The Influence of a Fungicide on the Epidemiology of Black Shank of Tobacco

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ABSTRACT

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Inoculum densities near 0.5 propagule of *Phytophthora parasitica* var. *nicotianae* per gram of soil resulted in 93 to 100% mortality of susceptible tobacco plants and apparent infection rates (r) of 0.08 to 0.11/unit/day in the plant growth room, greenhouse, or field. Disease was initiated sooner in the field but the time required for 50 and 90% mortality to occur was shorter under artificial conditions. No black shank occurred under plant growth room conditions with 1 or more mg/kg (ppm, w/w) of an experimental fungicide, CGA 48988 (CIBA-GEIGY Corp.), incorporated into soil containing 0.5 propagule per gram of soil; under greenhouse conditions 93, 60, 7, and 0% of the susceptible tobacco plants died at 0.5 propagule per gram of soil plus 0, 1, 10, or 50 ppm of the fungicide. After approximately 130 ml of a 150-μg/ml solution of the fungicide was added to individual susceptible transplants in the field at the time of planting, disease development initially was delayed, but after 50 days it progressed more rapidly (r = 0.22/unit/day) than in nontreated plants (r = 0.10/unit/day). All of the nontreated and treated susceptible plants and 61 and 10% of the nontreated and fungicide-treated resistant plants, respectively, were dead at harvest. The fungicide treatment resulted in significant increases ($P = 0.05$) in yields (based on six successive leaf harvests per treatment) of susceptible and resistant plants over nontreated plants. Increases in rhizosphere populations of *P. parasitica* var. *nicotianae* from an initial field population of 0.75 propagule per gram of soil, coincided with rapid disease development in each treatment.

Additional key words: *Nicotiana tabacum*, *Phytophthora nicotianae* var. *nicotianae*, Ridomil.

Black shank of tobacco (*Nicotiana tabacum* L.), which is caused by *Phytophthora parasitica* (Dast.) var. *nicotianae* (Breda de Hann) Tucker (= *P. nicotianae* Breda de Haan var. *nicotianae* Waterhouse), is a serious disease in most of the tobacco-growing areas of the United States (10). The incidence of black shank increases with increasing levels of inoculum (3, 5, 7, 10). Although factors that affect the epidemiology of black shank such as pH, temperature, and water relations have been investigated, little has been reported on the quantitative relationship of inoculum to the development of disease over time (10). Flowers and Hendrix (3) were unable to determine reliably the populations of *P. parasitica* var. *nicotianae* in fields at the time of planting and did not relate initial populations to specific amounts of disease development during the growing season; they showed, however, that populations of the fungus increase in the rhizospheres of host plants. They suggested that differences in the rate of black shank development could be due to differences in population densities at the beginning of the season. Plants of Burley 21, a cultivar of tobacco susceptible to *P. parasitica* var. *nicotianae*, were killed 3-4 wk after transplants were placed in a field where the inoculum density was less than 10 propagules per gram of soil (3), and after 2 wk in pots of naturally infested soil containing an initial population of 34 propagules per gram of soil (4).

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Control of black shank has been inadequate with available chemicals and even resistant cultivars often become diseased after exposure to high levels of inoculum. Recently, a group of acylalanine derivatives, which systemically control various diseases caused by fungi in the Oomycetes, have become available from CIBA-GEIGY Corp. for experimentation (1, 14, 15, 18). Diseases caused by species of *Phytophthora*, *Pythium*, *Bremia*, *Plasmopora*, *Pseudoperonospora*, and *Sclerospora* are among those controlled by these compounds. One acylalanine analogue was inhibitory to *P. parasitica* var. *nicotianae* in vitro and gave good control of black shank of tobacco in preliminary field tests in 1976 (Mitchell and Kannwischer, *unpublished*). This compound, which has the systematic chemical name of *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-alanine methyl ester, was tested as CGA 48988 (=GA-1-82) and given the trade name, Ridomil, in Europe (15, 18).

