). Changing the temperature during the infection period did not alter the relationship between these two isolates, except at 33 C where no lesions were produced (Fig. 4). These findings support the observations made during serial passage studies with Tx 83, Tx 84, and Ky 79 that the Texas isolates are more virulent to *N. repanda* and Ky 14 plants than is the Ky 79 isolate.

Our laboratory studies support the field observations made by Wolf (11) and Nesmith et al (4,5) that *N. repanda* is susceptible to blue mold for a long period of time and that the fungus sporulates profusely under favorable environmental conditions both in the field and under controlled environmental conditions. *N. repanda* survived and served as a continuing source of *P. tabacina* for up to 40–60 days after inoculation. The Texas isolates evaluated were more virulent on cultivated tobacco than was the Ky 79 isolate. The epidemiological significance of these findings have great importance. The frequency of infections of *N. repanda* in its native area of southwest Texas may be low, but once infection has occurred an abundance of inoculum may be produced through the systemic colonization that occurs during dry weather. Massive sporulation from these sources may suddenly occur even at a later date when favorable conditions are available. Such a host-pathogen system could play a major role in the epidemiology of blue mold in tobacco-growing regions from a climatic region normally thought of as relatively unfavorable for the disease.

Nesmith et al (4,5) hypothesized that *N. repanda* could be a source of *P. tabacina* for Kentucky epidemics of the disease. Studies (1,2) of meteorological events compatible with a Texas to Kentucky movement of sporangiospores supported this hypothesis. In this study, we obtained no evidence to support or contradict this hypothesis. Evidence was found that Ky 79 is different from Tx 83 and Tx 84. Previous research had indicated that the Ky 79 isolate probably originated from the southeastern United States, not from the southwest (2,3,10), indicating that the isolates we tested might be different. These studies show that more critical experiments are needed to evaluate the role of *P. tabacina* on *N. repanda* in Texas relating to the epidemics of blue mold in North America. Studies are in progress to directly compare Kentucky and Texas isolates from the same year.

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