Distinguishing Isolates of *Peronospora tabacina* from Geographic Regions Utilizing Tobacco Leaf Disks and Fluorescence Microscopy

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ABSTRACT

Wiglesworth, M. D., Nesmith, W. C., Siegel, M. R., Bonde, M. R., and Main, C. E. 1994. Distinguishing isolates of *Peronospora tabacina* from geographic regions utilizing tobacco leaf disks and fluorescence microscopy. Plant Dis. 78:456-460.

A laboratory bioassay to distinguish isolates of *Peronospora tabacina* D.B. Adam was developed utilizing 7-mm acetone-dipped disks cut from tobacco leaf panels from several cultivars of *Nicotiana tabacum,* sporangiospores of *P. tabacina,* a germination indicator (Calcofluor), and fluorescence microscopy. Sporangiospore germination percentage of each isolate × cultivar pair was determined. Mean germination of sporangiospores differed between domestic and international collection locations (except between the two Mexican isolates). These results suggest the presence of different pathotypes of *P. tabacina.*

Blue mold, caused by the obligate parasite *Peronospora tabacina* D.B. Adam, occurs in some species of the genus *Nicotiana* (1,20). In the United States prior to 1979, blue mold was found primarily confined to tobacco seedbeds, and damage to field tobacco was generally not severe (10). However, in 1979 blue mold became epidemic in the field and caused in excess of $250 million loss to the U.S. and Canadian tobacco industries. Lucas (11) hypothesized that this sudden appearance of the disease resulted from a change or shift in the population or pathotype of *P. tabacina*; however, evidence to support this hypothesis was inconclusive because no monitoring system to differentiate isolates of *P. tabacina* was in place at the time.

Accepted for publication 29 November 1993.

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Several workers investigated methods of differentiating pathotypes of *P. tabacina* (3,8,9,14,18,21). All of these approaches were successful in the specific and somewhat limited use for which each technique was developed. The objective of this research was to determine whether a standardized laboratory leaf disk bioassay utilizing disks from different cultivars of *Nicotiana tabacum* L. would express different characteristics from which pathotypes of *P. tabacina* could be identified. The research combined portions of previously tested methodologies and a microscopic laboratory bioassay. The interaction of the pathogen and host were the only variables.

MATERIALS AND METHODS

Plant production. Cultivars (seed courtesy of R. Delon, CORESTA, Bergerac, France) were entries from the CORESTA (Centre De Cooperation pour les Recherches Scientifiques Relative au Tabac) trap collection (Table 1). The CORESTA trap collection is a set of *N. tabacum* cultivars that have been distributed to tobacco-growing countries since 1962 to detect changes in the pathogenicity and/or virulence of *P. tabacina.* In addition, the burley cultivars Kentucky 14 (Ky14) and Tennessee 86 (TN86) were used. Natural light intensity ranged from 75 (in the winter months) to 1,700 $\mu E \cdot m^{-2} \cdot s^{-1}$ (in the summer months). In winter (November–March), natural radiation was supplemented with fluorescent or sodium vapor light to provide a 14-hr photoperiod. Temperatures ranged from 16 to 26 C in winter and from 20 to 30 C in summer. *Nicotiana* cultivars were seeded in flats (20 × 31 × 8 cm) containing a peat–perlite potting mix that had been saturated with tap water. When seedlings were approximately 2.5 cm in height, they were transplanted to seedling flats (20 seedlings per flat) which had been filled with a 3:1 combination of potting mix and Maury silt loam soil collected from Spindletop Farm, University of Kentucky, and steam-treated or methyl bromide fumigated and immediately transferred to the growth chamber for preconditioning and growth as described by Reuveni et al (13). Growth chamber conditions were 23 C, light intensity of 125 $\mu E \cdot m^{-2} \cdot s^{-1}$, and a 12-hr photoperiod. Plants were watered daily and fertilized

biweekly with Peters fertilizer solution (14.8 ml of 20-20-20 [N-P-K] soluble fertilizer in 3.78 L of water).

