Race 3 of Phytophthora parasitica var. nicotianae

John L. McIntyre and G. S. Taylor

Assistant Plant Pathologist and Plant Pathologist, The Connecticut Agricultural Experiment Station, New Haven, CT 06504, and Windsor, CT 06095, respectively.

The authors thank S. Rich for allowing the use of unpublished data from transmission electron microscope observations of zoospores. We also acknowledge the excellent technical assistance of M. Reisner and B. Wooding.

Accepted for publication 15 July 1977.

ABSTRACT

MC INTYRE, J. L., and G. S. TAYLOR. 1978. Race 3 of Phytophthora parasitica var. nicotianae. Phytopathology 68:35-38.

Connecticut (CT) isolates of *Phytophthora parasitica* var. *nicotianae* cause typical black shank symptoms on tobacco and have similar morphology to isolates of races 0 and 1. It differs, however, in that the symptom response of the differential tobacco *Nicotiana nesophila* to CT is similar to that caused by race 1, but on cultivar 1071 the symptom response is similar to that caused by race 0. Tobacco cultivar A23, which is resistant to race 2, is susceptible to CT, and cultivar L8 is moderately susceptible to CT. After 5 days of growth in liquid culture, the dry weight of CT mycelia is 50% less than that of races 0 or 1. Races 0 or 1 cause the pH of culture broth to change from 7.2 to about 5.3 but in broth cultures of CT pH remains essentially unchanged. On an agar medium containing sucrose and 2,3,5-triphenyl tetrazolium chloride (TTZ), the TTZ is reduced by races 0 and 1 but not by CT. Isolates of CT qualify as a new race, race 3, of *P. parasitica* var. *nicotianae*.

Physiologic races of a fungus are defined as being similar in morphology but unlike in certain cultural, physiological, biochemical, pathological, or other characters (1).

Races 0 and 1 of *Phytophthora parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker [=*P. nicotianae* var. *nicotianae* (Breda de Haan) Waterhouse (21)] the causal organisms of tobacco black shank, occur in most tobacco growing regions of the world (10). Race 2 of this pathogen has been reported from South Africa (16). These races can be separated by their pathogenicity to differential tobacco cultivars or breeding lines (5, 7, 8, 23, 24). In 1973, black shank of tobacco was observed for the first time in Connecticut (20), and by 1976 the causal organism had spread to at least 24 fields in the Connecticut River Valley. We have reported (14) that Connecticut (CT) isolates of this pathogen do not produce symptoms similar to those caused by races 0 or 1 when stem-inoculated into differential tobacco cultivars. McIntyre and Hankin (12) also have shown a biochemical difference in that races 0 and 1, but not CT, produce kestoses when grown in a broth containing sucrose. We report here on experiments to determine other differences between CT and races 0 and 1.

MATERIALS AND METHODS

Connecticut isolates.—*Phytophthora parasitica* var. *nicotianae* was isolated from *Nicotiana tabacum* L. 'Windsor Shade 117' (WS 117) in Connecticut during the summers of 1973, 1974, and 1975. Isolations were made from infected tissue onto a *Phytophthora*-selective medium (19), and mycelia from pure cultures were stem-inoculated (5, 24) into WS 117 to verify pathogenicity. The pathogen was reisolated as before from plants with symptoms. Pure cultures of these isolates were maintained in the dark at 25 C on agar slants of a mineral salt medium (MM) (25) containing vitamins (3), 0.1% casamino acids (Difco Laboratories, Detroit, MI 48232), and 1.5% (w/v) glycerol, or on P-1 medium containing 3% (w/v) sucrose and 0.2% (w/v) lecithin (Type II-S from soybeans; Sigma Chemical Co., St. Louis, MO 63178 (6).

To maintain virulence, the isolates were stem-inoculated into WS 117 at least once every 6 mo, and then reisolated and purified as before. Ten different CT isolates were used for these studies.