Most evaluations of black shank are based on yield losses, disease indices, or the percentages of plants dead at the end of the season. Other quantitative parameters such as the times required for disease to reach specific severities and apparent infection rates (19) allow more accurate interpretation of the influence of a fungicide on disease progression. The objective of this study was to determine the influence of CGA 48988 on the relationship of inoculum density to the rate and severity of black shank development in susceptible and moderately resistant cultivars of tobacco under plant growth room, greenhouse, and field conditions. Portions of this work have been reported (8, 12).

MATERIALS AND METHODS

The isolate of *P. parasitica* var. *nicotianae* (P-230) from tobacco used in this study was obtained from the collection of *Phytophthora* spp. maintained at the Department of Plant Pathology, University of California, Riverside. It was maintained on V-8 juice agar and transferred monthly.

Chlamydospores produced by the method of Tsao (16) were used as inoculum in growth room and greenhouse studies. Known numbers of chlamydospores, free of hyphal fragments and other spores, were obtained with the method described by Ramirez and Mitchell (13).

Growth room experiments were conducted using raw or autoclaved (2 hr on two successive days) Astatula sand (pH of 6.2 in a 1:2 suspension of soil in 0.01 M $CaCl_2$). The infested-soil-layer technique used by Mitchell (11) was used to allow noninjured plant roots to grow into infested soil. Soil was infested with known numbers of chlamydospores to establish various inoculum densities, mixed thoroughly, and adjusted to a final water content of 5% (w/w). Sixty-five grams of soil infested with 0, 0.5, 5, or 50 chlamydospores per gram of soil were layered over 15 g of autoclaved builder's sand in 100-ml polypropylene beakers which had small drainage holes. The infested layer was covered with 35 g of noninfested soil into which a 1-mo-old tobacco seedling of the susceptible cultivar Hicks was transplanted. The soil surface was covered with autoclaved vermiculite and the beakers were place on wire screen frames to prevent water movement among cups. The seedlings had been grown for 1 mo in autoclaved vermiculite and fertilized weekly with 50-ml of a nutrient solution (6) diluted to half strength. The fungicide was incorporated at 0, 1, 5, or 25 mg active ingredient (ai) of a 25% WP of CGA 48988 per kilogram of air-dried soil (ppm) into both the infested and noninfested soil at each inoculum density. Experiments included 15 replicates of each treatment and were repeated three times. Plants were maintained in a growth room for 50 days at 25-27 C and 12 hr of light (4,000 lux at the level of the plants). Plants were watered from the top with 15 ml of tap water per plant daily and 15 ml of half-strength nutrient solution twice a week.

Disease development, as related to inoculum density and fungicide treatment, also was evaluated under greenhouse conditions in 10-cm-diameter pots. Astatula sand, treated with 1 kg of methyl bromide per 200 kg of soil was infested with 0, 0.5, 5, or 50 chlamydospores per gram of soil. Two hundred grams of infested soil were placed in each pot and covered with 200 g of noninfested soil, into which a 6- to 8-wk-old Hicks seedling was transplanted. Tobacco transplants had been grown in methyl bromide-treated soil in 10-cm-square trays and fertilized twice a week with 50 ml of the nutrient solution per try. The fungicide was incorporated at 0, 1, 10, and 50 ppm into both the infested and noninfested soil layers in the pots. Plants were watered daily with 50 ml of water and twice a week with 50 ml of the nutrient solution per plant. Temperatures in the greenhouse fluctuated from 19 to 32 C. Treatments consisted of 15 plants each and the experiments were conducted three times.

Plants in beakers and pots were inspected daily for wilting and discoloration of the stem. Dying plants were harvested when observed and the remainder of the plants were harvested 50 or 100 days after transplanting from beakers or pots, respectively. Plant roots were washed, dipped in 70% ethyl alcohol, rinsed in sterile distilled water, blotted dry, and plated on the $P_{10}VP$ selective medium of Tsao and Ocaña (17). The plates were observed for growth of *P. parasitica* var. *nicotianae* from the roots after incubation for 48 hr in the dark at 25 C.