Isolates and inoculum. Isolates of *P. tabacina* used in this research were collected as follows: Texas 1983 isolate (Tx83) collected from fiddle leaf tobacco, *N. repanda,* growing in Uvalde County, Texas; Kentucky 1979 (Ky79) collected in Scott County, Kentucky, during the blue mold epidemic of 1979; and Mexican isolates collected from *P. tabacina*-infected commercial cigar tobaccos in Papantla (Mx87P) and San Andres Tuxtla (Mx87S), Veracruz, Mexico. The Bulgarian isolate (Bul88) was collected by C. E. Main from a formerly blue mold resistant, oriental hybrid 0297 during July 1988 from the Tobacco Research Station, Gotche Delcher, Bulgaria, near the border with Greece.

The methods of Reuveni et al (16) were used for inoculum collection. Fungal spores were cryogenically stored as described by Bromfield and Schmitt (2,5). Spores were suspended in 1.8 ml of 15% dimethylsulfoxide (DMSO) and placed at −20 C for 24 hr then transferred to liquid nitrogen (−196 C).

Individual samples were removed from liquid nitrogen and warmed in a water bath at 40 C. The solution was poured through a 3-μm Millipore filter and rinsed with distilled water to remove the DMSO. The sporangiospores then were resuspended in distilled deionized water and inoculated onto 3- to 4-wk-old *N. tabacum* cv. Ky14 seedlings. Sporangia from the inoculated plants were produced as described previously (16). Inoculum of each isolate was washed using distilled water and a Millipore filtration system with a 3-μm filter to remove autoinhibitors of the sporangiospores. Spores then were resuspended in chilled distilled deionized water, and the concentration was adjusted using a hemacytometer according to the assay protocol.

Isolates from Mexico and Bulgaria were collected as freshly sporulating lesions on commercial tobacco in Mexico (cultivar Viena Amarilla) and from a formerly resistant oriental hybrid being increased for release in Bulgaria. The lesions were collected early in the morning and immediately placed inside plastic bags together with moistened toweling to prevent dehydration. All experiments with foreign isolates were completed in the plant disease containment facility at Frederick, Maryland, with the permission of state and federal authorities.

Large disk assay. Initial experiments were performed on tobacco leaf disks as described by Reuveni et al (15). Tobacco leaves were excised starting from the third expanded leaf from the apical bud of 6-wk-old plants of the tobacco cultivars. Excised leaves from 2- to 3-wk-old Ky14 seedlings were used as a susceptible inoculation control. Leaves then were cut with a razor blade into two panels, each approximately 30 × 40 mm in length. One leaf panel was dipped in acetone for 1 sec, followed by two rinses in distilled deionized water. The acetone treatment was used in this study to alter the susceptibility of the leaf disks by removing surface inhibitory compounds, most notably the α and β 1,3 duvatrienediols (4,16). The other leaf panel was dipped in distilled deionized water. After the acetone and water treatments, thirty 18-mm-diameter disks (no. 13 corkborer) per isolate–cultivar combination were cut from leaf panels. Ten disks were placed in petri dishes containing Whatman no. 1 filter paper. The filter paper was moistened with a 1-mg/ml-concentration kinetin solution, and the dish was further moistened with distilled water.

A sporangiospore suspension of *P. tabacina,* at a concentration of 1.3×10^4 sporangiospores per milliliter, was deposited uniformly on leaf disks by using an airbrush sprayer (Type H1 W/H-3-OZ, Paasche Airbrush Co., Chicago, IL). The dishes were transferred to a dark, moist chamber at 15 C for 24 hr and then maintained in a growth chamber (21 C, 75–80 $\mu E \cdot m^{-2} \cdot s^{-1}$, 12-hr photoperiod) for the completion of the infection cycle. Five days after inoculation, the leaf disks were transferred to glass petri dishes containing water-saturated sponges. The disks were placed over 15-mm-diameter holes in the sponge, misted with distilled water, covered with the glass petri dish top, and returned to the growth chamber. After 2 days, disks were rated for disease severity and sporulation. Disease severity was evaluated on a 0–4 scale as previously described by Reuveni et al (14), based on area of leaf chlorotic or covered with spores, where 0 = no sporulation, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100%.