Race 0 and 1 isolates.—The following isolates of races 0 and 1 of *P. parasitica* var. *nicotianae* were used: race 0 (isolate 1587, from C. Litton, University of Kentucky, Lexington, KY 40506; isolate 1189-A-2 from J. Apple, North Carolina State University, Raleigh, NC 27607; isolates 1189-A-3, A-10-2, M-16-(9), and D-Barrow-1, from G. Lucas, North Carolina State University, Raleigh, NC 27607; isolates 42 and 230, from R. Flowers, Georgia Coastal Plains Experiment Station, Tifton, GA 31794; isolates M-15E, M-15H, and M-15I, source unknown); and race 1 (isolate 1668-1 from C. Litton, isolate 1452-1-2-2 from J. Apple, isolate 85-B-1-75 from G. Lucas, isolates 43 and 228, from R. Flowers, and isolates M-15F and M-15G, source unknown). Isolates were stored and virulence maintained as described above. All isolates were used for each study unless otherwise noted.

Morphology.—Single isolates of the test organisms were induced to form sporangia as previously described (14). Microscopic examination (×450 magnification) of the mycelia, mature sporangia, and sporangiophores of 5-day-old cultures was made. To obtain an average, 50 individual mycelia or sporangia and 25 sporangiophores of each isolate were examined.

0032-949X/78/000005 $03.00/0

Copyright © 1978 The American Phytopathological Society, 3340 Pilot Knob Road, St. Paul, MN 55121. All rights reserved.

Pathogenicity.—*Nicotiana nesophila* [resistant to race 1, susceptible to race 0 (9)], and *N. tabacum* cultivar WS 117 [susceptible to races 0, 1, and CT (14)] were used. Also used were *N. tabacum* cultivars L8 [resistance from *Nicotiana plumbaginifolia* (23)] and 1071 [resistance from *Nicotiana longiflora* (23)], both of which are resistant to race 0 and susceptible to race 1 (23). Included in this study was tobacco cultivar A23, which is susceptible to races 0 and 1 and resistant to race 2 (7, 8). Resistance of A23 to race 2 is from breeding lines M.K. 95 or M.K. 165, both of which confer to their progenies the Delcrest 202 type of resistance (conferred by a single dominant gene) to infection by race 2 the black shank pathogen (7, 8). The seed of A23 was obtained from M. P. Lamprecht, Tobacco Research Institute, Rustenberg, South Africa. Tobacco seedlings were grown as previously described (13), and 12- to 16-wk-old plants were stem-inoculated with mycelia and observed 5 days later for black shank symptoms. Five plants were stem-inoculated with each isolate, and the experiment was repeated once.

Some cultural and biochemical features unique to CT.—*Growth in culture broth.*—We have observed on several solid media that CT grows more slowly than races 0 or 1. To quantitate this apparent difference, isolates (five isolates each of races 0, 1, and CT) were grown on solid P-1 medium and mycelial plugs were cut with a sterile No. 2 cork borer from 5-day-old cultures. One mycelial plug was placed into a 125-ml Erlenmeyer flask (five replicates) containing 25 ml of P-1 broth (6). Cultures were incubated in the dark at 25 C and mycelia from each flask were harvested after 5 days onto oven-dried (100 C, 24 hr) and weighed Whatman No. 1 filter paper disks (vacuum filtration using a Büchner funnel). The mycelium on the paper disk was oven-dried for 24 hr at 100 C, weighed, and the mycelium dry weight was determined by difference. The initial and final pH of the P-1 broth was also determined.