The development of black shank was observed in a field near Quincy, FL, that had been artificially infested with *P. parasitica* var. *nicotianae* 7 yr prior to this study and then planted with tobacco in subsequent years. Split-plot experiments with a randomized complete block design of three replications were used. Fungicide treatments were whole plots of four rows each and plant cultivars were subplots. Each subplot consisted of two rows (15 plants per row) of the susceptible tobacco cultivar, Hicks, or two rows of the moderately resistant cultivar, Speight G-28. Plants were watered with an overhead irrigation system and cultural practices were similar to those used by commercial growers in the area. Nine-wk-old tobacco plants from fumigated plant beds were transplanted into the field.

Approximately 130 ml of a transplant solution that contained 150 μg/ml ai of 25% CGA 48988 WP were added to the hole in which each plant was set in the field. Plants were inspected weekly for symptoms of black shank and considered to be dead when they had blackened stems and had wilted permanently. Soil samples from the top 10-cm of the soil were taken from the rhizospheres of 12 plants in each subplot and combined. The position of each plant was marked to permit sampling after plant death. Twenty grams of each combined sample were diluted 1:10 in 0.25% water agar and 1-ml aliquots were spread over the surface of 10 agar plates of a selective medium containing: 10 mg pimaricin (Delvocid, 50% ai, Gist-Brocades N.V., Delft, Holland), 250 mg ampicillin (Polycillin-N, 81% ai, Bristol-Myers Co., Syracuse, NY 13201), 10 mg rifampicin (Rifamycin SV, 100% ai, Sigma Chemical Co., St. Louis, MO 63178), 100 mg pentachloronitrobenzene (Terraclor, 75% ai, Olin Mathieson Chemical Corp., Little Rock, AR 72203), and 17 g Difco cornmeal agar in 1.0 liter deionized water. After incubation for 48 hr at 25 C in the dark, the soil suspension was washed from the agar surface under a slow stream of tap water and fungal colonies were counted.

RESULTS

In the growth room, greenhouse, or field, an inoculum density at planting of approximately 0.5 propagule of *P. parasitica* var. *nicotianae* per gram of soil resulted in 93 to 100% mortality of Hicks plants and apparent infection rates (r) of 0.08-0.11/unit/day (Table 1). Apparent infection rates were determined by linear regression analyses of time in days versus the percentages of mortality (dead or moribund plants) corrected for multiple infections. $\text{Log}_e [1/(1-x)]$, where x equaled the proportion of dead plants, was used for the growth room and greenhouse data because individual plants were exposed to defined initial inoculum in individual pots. $\text{Log}_e [x/(1-x)]$ was used for field data because secondary infection could have resulted from the spread of inoculum in the field. When dead plants were plated on $P_{10}VP$, *P.*

parasitica var. *nicotianae* grew from the roots and blackened stems of all plants counted as dead. Although raw soil was not used routinely in this study, the 87% mortality and r = 0.08/unit/day at 0.5 chlamydospore per gram of raw soil in beakers or pots obtained in two tests were similar to values obtained at 0.5 chlamydospore per gram of soil under the same conditions in autoclaved or methyl bromide-treated soil.

When inoculum densities were increased from 0.5 to 5 and 50 chlamydospores per gram of soil, r was progressively greater in the growth room but not in the greenhouse (Table 1). Mortality occurred sooner in both the growth room and greenhouse at the higher inoculum densities. The times required for 10% of the plants to die (t_{10}) (as determined by linear regression analyses) were 23, 20, and 17 days at 0.5, 5, and 50 chlamydospores per gram of soil, respectively, in the growth room; the corresponding values at these propagule densities in the greenhouse were 35, 24, and 21 days. Although symptoms occurred sooner in the growth room than in the greenhouse, the times required for plant death to increase from 10 to 50% (t_{10-50}) at each inoculum density were similar in the two environments. The number of days required for mortality to increase from 10 to 90% (t_{10-90}) was about the same at 0.5 chlamydospore per gram of soil in both the growth room and the greenhouse, but t_{10-90} was shorter at 5 and 50 chlamydospores per gram of soil in the growth room than in the greenhouse. The t_{10-90} was more rapid at 50 than at 5 chlamydospores per gram of soil in the growth room, but not in the greenhouse.