Small disk assay. The large leaf disk bioassay was initially used to establish baseline disease severity ratings on the CORESTA cultivars. CORESTA cultivars were seeded as described earlier. For each cultivar, six randomly chosen leaves were excised from 5- to 6-wk-old plants. The third leaf from the apical bud was used. Leaf sections were treated with acetone as previously described.

For each cultivar–isolate–treatment combination, five leaf disks were randomly excised from each of the six leaves. Disks were removed using a no. 4 corkborer, 7 mm diameter, from areas between veins with the veinal structures avoided. The 30 disks for each cultivar–isolate–treatment combination were transferred to petri dishes containing a 9.0-cm Whatman no. 1 filter paper saturated with distilled water.

A drop (3–5 μl) of a *P. tabacina* sporangiospore suspension, at a concentration of 50,000 sporangiospores per milliliter, was placed in the center of each disk and incubated for 24 hr at 15 C. After the dark period, the disks were transferred to microscope slides (25 × 76 × 1 mm), 10 disks of each isolate–cultivar–treatment combination per slide. Ten disks were chosen to be placed on each slide for maximal fit.

Sporangiospore germination was observed using a Calcofluor solution, i.e., a fluorescent cellulose marker (17) as described by Cohen et al (4). In the center of each leaf disk, we placed a 3- to 5-ml drop of 0.01% Calcofluor (Calcofluor White M2R, 4,4′-bis(4-anilino-6-diethylamino-*s*-triazin-2-ylamino)-2, 2′-stilbene disulfonic acid; Polysciences, Inc., Warrington, PA 18976). Immediately, approximately 100 μl of Cytoseal (60% toluene, acrylic resin, and antioxidant; Stephens Scientific, Denville, NJ 07834) was added to the surface of each leaf disk to prepare a permanent microscopic mount. Finally, a coverslip (24 × 50 mm, no. 1) was placed over the disks and they were observed microscopically with an Olympus Model BH-2 microscope equipped with a BH-2-RFL fluorescent attachment and an Osram mercury lamp (HBO 100/2), a DM-400 dichroic beam splitter, and a DM-455 barrier filter. Objectives used were D Plan Apochromatic at 10×, NA 0.40, and 20×, NA 0.70, with a 12.5× eyepiece.

Germination was recorded for each tobacco cultivar–fungal isolate combination. Sporangiospores were considered to have germinated when they had produced a germ tube and an appressorial structure. The total number of germinating spores was counted and divided by the total number of sporangiospores present. The sporangiospores that were lysed and/or damaged were not counted as part of the total number. Statistical significance was determined by the GLM procedure of SAS (SAS Institute, Cary, NC) and Duncan's multiple range test ($P < 0.05$). Spearman correlation was used to determine R^2 values.

Table 1. Sources of blue mold resistance for cultivars of *Nicotiana tabacum* used in this study. All cultivars are from the CORESTA trap collection, except Ky14 and TN86

Cultivar	Source of resistance
Ky14	None
TN86	None
Bel 61-10	*N. debneyi*
Bergerac C	None
Chemical Mutant	*N. tabacum* chemically mutated by triethylene iminotrazine
GA 955	*N. excelsior*
R × T	*N. tabacum* × *N. rustica*
Samsoun	None
Trumpf	*N. goodspeedii*
Pobeda 2	*N. debneyi*
Tu 8.4-7.2	*N. debneyi* via Bel 61-10
Ovens 62	*N. velutina*