Carbohydrate utilization.—Shepherd and Pratt (18) reported growth of Australian isolates of *P. parasitica* var. *nicotianae* on arabinose and raffinose. However, Wills (22) did not observe any growth of a North American isolate of the fungus on these carbohydrates. Therefore, an attempt was made to differentiate isolates by their ability or inability to grow on various carbohydrates. Isolates were grown for 5 days at 25 C in the dark on the mineral medium (MM) containing vitamins but no carbohydrate. Plugs of these carbohydrate-starved mycelia were cut from these plates with a No. 2 cork borer and transferred to MM or MM containing 0.2% w/v of either glucose, maltose (Mallinckrodt, St. Louis, MO 63160), arabinose, raffinose, cellobiose, or N-acetyl-glucosamine (Sigma Chemical Co.). Cultures were incubated in the dark for 4 days at 25 C at which time the radius of mycelial growth was measured.

Mycelial plugs also were transferred to nutrient agar (Difco) which was overlaid with 5 ml of 1.1% Noble agar (Difco) containing either 0.2% w/v chitin, inulin, dextran, xylan (Sigma Chemical Co.), galactan (Calbiochem, San Diego, CA 92037), or araban (Pierce Chemical Co., Rockford, IL 61105). Cultures were incubated for 8 days at 25 C in the dark and the mycelial growth was measured. Clear zones around the mycelium in the otherwise opaque medium indicated hydrolysis of the substrate.

TABLE 1. Dimensions of sporangiophores, sporangia, hyphae, and zoospores of races 0, 1, or Connecticut (CT) isolates of *Phytophthora parasitica* var. *nicotianae*[a]

Structure	Isolate and dimensions ($\mu m \pm S_{\bar{x}}$)		
	Race 0	Race 1	CT
Sporangiophore	173.2 ± 12.0	190.8 ± 11.5	170.4 ± 9.7
Sporangia	(45.8 ± 0.7) × (37.8 ± 0.6)	(43.6 ± 0.6) × (35.4 ± 0.4)	(41.3 ± 0.6) × (31.7 ± 0.4)
Hyphae (width)	5.2 ± 0.2	5.1 ± 0.1	5.2 ± 0.1
Zoospores[b]	10-14 × 4-7	10-14 × 4-7	10-14 × 4-7

[a]Individual isolates of race 0, 1, or CT were induced to form sporangiophores and sporangia. These structures and hyphae of 5-day-old cultures were observed at ×450 magnification. Values were determined from 50 observations except for sporangiophores, for which 25 observations were made.

[b]Zoospore sizes were determined by transmission electron microscopy.

TABLE 2. Percentage of tobacco plants with stem necrosis 5 days after stem-inoculation of tobacco cultivars and lines with mycelia from races 0, 1, or Connecticut (CT) isolates of *Phytophthora parasitica* var. *nicotianae*[a]

Tobacco cultivar, species, or line[b]	Plants with symptoms ($\% \pm S_{\bar{x}}$)		
	Race 0[c]	Race 1[c]	CT[c]
L8	0	100	56.0 ± 4.0
1071	0	100	0
Nicotiana nesophila	83.6 ± 3.1	27.1 ± 3.4	24.0 ± 2.3
A23	100	100	100
WS 117	100	100	100

[a]Five plants of each tobacco cultivar or line were individually stem-inoculated with mycelium from race 0 (11 isolates), race 1 (seven isolates), or CT (10 isolates), and the experiment was repeated once. Data are presented as averages for all observations.

[b]Twleve- to 16-wk-old tobacco cultivars or lines were used in this study.

[c]Total number of observations: race 0 = 110; race 1 = 70; and CT = 100.

Reaction on 2,3,5-triphenyl tetrazolium chloride (TTZ).—The MM was prepared to contain 0.1% w/v sucrose and 0.05% w/v TTZ (Sigma Chemical Co.). Plugs were cut with a No. 2 cork borer from 5-day-old cultures growing on the P-1 medium and transferred to the TTZ medium. Cultures were incubated in the dark at 25 C and observed at 24 and 120 hr. Reduction of TTZ is indicated if the mycelial plug becomes red.

RESULTS

Morphology.—Races 0, 1, and CT were morphologically similar (Table 1). Sporangia (average sizes = 41.3 to 45.8 × 31.7 to 37.8 μm) typically had an apical "beak" and were borne terminally on the sporangiophores (average lengths = 170.4 to 190.8 μm).