Although black shank occurred sooner in the field (t_{10} = 18 and 6 days in 1976 and 1977, respectively) than in the greenhouse (t_{10} = 35 days) or growth room (t_{10} = 23 days), t_{10-50} and t_{10-90} were longer in the field than under artificial conditions (Table 1). The t_{10-50} (24 and 22 days in 1976 and 1977, respectively) and t_{10-90} (49 and 44 days in 1976 and 1977, respectively) in the field experiments were similar for each of the two years.

No plants were killed under growth room conditions at 0.5 chlamydospore per gram of soil when 1 or 5 ppm of CGA 48988 were incorporated into the soil at the time of planting (Table 1). Percentages of mortality in pots in the greenhouse were 93, 60, 7, and 0% at 0.5 chlamydospore per gram of soil with 0, 1, 10, and 50 ppm of the fungicide, respectively. No infection occurred in plants grown in pots containing 50 ppm of CGA 48988 after 100 days at inoculum levels up to 50 chlamydospores per gram of soil. Phytotoxicity due to the fungicide was not evident at concentrations that controlled black shank in greenhouse or growth room studies. Root weights were not reduced in

TABLE 1. The influence of inoculum density of *Phytophthora parasitica* var. *nicotianae* and the fungicide CGA 48988[a] on the development of black shank in the susceptible tobacco cultivar Hicks under plant growth room, greenhouse, and field conditions

Conditions[b]	Propagules[c] /g of soil (no.)	CGA 48988 (ppm)	Disease development[d] t_{10} (days)	t_{10-50} (days)	t_{10-90} (days)	Mortality at harvest[e] (%)	r[f] /unit/day
Growth room	0.5	0	23	7	24	93	.09
	0.5	1	—[g]	—	—	0	0
	0.5	5	—	—	—	0	0
	5.0	0	20	4	16	100	.14
	50.0	0	17	4	13	100	.17
Greenhouse	0.5	0	35	7	25	93	.09
	0.5	1	36	30	110	60	.02
	0.5	10	—	—	—	7	—
	0.5	50	—	—	—	0	0
	5.0	0	24	5	19	100	.10
	50.0	0	21	5	19	100	.10
	50.0	50	—	—	—	0	0
Field 1976	Nil[h]	0	18	24	49	100	.09
Field 1977	0.75	0	6	22	44	100	.10
	0.75	150	53	10	20	100	.22[i]

[a]A 25% wettable powder formulation of *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-alanine methyl ester (CGA 48988 = GA-1-82, CIBA-GEIGY Corp.) was incorporated into soil (w/w) for greenhouse and growth room tests and was added as 130 ml of a 150 μg/ml solution to the soil around each transplant at the time of planting in the field.

[b]Growth rooms provided 12 hr of light (4,000 lux at the level of the plants) at 25 C; temperatures varied from 19-32 C in the greenhouse.

[c]Inoculum for growth room and greenhouse tests established as chlamydospores per gram of soil; populations in field soil based on dilution plate assays and converted to propagules per gram of soil.

[d]Days (interpolated from linear regression analyses) required for 10% plant death (t_{10}) or for mortality to increase from 10 to 50% (t_{10-50}) and from 10 to 90% (t_{10-90}).

[e]Percent plant mortality with 15 plants per treatment 50, 100, and 94 days after planting in the growth room, greenhouse, or field, respectively.