RESULTS

Disease severity was determined, using the Ky79 isolate, on large leaf disks removed from acetone-treated or non-treated leaf sections of individual 5- to 6-wk-old CORESTA cultivars (Table 2). Following water treatment, the cultivars Pobeda, Chemical Mutant, Ga955, R × T, Trumpf, Bel 61-10, Tu 8.4-7.2, and Samsoun exhibited low disease severity, ranging from 0.5 to 1.5; while the cultivars TN86, Ky14, and Bergerac produced more disease than did all other cultivars, 2.0 and 2.2, respectively. Information from CORESTA (17,18) indicates that all cultivars are resistant to medium-resistant to the blue mold pathogen, with the exception of the susceptible cultivar Bergerac. The water treatment yielded results which followed this trend. Furthermore, the burley cultivars (Ky14 and TN86) added to this system were as susceptible as the CORESTA susceptible cultivar, Bergerac. The acetone treatment resulted in more disease than did the water treatment; the range of disease ratings were 1.6 to 3.1 (Table 2).

Small leaf disk bioassay. The small and large disk bioassays were directly compared using leaf tissue from the same source and the results given in Table 3 and Figure 1. There was a significant positive correlation ($P \leq 0.001$, $R^2 = 0.82$ for water and $R^2 = 0.69$ for acetone treatment) between disease severity and germination percentage. This correlation indicates disease severity and sporangiospore germination (for acetone and water treated leaf disks) using the Ky79 isolate were related. Also, either germination or severity alone may be used to measure potential differences in isolates of *P. tabacina*.

The small leaf disk experiment was initially conducted with both acetone and water treatments of leaf sections of the CORESTA cultivars. In the water treatment (Fig. 2), the overall (across cultivars) germination percentages of the Ky79 and Tx83 isolates were not significantly different ($P < 0.05$). In these particular cultivars, sporangiospore germination was greater with the Tx83 isolate than with the Ky79 isolate. These data support the findings of Reuveni et al (13), who stated that the Tx83 isolate caused greater disease severity on Ky14 than did Ky79.

In the acetone treatments (Fig. 3), the differences in germination between Tx83 and Ky79 were more substantial than on water treated disks. In only one cultivar, Ky14, were the differences not significant ($P < 0.05$). In all other cultivars, Tx83 had a greater percentage of germination on the cultivar leaf surface than did the Ky79 isolate. The Ky79 isolate germinated less frequently on the more resistant cultivars, e.g., Bel 61-10, than on the susceptible cultivars, e.g., Ky14.

Geographically distinct isolates of *P. tabacina*. Isolates from Mexico, Bulgaria, and Kentucky reacted significantly

Table 2. Differences in disease severity on leaf disks of specific cultivars of *Nicotiana tabacum* treated with acetone or water, followed by inoculation with sporangiospores of isolate Ky79 of *Peronospora tabacina*

	Treatment[y]	
Cultivar	Water[z]	Acetone
Ky14	2.1 a	3.0 a
TN86	2.2 a	3.1 a
Samsoun	1.3 b	2.8 a
Trumpf	1.5 ab	2.7 a
Pobeda 2	0.9 b	2.6 ab
Chemical Mutant	0.6 b	2.0 bc
Bergerac	2.0 a	2.8 a
Ga955	1.1 b	1.6 c
Bel 61-10	0.5 bc	1.7 c
R × T	0.8 b	2.4 b
Tu 8.4-7.2	0.8 b	2.2 bc

[y] Four leaves per cultivar-treatment were used for the disease severity rating. Disease severity ratings were based on a 0–4 scale where 0 = 0% disease, 1 = 1–25% of the leaf surface chlorotic; 2 = 26–50% chlorotic, 3 = 51–75% chlorotic, and 4 = 76–100% chlorotic. All acetone treatments are significantly different ($P > 0.01$) from the water treatments except for Ga955. The severity rating on the Ky14 control (2–3 wk old) was 4.0 for both treatments.

