Free Sterol and Total Lipids in Stems of Susceptible and Resistant Tobacco Cultivars Colonized by *Phytophthora parasitica* var. *nicotianae*

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ABSTRACT

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The susceptibility or resistance of plants of two flue-cured tobacco cultivars and one breeding line to *Phytophthora parasitica* var. *nicotianae* was not related to the total lipid or free sterol concentrations in the stems of control plants. Significant increases in lipid concentrations were recorded throughout the 12-day test period, as lesions developed in stems of highly susceptible cultivar Virginia Gold. Only at the second and 12th day did plants of moderately resistant cultivar North Carolina 88 and the highly resistant cultivar North Carolina 1071, respectively, contain significantly higher concentrations of total lipid. Early in disease development (day 2), colonized stems of Virginia Gold plants also had significantly higher concentrations of free sterols than stems of control plants. The free sterols cholesterol, campesterol, stigmasterol, and sitosterol were present in both control and colonized stems of each cultivar and line. Together, stigmasterol and sitosterol constituted 78–84, 63–78, and 64–77% of the free sterol concentrations in the stems of control plants of Virginia Gold, North Carolina 88, and North Carolina 1071, respectively. Although the free sterols and total lipids are not involved in either disease susceptibility or resistance, the increased concentrations of free sterols in the highly susceptible cultivar early in disease development may influence the rate of colonization by *P. parasitica* var *nicotianae*.

Additional key words: black shank.

Pythiaceous fungi require an exogenous source of sterols for growth and reproduction (3,5–7,9). Only a few studies have focused on the role of plant host sterols in resistance or susceptibility to pythiaceous fungi. Elliott and Knights (3) reported that the ratio of the sterol precursor, cycloartenol, to sitosterol differed in tubers of six potato cultivars and that their resistance to *Phytophthora infestans* was related to these ratios. Langcake (12) and Stössel and Hohl (17), however, analyzed potato leaves infected with *P. infestans* and reported that, although the relative amounts of major sterols in leaves differed, there was no relationship between resistance and the ratio of cycloartenol to sitosterol.

Mellano et al (16) reported a correlation between susceptibility to *Pythium ultimum* in snapdragon plants and the occurrence of sitosterol in the host roots. The correlation was based partially on depressed pectic enzyme activity in culture filtrates of *P. ultimum* grown in the presence of sitosterol. Reduction in pectic enzyme activity coincided with the appearance of oospores. They suggested the site of infection was an important factor in determining which type of resistance factor might be involved and that substances (such as sitosterol) present in tolerant host tissues, might switch fungal development from an aggressive vegetative proliferation to a "resting state" of reproductive activity.

None of the above studies were designed to determine whether the concentration of sterols or lipids in the host plant changed during the course of disease development. To our knowledge, the only study in which this was attempted was that of Hoppe and Heitefuss (10). No differences were detected in sterol composition between healthy and infected leaves of *Phaseolus vulgaris* 3, 6, or 9 days after inoculation with *Uromyces phaseoli*. Since *U. phaseoli* is a biotroph and most pythiaceous fungi are necrotrophs, differences may exist in host response to infection. Lösel (14) recently warned against the dangers of generalizing about the reactions of even a single fungus on different hosts.

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Resistance to *Phytophthora parasitica* Dast. var. *nicotianae* (B. de Hann) Tucker, the cause of black shank in tobacco, varies among tobacco species, cultivars, and lines (21). Stems are a primary site of infection in field-grown tobacco. Moreover, black shank resistance in tobacco stems is closely correlated with whole plant resistance (21).

The purpose of this study was to determine the nature and alteration of total lipids and free sterols in the basal stem region during black shank disease development of race 0 susceptible and resistant flue-cured tobacco.

MATERIALS AND METHODS

Two tobacco (*Nicotiana tabacum* L.) cultivars, the highly susceptible Virginia Gold and the moderately resistant North Carolina 88, and one breeding line, the highly resistant North Carolina 1071, were used. The black shank resistance of North Carolina 88 and 1071 was derived from Florida 301 tobacco and *N. plumbaginifolia* Viv., respectively (N. T. Powell, North Carolina State University, *personal communication*). Plants were grown in a greenhouse with a mean temperature of 25 C and inoculated with race 0 of *P. parasitica* var. *nicotianae* when the plants had 8–10 expanded leaves. The stems were inoculated between the second and third node above the soil line by depositing a mycelial suspension into a 4-mm wound in the cortical tissue (21). Control stems were wounded and treated with water. The wounded internodes of inoculated and control stems were covered with gauze and Parafilm® (American Can Co., Greenwich, CT 06830) for 48 hr to prevent drying. The internodes were approximately 46 mm long and 30 mm in circumference.