Pathogenicity.—*Nicotiana nesophila* and *N. tabacum* L8 and 1071 all exhibited typical differential responses to races 0 and 1 (Table 2). *Nicotiana nesophila* and 1071 both were resistant to CT, and A23 and WS 117 both were susceptible to races 0, 1, and CT. A typical susceptible response was evident within 48 hr of inoculation by necrotic tissue which extended 1 to 2 cm around the inoculation site. Within 4 to 5 days after inoculation the necrotic tissues extended at least 5 cm above and below the inoculation site and the tissues were collapsed. About 50% of the L8 plants exhibited black shank symptoms within 5 days of inoculation with CT. However, the symptom response of L8 to CT was moderate; stem necrosis usually extended only several cm above and below the inoculation site.

Some cultural and biochemical features unique to CT.—*Growth in culture broth*.—After 5 days growth in P-1 broth, the average dry weight of CT mycelia was 23.4 mg while that for races 0 or 1 was 47.2 and 50.4 mg, respectively (Table 3). This difference was significant at $P = 0.01$ as indicated by Duncan's new multiple range test.

Connecticut isolates reduced the pH of P-1 broth from 7.22 to 6.78 while races 0 and 1 reduced the pH to 5.38 and 5.25, respectively (Table 3). This difference was statistically significant, $P = 0.01$.

Carbohydrate utilization.—Races 0, 1, and CT grew on all of the carbohydrates tested. Of the mono-, di-, and tri-saccharides tested, race 0 grew best on maltose (3.70 ± 0.40 mm radial growth = average radial growth for all observations ± $S_{\bar{x}}$) and poorest on N-acetyl-glucosamine (2.36 ± 0.28 mm). Race 1 grew best on glucose (4.00 ± 0.31 mm) and poorest on cellobiose (2.29 ± 0.42 mm), and CT grew best on maltose (4.50 ± 0.45 mm) and poorest on raffinose (2.70 ± 0.26 mm). On the polysaccharides, races 0 and 1 grew best on inulin (15.20 ± 0.85 mm and 19.29 ± 1.57 mm, respectively), whereas CT grew best on galactan (14.20 ± 1.20 mm). Poorest growth of race 0 was on chitin (8.09 ± 0.50 mm) whereas race 1 grew poorest on araban (11.14 ± 1.10 mm) and CT on xylan (6.90 ± 0.60 mm). Zones indicating substrate hydrolysis were not evident.

Reaction on 2,3,5-triphenyl tetrazolium chloride (TTZ).—Races 0 and 1 reduced TTZ within 24 hr of incubation, and a slight reddening of the plug was evident within 60 min of incubation. Connecticut isolates did not reduce this dye even after 120 hr of incubation.

DISCUSSION

Phytophthora parasitica var. *nicotianae* was isolated from tobacco growing in fields in Connecticut and produced typical black shank symptoms, including "disking" of the pith (10), on inoculated tobacco plants. The morphology of CT was similar to the descriptions of this fungus by Waterhouse (21).

We have confirmed the results of our earlier experiments (14) on the pathogenicity of CT on 8-wk-old tobacco plants. In addition, we have shown that tobacco cultivar A23, resistant to race 2 (7, 8), is susceptible to CT. Results of our earlier study, in which immature tobacco seedlings were used (14), suggested that CT may be more pathogenic than races 0 and 1. This could explain, at least in part, the results obtained with L8, which was moderately susceptible to CT, since Apple (2) has reported that highly pathogenic isolates could parasitize "resistant" plants. However, the response of *N. nesophila* and 1071 to CT (race 1 and race 0, respectively) indicates that CT is pathogenically unlike races 0 or 1.