[z] Different letters within columns denote significant differences, $P < 0.01$, among cultivars as determined by Duncan's multiple range test.

Table 3. Disease severity and germination percentage of *Peronospora tabacina* sporangiospores after inoculation with the Ky79 isolate onto 6-wk-old CORESTA *Nicotiana tabacum* cultivars treated with acetone or water

	Water treatment		Acetone treatment	
Cultivars	DS[y]	GP	DS	GP
Ky14	2.0 a[z]	30 a	3.0 a	60 b
TN86	2.3 a	28 a	3.2 a	62 b
Bergerac	2.0 a	31 a	2.8 b	58 c
Samsoun	1.3 b	16 b	2.0 c	40 d
Trumpf	1.0 b	21 b	3.2 a	70 a
Pobeda	0 d	3 c	2.6 b	60 b
Chemical Mutant	0 d	0 d	2.0 c	54 c
Ga955	0 d	0 d	1.5 d	39 d
Bel 61-10	1.1 b	20 b	1.5 d	50 c
Tu 8.4-7.2	0 d	3 c	2.0 c	53 c
R × T	0.5 c	20 b	2.5 b	50 c

[y] DS denotes the disease severity on either water- or acetone-treated inoculated leaf disks, 13 mm in diameter, 30 per cultivar. DS was on a 0–4 scale where 0 = 0% disease, 1 = 1–25% chlorotic, 2 = 26–50% chlorotic, 3 = 51–75% chlorotic, and 4 = 76–100% chlorotic. GP denotes germination percentage on the leaf surface of at least 10 disks per cultivar. GP was determined by observation of sporangiospores stained with 0.01% Calcofluor using fluorescence microscopy. DS of Ky14 untreated controls was 4.0; GP was 76 for water and 81 for acetone treatment.

[z] Different letters within columns denote significant differences, $P < 0.01$, among cultivars as determined by Duncan's multiple range test.

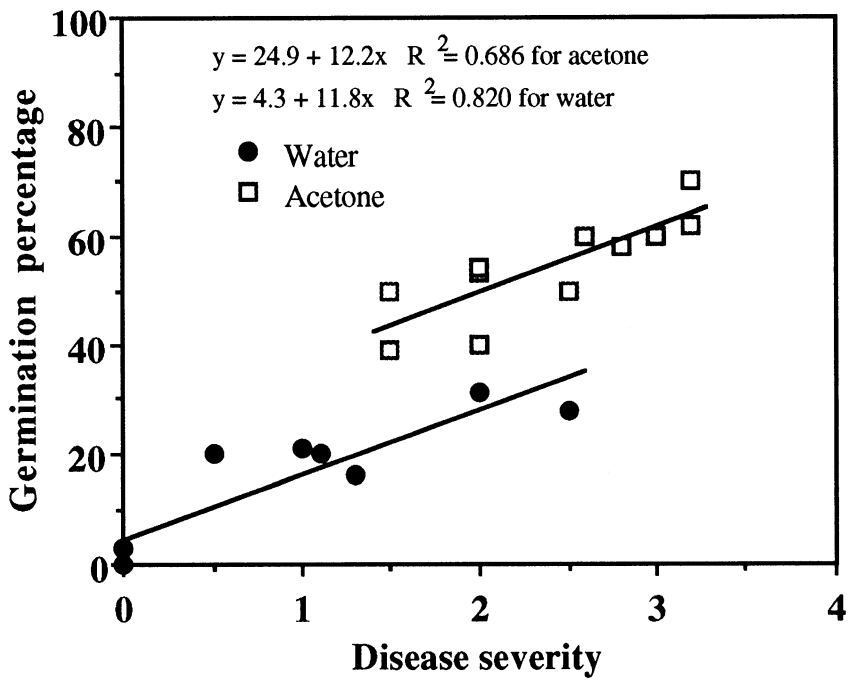

Fig. 1. Relationship between disease severity and germination percentage comparing the small leaf disk germination bioassay and the large leaf disk disease severity bioassay using all cultivars.