[f]Apparent infection rates (r), were slopes of linear regression lines of $\log_e [1/(1-x)]$ on time in the growth room and greenhouse and $\log_e [x/(1-x)]$ in the field; x = percent death due to black shank.

[g]The symbol — = insufficient disease for calculation.

[h]*Phytophthora parasitica* var. *nicotianae* was not detected before planting.

[i]Calculation for r for the fungicide-treated plants in 1977 was based on disease incidence from 49 to 70 days after planting.

the absence of the fungus at fungicide concentrations up to 10 ppm in growth room experiments, and at 0 or 0.5 chlamydospore per gram of soil there were no differences in root weights with 0, 5, or 25 ppm of the fungicide incorporated into autoclaved or raw soil. In the field, however, plants treated with the fungicide appeared to be stunted at the beginning of the season, but no differences between the treated and nontreated plants were evident at the end of the season.

An arithmetic plot of percent mortality of nontreated susceptible plants in the field versus time results in an s-shaped disease progress curve; disease development was most rapid between 21 and 42 days after planting (Fig. 1-A). The development of black shank was delayed by the fungicide treatment until 56 days after planting, but then the disease progressed more rapidly than in the control. Black shank developed more slowly in the resistant plants than in the susceptible plants; at the end of the season 61% of the nontreated resistant plants and only 10% of the fungicide-treated resistant plants were dead. Although 100% of the treated and nontreated susceptible plants were dead at the end of the season (94 days after transplanting), the delay in disease development in treated plants resulted in a dry-leaf yield from six leaf harvests of 1,964 kg/ha, which was significantly (P = 0.05) greater than the yield of 246 kg/ha in the control. Yield of resistant plants also was increased significantly (P = 0.05) from 2,882 kg/ha for nontreated plants to 4,102 kg/ha for plants treated with the fungicide.

The apparent infection rates were lower for resistant plants in the field than for susceptible plants (Fig. 1-B). Excellent protection from black shank was provided by treating the resistant cultivar Speight G-28 with CGA 48988. As indicated above, disease was delayed in susceptible plants treated with the fungicide, but after 56 days it progressed faster (r = 0.22/unit/day) than in the nontreated susceptible plants (r = 0.10/unit/day). The apparent infection rate for the fungicide-treated susceptible plants was calculated from disease observations made from 49 to 70 days after planting.

The population of *P. parasitica* var. *nicotianae* in the field at the time of planting was 0.75 propagule per gram of soil, but populations increased to a maximum of over 250 propagules per gram of soil in samples from the rhizospheres of nontreated, susceptible plants between 13 and 46 days after planting (Fig. 2). Maximum populations of the fungus in the rhizospheres of fungicide-treated, susceptible plants and nontreated, resistant plants were 145 and 112 propagules per gram of soil, respectively, but they were not attained until approximately 65 days after planting. Populations of *P. parasitica* var. *nicotianae* did not reach the maximum of 105 propagules per gram of soil in the rhizosphere of resistant plants treated with the fungicide until after the time of final leaf harvest. After the death of infected plants, populations decreased in all treatments. Soil samples collected from the rhizospheres of Hicks plants in the field in 1976 were plated on the gallic acid medium developed by Flowers and Hendrix (2), but very low levels of *P. parasitica* var. *nicotianae* were detected. The maximum rhizosphere population of 32 propagules per gram of soil was detected 70 days after planting.