The physiological and biochemical studies indicated additional differences between CT and races 0 and 1. Races could not be separated by their ability or inability to use various carbohydrates. However, the growth of races 0 and 1 in P-1 broth, as determined by mycelial dry weights, was about twice that of CT. The growth of races 0 and 1 also caused the culture broth to become more acid (final pH = 5.38 and 5.25, respectively) than did CT (final pH = 6.78). This difference may be due to the reduced growth of CT as compared to races 0 and 1. However, differences in growth and pH can be used as criteria to separate races 0 and 1 from CT.

All of the race 0 and 1 isolates that we have studied can be separated from CT isolates by the reaction on the TTZ medium. In fact, this observation has now been made for

TABLE 3. Dry weight of mycelium of races 0, 1, and Connecticut (CT) isolates of *Phytophthora parasitica* var. *nicotianae*, and final pH of culture broth, after 5 days of growth in liquid medium[a]

Isolate	Determinations (no.)	Dry weight of mycelium (mg ± $S_{\bar{x}}$)[b]	Final pH ± $S_{\bar{x}}$[bc]
Race 0	25	47.2 ± 2.0 a	5.38 ± 0.05 a
Race 1	25	50.4 ± 2.0 a	5.25 ± 0.03 a
CT	25	23.4 ± 1.0 b	6.78 ± 0.09 b

[a]Five isolates each of race 0, race 1, and CT were grown for 5 days in 25 ml of P-1 broth (6). Mycelia were harvested on dried and tared Whatman No. 1 filter paper disks and dry weight determined. The final pH of the culture broth was also determined.

[b]Data were analyzed by Duncan's new multiple range test. Values followed by different letters are significantly different, $P = 0.01$.

[c]Initial pH ± $S_{\bar{x}}$ of the P-1 broth was 7.22 ± 0.03.

a total of 13, 9, and 12 isolates of race 0, 1, and CT, respectively. Tetrazolium salts, normally colorless, are reduced to colored formazans by several dehydrogenases in metabolically active cells (11). This dye has been used to separate respiratory efficient (reduce the dye) from respiratory inefficient (do not reduce the dye) microorganisms (15, 17). Our results suggest that races 0 and 1, which reduce this dye, may be respiratory efficient, while CT isolates are not. If true, this could also explain the differences in the rate of growth of CT as compared to races 0 and 1.

Hankin and McIntyre (4) have demonstrated that races 0 and 1, when grown in a broth containing 3% sucrose, produce a trisaccharide, kestose, whereas CT does not (12). This provides a biochemical method to separate CT isolates from races 0 and 1.

Our results show that although CT causes typical black shank symptoms and has a similar morphology to races 0 and 1, it differs significantly from these races in pathological, physiological, and biochemical traits. Thus, according to the currently accepted definition of physiologic races of Ainsworth (1), CT isolates qualify as race 3 of *P. parasitica* var. *nicotianae*.