differently ($P < 0.05$) in the small leaf disk assay. Among all cultivars, the Mexican isolates had an overall germination of 83 ± 5.0% and 89 ± 4.1%, the Bulgarian 17 ± 9.5%, and the Ky79 isolate 37 ± 3.5% (Table 4). The overall germination–penetration percentage easily distinguishes all of the geographically distinct isolates. Furthermore, with the exception of the cultivar Chemical Mutant, this ranking held true among isolates from Mexico, Kentucky, and Bulgaria. The two Mexican isolates in this test were not significantly different when the overall mean was calculated, although reactions on four cultivars were significantly different ($P < 0.05$), indicating a detectable difference between the two Mexican isolates.

DISCUSSION

Worldwide, the CORESTA trap collection has been the preferential assay for differentiating changes in virulence in *P. tabacina* (6,7,10). However, the collection has its limitations (8,13,19).

In the present investigation, the CORESTA assay was modified so that 1) the soil, plant growth, and laboratory assays would be under controlled environments; 2) specified inoculum concentrations would be applied; 3) a 1-sec acetone leaf-wash treatment would be included to eliminate leaf-surface inhibitors; and 4) burley cultivars Ky14 and TN86 would be included to make the results applicable to burley tobacco production areas.

The large leaf bioassay was used previously to show that differences existed in pathogenicity between the *P. tabacina* isolates Ky79 and Tx83 (13). The large leaf system differentiated isolates based on pathogenicity, and the experiments required only a small amount of space. However, the large leaf disk system had several limitations, including low disease severity in water treatments, subjectivity of the disease ratings, and the need for sufficient knowledge of the disease symptomology for evaluation. Consequently, to utilize the bioassay worldwide, the rating system must be more concise and less subjective.

Cohen et al (4) used a similar technique to observe sporangiospores of the blue mold fungus on the tobacco leaf surface, and we have adapted this technique for our purposes. In our small leaf disk assay, the smaller diameter leaf disk and inoculation point facilitated locating the spores and the determination of germination percentage, thus increasing the number of observations and replication attainable over a period of time. The germination percentage in the small disk bioassay was determined by counting the number of germinating and penetrating sporangiospores, and dividing by the total number of intact sporangiospores. Damaged sporangiospores were not counted, as they had no potential to initiate infection; in addition, the numbers of such sporangiospores were extremely low.

When the small leaf disk bioassay was compared to the large leaf bioassay using the Ky79 isolate of *P. tabacina,* there was a correlation between disease severity and sporangiospore germination. Based on this result, it was speculated that either bioassay could be used for differentiation of isolates, although only one isolate of *P. tabacina* was used. Furthermore, the slopes of the regression lines of the water and acetone treatments are nearly parallel, 11.8 for the water treatment and 12.2 for the acetone treatment. The acetone treatment resulted in greater disease on all CORESTA cultivars, and the parallel nature of the regression lines strongly suggested that the removal of acetone-soluble compounds did not eliminate all disease inhibitory factors. Inhibitory compounds other than duvatrienediols may be present on the surface of the tobacco leaf (12).

There are several advantages of the small leaf disk assay that make it preferable to the large leaf assay. The small leaf disk assay was more quantitative than the large leaf assay. The observer could directly observe and count the fluorescing sporangiospores, germ tubes,