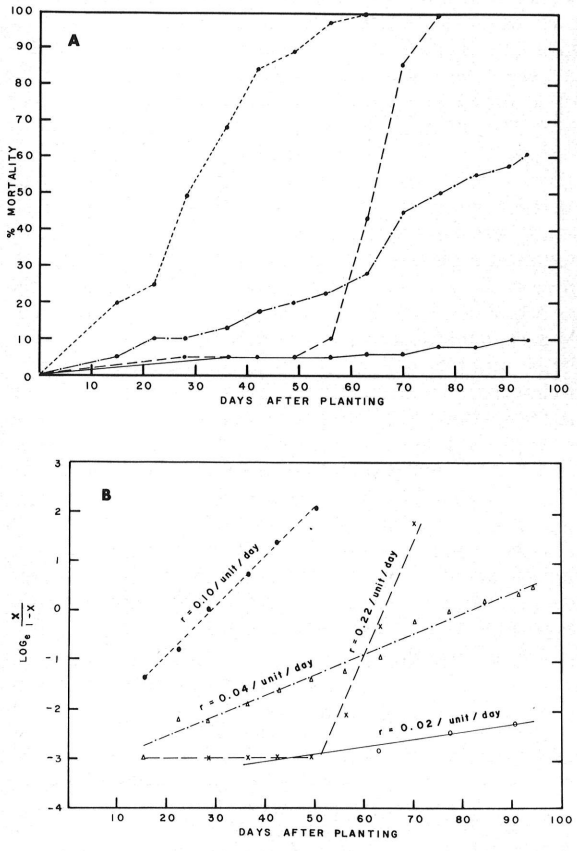

Fig. 1–(A, B). Rate of plant death in the field for tobacco plants of the susceptible cultivar Hicks (• - - - •), CGA 48988-treated Hicks (× - - - ×), resistant cultivar Speight G-28 (Δ · - · - ·Δ), and CGA 48988-treated Speight G-28 (O——O). **A)** Percentage mortality and days after planting. **B)** Regression lines for mortality (x = proportion of plants dead) and days after planting.

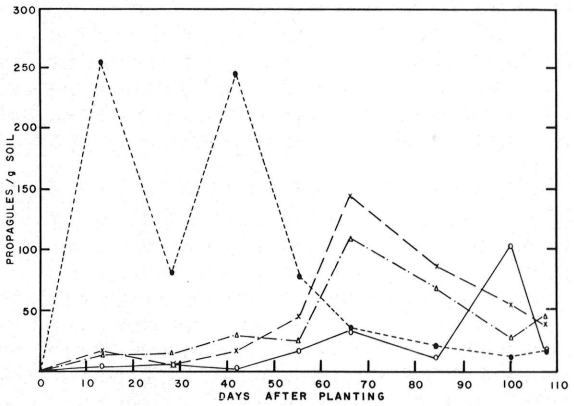

Fig. 2. Populations of *Phytophthora parasitica* var. *nicotianae* in the rhizospheres of tobacco plants of the susceptible cultivar Hicks (• - - - •), CGA 48988-treated Hicks (× - - - ×), resistant cultivar Speight G-28 (Δ · - · - · Δ), and CGA 48988-treated Speight G-28 (O——O).

DISCUSSION

This study confirms that severe black shank can occur in soil with very low initial inoculum levels of *P. parasitica* var. *nicotianae*. All of the susceptible Hicks and 58-61% of the moderately resistant Speight G-28 plants died in three separate fields in north Florida which had initial populations of *P. parasitica* var. *nicotianae* of 0.2, 0.4, and 0.75 propagule per gram of soil (Mitchell, *unpublished*). Flowers and Hendrix (3, 4) also reported severe disease under greenhouse or field conditions where initial populations of *P. parasitica* var. *nicotianae* were low. Kannwischer and Mitchell (*unpublished*) found that approximately 0.2 chlamydospore per gram of soil was required for 50% infection of tobacco seedlings by *P. parasitica* var. *nicotianae* under growth room conditions. Studies with other *Phytophthora* spp. indicate that 0.1 to 1 chlamydospore per gram of soil can cause 50% infection of papaya or milkweed vine (13). In this study initial inoculum densities of 0.5 chlamydospore per gram of soil resulted in mortality and apparent infection rates under growth room and greenhouse conditions comparable to those in the field at approximately the same initial inoculum densities.