LITERATURE CITED

1. AINSWORTH, G. C. 1971. Ainsworth and Bisby's dictionary of the fungi, 6th ed. Commonwealth Mycological Institute, Kew, Surrey, England. 663 p.
2. APPLE, J. L. 1957. Pathogenic, cultural, and physiological variation within Phytophthora parasitica var. nicotianae. Phytopathology 47:733-740.
3. BEADLE, G. W., and E. L. TATUM. 1945. Neurospora. II. Methods of producing and detecting mutations concerned with nutritional requirements. Am. J. Bot. 32:678-686.
4. HANKIN, L., and J. L. MC INTYRE. 1977. Production of kestoses (fructosylsucroses) by Phytophthora parasitica var. nicotianae. Appl. Environ. Microbiol. 33:522-524.
5. HENDRIX, J. W., and J. L. APPLE. 1967. Stem resistance to Phytophthora parasitica var. nicotianae in tobacco derived from Nicotiana longiflora and N. plumbaginifolia. Tob. Sci. 11:148-150.
6. HOHL, H. R. 1975. Levels of nutritional complexity in Phytophthora: lipids, nitrogen sources and growth factors. Phytopathol. Z. 84:18-33.
7. LAMPRECHT, M. P. 1973. Breeding flue-cured tobacco resistant to South African black shank (Phytophthora nicotianae (B. de Haan) var. nicotianae). Agroplantae 5:67-72.
8. LAMPRECHT, M. P., G. C. PRINSLOO, and R. J. VAN WYK. 1974. Inheritance of resistance to race 2 of the black shank fungus, Phytophthora nicotianae (B. de Haan) var. nicotianae, of tobacco. Agroplantae 6:73-76.
9. LITTON, C. C., G. B. COLLINS, and P. D. LEGG. 1970. Reactions of Nicotiana tabacum and other N. species to race 0 and 1 of Phytophthora parasitica var. nicotianae. Tob. Sci. 14:128-130.
10. LUCAS, G. B. 1975. Diseases of tobacco, 3rd ed. Biological Consulting Associates, Raleigh, North Carolina. 621 p.
11. MARTINEZ-RODRIQUEZ, R., A. TOLEDANO, and M. GONZALEZ. 1972. Reduction of the tetrazolium salts in cells and tissue sections incubated without substrate. Ann. Histochim. 17:233-242.
12. MC INTYRE, J. L., and L. HANKIN. 1977. Lack of kestose (fructosylsucrose) production by race 3 of Phytophthora parasitica var. nicotianae differentiates it from races 0 and 1. Mycologia 69:756-760.
13. MC INTYRE, J. L., and P. M. MILLER. 1976. Competitive interaction of Tylenchorhynchus claytoni and Pratylenchus penetrans in tobacco roots. Phytopathology 66:1427-1430.
14. MC INTYRE, J. L., and G. S. TAYLOR. 1976. Screening tobacco seedlings for resistance to Phytophthora parasitica var. nicotianae. Phytopathology 66:70-73.
15. OGUR, M., R. ST. JOHN, and S. NAGAI. 1957. Tetrazolium overlay technique for population studies of respiration deficiency in yeast. Science 125:928-929.
16. PRINSLOO, G. C., and G. D. C. PAUER. 1973. Die identifikasie van rasse van Phytophthora nicotianae (B. de Haan) Nicotianae wat in Suid Afrika voorkom. Phytophylactica 6:217-220.
17. RAUT, C. 1953. A cytochrome deficient mutant of Saccharomyces cerevisiae. Exp. Cell Res. 4:295-305.
18. SHEPHERD. C. J., and B. H. PRATT. 1973. Characterization of an isolate of Phytophthora nicotianae var. nicotianae from North Queensland. Austr. J. Biol. Sci. 26:1087-1094.
19. TSAO, P. H., and G. OCANA. 1969. Selective isolation of species of Phytophthora from natural soils on an improved antibiotic medium. Nature (Lond.) 223:636-638.
20. WALTON, G. S., and S. RICH. 1974. Serious and unusual plant diseases in Connecticut in 1973. Plant Dis. Rep. 54:428-429.
21. WATERHOUSE, G. M. 1970. The genus Phytophthora de Bary. Mycol. Pap., No. 122. Commonwealth Mycological Institute. Kew, Surrey, England. 59 p.
22. WILLS, W. H. 1954. The utilization of carbon and nitrogen compounds by Phytophthora parasitica Dasture var. nicotianae (Breda de Haan) Tucker. J. Elisha Mitchell Sci. Soc. 70:231-235.
23. WILLS, W. H. 1971. A comparison of black shank resistance of tobacco measured in inoculated whole plants and excised leaf tissue. Tob. Sci. 15:47-50.
24. WILLS, W. H., and L. D. MOORE. 1971. Response of some cultivars and lines of tobacco to stem inoculation with Phytophthora parasitica var. nicotianae. Tob. Sci. 15:51-53.
25. ZUCKER, M., and L. HANKIN. 1970. Regulation of pectate lyase synthesis in Pseudomonas fluorescens and Erwinia carotovora. J. Bacteriol. 104:13-18.