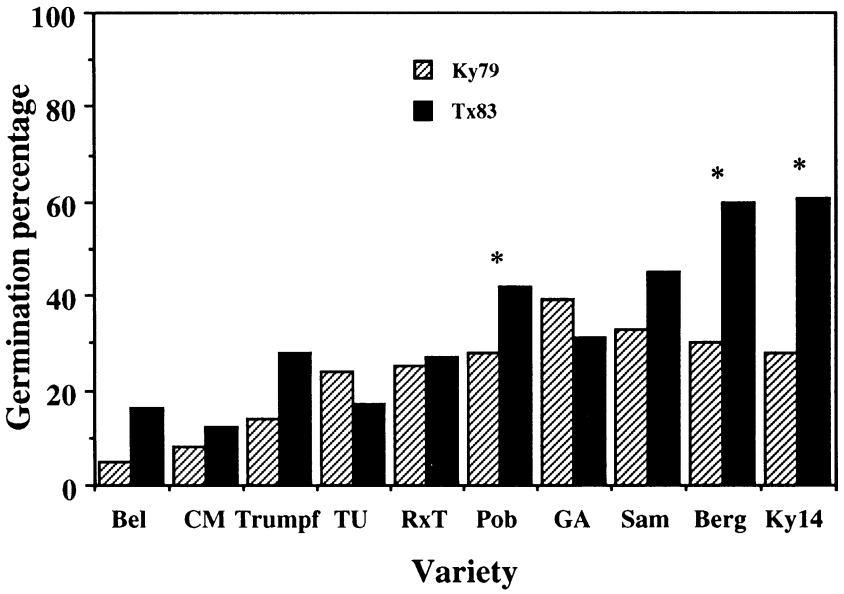

Fig. 2. Germination percentages of sporangiospores of the Ky79 and Tx83 isolates of *Peronospora tabacina* on small leaf disks from water-treated leaf sections of individual cultivars of *Nicotiana tabacum.* Significant differences ($P > 0.05$) between the Ky79 and Tx83 isolates in individual cultivars are denoted by *.

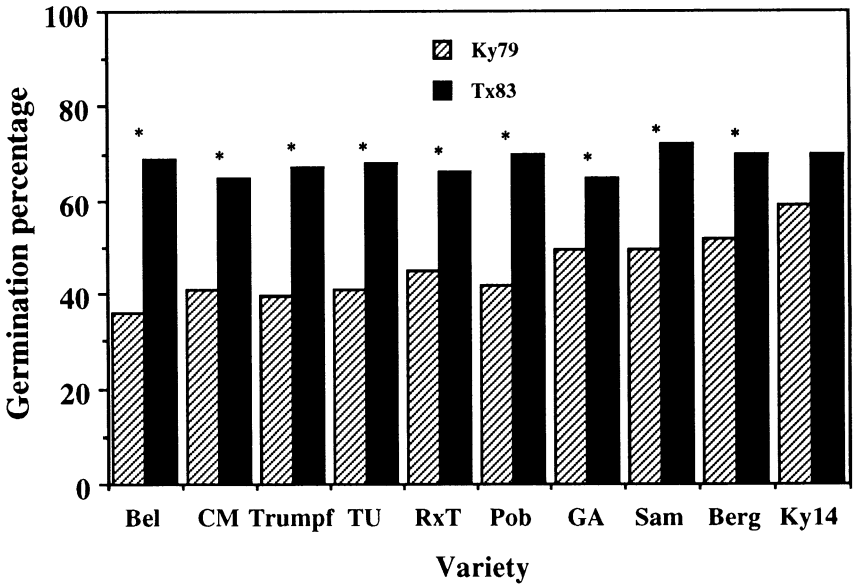

Fig. 3. Germination percentage of sporangiospores of the Ky79 and Tx83 isolates of *Peronospora tabacina* on small leaf disks from acetone-treated leaf sections of individual cultivars of *Nicotiana tabacum.* Significant differences ($P > 0.05$) between the Ky79 and Tx83 isolates in individual cultivars are denoted by *.