The similarity in the values obtained under controlled and field conditions may be due in part to the use of inoculum in the growth room and the greenhouse in the form of chlamydospores, which most closely represent the type of survival or resting structures that are present in the soil or plant debris in the field at the time of planting. The use of autoclaved or methyl bromide-treated soil in these tests is considered reliable because results of experiments using raw, methyl bromide-treated, or autoclaved soil, did not differ greatly. Earlier initiation of disease and higher r at high inoculum densities in the growth room than in the greenhouse may be due to the more favorable environmental conditions for disease development in the growth room or to greater susceptibility of the younger seedlings in the growth room than in greenhouse experiments. The times required for mortality and r in the field were more closely simulated in the greenhouse than in the growth room.

Model systems in the growth room and greenhouse make possible the evaluation of a large number of plants in a relatively small area under controlled environmental conditions. Systems such as those used in this study can be useful in the quantitative evaluation of factors affecting disease such as environmental conditions, host resistance or tolerance, and biological or chemical control.

In this study, CGA 48988 was efficacious in the control of black shank at very low concentrations in the growth room and greenhouse. The fungicide at 1 ppm gave complete control of black shank in seedlings grown in small volumes of artificially infested soil in the growth room for 50 days. When plants were grown in larger volumes of infested soil in pots in the greenhouse for 100 days, only 7% of the plants were killed at 10 ppm of CGA 48988; even at an initial inoculum density of 50 chlamydospores per gram of soil none of the plants was killed after 100 days in pots and *P. parasitica* var. *nicotianae* was not isolated from the roots of plants exposed to the fungicide at 50 ppm of soil. *Phytophthora parasitica* var. *nicotianae* was not completely inhibited at similar levels in vitro; although *P. cryptogea*, *P. palmivora*, and *P. parasitica* were inhibited almost completely at 5 μg/ml of CGA 48988 in V-8 juice agar, five isolates of *P. parasitica* var. *nicotianae* were inhibited only 60% at 5 to 50 μg/ml (Mitchell, *unpublished*). Kelley (9) observed that radial growth of four other *Phytophthora* spp. was reduced in V-8 juice agar from 25-70% by 1 μg/ml of CGA 48988. Under field conditions 150 ppm of the fungicide delayed disease in susceptible plants for approximately 50 days, but then disease progressed rapidly. Good control was obtained when moderately resistant plants were treated with CGA 48988. Increases in rhizosphere populations of *P. parasitica* var. *nicotianae* corresponded to the time of rapid disease development in each treatment. Flowers and Hendrix (3, 4) also concluded that increases in rhizosphere populations were associated with pathogenesis.

The quantitative evaluation of disease over time should prove useful in recommendations for the application of control measures. The lack of disease control in susceptible plants beginning about 50 days after planting was due possibly to a reduction in efficacy of the fungicide over time, to changes in the physiology of the plant, or to the movement of roots into infested soil outside of the treated area and subsequent infection. It is of interest that both the first leaf harvest and flowering occurred about 50 days after transplant. Further considerations for the use of CGA 48988 to control black shank should include the application of a second fungicide treatment about 40-45 days after planting or the use of a broadcast treatment to increase the volume of treated soil.

The logit transformation $\log_e[x/(1-x)]$ was used to correct for multiple infections in the field because black shank was considered to be a multiple cycle [="compound interest" (19)] disease, and because this transformation allowed interpolations for t_{10}, t_{10-50}, and t_{10-90} that corresponded closely to those that actually occurred. Apparent infection rates have not been determined for other multiple cycle, plant diseases caused by soilborne pathogens, and the treatment of data in this study for the determination of r should be viewed as an initial attempt to apply a known transformation to such a disease. Additional work will be required to determine which transformation(s) will best express data for the epidemiological characterization of multiple cycle, soilborne diseases. Since the significance of secondary dissemination of inoculum by movement of infested soil or water has not been determined critically, it will be particularly important to evaluate disease gradients from point sources of inoculum.

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