Table 4. Germination percentage of sporangiospores of *Peronospora tabacina* isolates collected from Kentucky, Mexico, and Bulgaria. The isolates were tested using the small leaf disk assay on 6-wk-old acetone-treated cultivars from the CORESTA tobacco collection

	Isolate			
Cultivar	**Mx87P**	**Mx87S**	**Ky79**	**Bul88**
Ky14	93.1 a[y]	79.3 b	54.0 c	2.2 d
Bergerec C	90.8 a	82.4 b	28.9 c	1.3 d
R × T	73.3 a	69.8 a	33.3 c	3.4 d
Samsoun	90.7 a	88.8 a	43.9 b	12.1 c
Pobeda 2	89.3 a	82.9 b	53.1 b	35.0 c
Ga955	91.3 a	83.8 b	48.3 c	29.2 d
Chemical Mutant	71.4 a	74.7 a	36.7 b	38.0 b
Trumpf	76.9 a	82.5 a	30.6 b	2.1 c
Ovens 62	92.8 a	89.5 a	25.3 b	0.8 c
Mean[z]	83 a	89 a	37 c	7 b

[y] Percentages represent mean of three replications of the isolate–cultivar combinations.
[z] Lowercase letters across rows denote significant differences, $P < 0.01$, among cultivars within an isolate as determined by LSD.

and appresorial point. Secondly, with the use of Cytoseal, slides can be preserved as a permanent mount and data could be collected over time. The large leaf disk assay required a rigid timetable for recording disease data. And lastly, the small disk assay requires considerably less growth chamber space than the large leaf disk assay. Moreover, the smaller leaf disk bioassay requires only overnight dark incubation at 18 C.

Reuveni et al (14), using the large leaf disk assay, determined that isolates Tx83 and Ky79 were different in disease severity on the cultivar Ky14, with Tx83 producing more disease than Ky79. The small leaf disk assay was used to verify this observation and to further evaluate the similarity of the two leaf bioassays. Because the response of the TN86 cultivar was very similar to Ky14 in all previous tests, it was removed from the assay collection.

Isolates collected from Mexico in 1987 and Bulgaria in 1988 were compared to the burley tobacco isolate, Ky79. Whereas the Mexican isolates germinated well on all cultivars, the Bulgarian isolate germinated well only in water or on leaf surfaces of highly resistant cultivars, e.g., Chemical Mutant. This is somewhat confusing, as the isolate collected in Bulgaria was noted to be extremely virulent to a previously known resistant tobacco hybrid (C. E. Main, *unpublished*).

The experiments conducted at Ft. Detrick indicated a couple of positive aspects of the small leaf disk assay. One was that the bioassay can be used in locations without compromising the integrity of the system. The other was that it gives yet another marker for differentiation of the Mexican isolates. Until now, Mexican isolates only could be distinguished from other *P. tabacina* isolates by their metalaxyl resistance (21).

The goal of our research was to determine whether an isolate differentiation bioassay could be utilized with minimal equipment in the laboratory and standardized for worldwide use. The small leaf disk bioassay described here suits our original intentions by using a cultivar system in a laboratory setting and eliminating problems associated with field experiments.

One disadvantage of the small leaf disk bioassay is the large number of observations required in a given experiment. This can be tedious and time-consuming. Although the small disk and large disk assays were correlated in terms of ratings, questions can be raised as to the reality of a system that uses a single aspect of a disease cycle. Incubation period, lesion expansion (invasion), sporulation, latent period, and infection period have all been studied in other host–pathogen systems. The acetone wash treatment contributes to the artificiality of this assay because the natural chemical barriers have been mitigated or diminished. Reuveni et al (14) assessed the severity of infection while the CORESTA trap collection evaluated the incidence of severity and sporulation. If all assays are utilized, the complexity of the host–pathogen relationship may be better understood.

ACKNOWLEDGMENTS

We thank William Dowler for permission to use the facilities at the USDA–Foreign Disease–Weed Science Research Unit. We also thank Gary Peterson, Moshe Reuveni, Said Ghabriel, J. Juarez, Paul Shoemaker, and David Lemke for invaluable assistance. This research was supported in part by the R.J. Reynolds tobacco company. This paper is a Kentucky Agricultural Experiment Station Journal Series Paper, no. 92-11-218.

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