Tobacco stems of each cultivar or breeding line were collected 2, 4, 8, and 12 days after inoculation. The extent of colonization was determined by measuring the length and circumference of the lesions. Only the lesions of inoculated stems and the wounded internode of control stems were collected, quick-frozen in dry ice and methanol, freeze-dried, ground to pass through a 0.97-mm screen, and stored in a desiccator until they were analyzed. A randomized complete block design was used with five replications of three plants each within each treatment harvested at each date.

The total lipids were Soxhlet extracted for 16 hr in glass-distilled chloroform-methanol (2:1, v/v). The solvents containing the lipids were evaporated, the extracted material was dissolved in glass-distilled chloroform, filtered through Whatman No. 1 filter paper, evaporated to dryness, and the lipid weights were determined gravimetrically. Lipids were separated by thin-layer chromatography (TLC) in Silica gel-G with a solvent system according to Trevathan et al (18). The free sterols were removed from the TLC plates and prepared for gas-liquid chromatography (18). Identification and quantification of the free sterols were achieved with a Bendix Model 2600 gas chromatograph (Bendix Process Instruments Division, Ronceverte, WV 24970) and an Autolab minigrator (Spectra Physics, Santa Clara, CA 95051). An external standard of cholesterol, campesterol, stigmasterol, and sitosterol was used for free sterol identification and quantification.

The results of three experiments were expressed as the means of all replications. Data were compared using Student's t test and significant differences were determined at $P \leq 0.05$.

RESULTS

Within 2 days after inoculation, lesions developed on all inoculated stems (Fig. 1). The rate of lesion development was correlated directly with the degree of susceptibility of the cultivar or line. Symptom development progressed rapidly along the stems of highly susceptible Virginia Gold plants and the entire circumference of each stem was colonized by the pathogen. Visible lesion development was four to five internodes long. Moderately resistant North Carolina 88 plants had lesions one to two internodes long that encompassing two-thirds of the circumference of the stems, whereas highly resistant North Carolina 1071 plants exhibited visible stem lesions that were limited to the inoculated internodes. Pith tissues of inoculated North Carolina 1071 plants did not exhibit visible symptoms of black shank disease development. Pith tissues of the other cultivars were colonized by the pathogen. By day 8, extensive lesion development and stem collapse were observed in susceptible Virginia Gold plants but not in those of the more resistant North Carolina 88 or 1071.

No significant differences were observed in concentrations of total lipids (percent dry weight) (Fig. 2) and free sterols (microgram per milligram, dry weight) (Fig. 3) among the control plants within each cultivar. At each harvest date, diseased stems of Virginia Gold plants contained significantly higher concentrations of total lipids than comparable control stems. Only at day 2 was the total lipid concentration significantly higher in colonized North Carolina 88 stems. By day 12, however, inoculated stems of North Carolina 1071 contained significantly higher concentrations of total lipid compared to controls.

No significant differences in the concentrations of free sterols occurred between inoculated stems and control of either North Carolina 88 or 1071 (Fig. 3). The free sterol concentration in colonized Virginia Gold stems was significantly higher than the controls only at 2 days after inoculation.

The relative abundance of cholesterol, campesterol, stigmasterol, and sitosterol in both control and colonized stems of the three cultivars is presented in Table 1. Cholesterol and campesterol were least abundant. The relative percentage of these sterols present in both colonized and control stems either remained constant or had decreased by the end of the 12 days. The predominant sterols detected in both control and colonized tobacco stems were stigmasterol and sitosterol.

Concentrations of both stigmasterol and sitosterol were

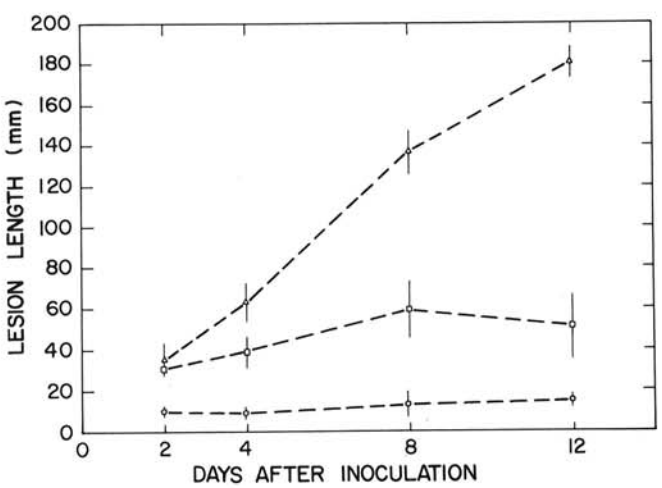

Fig. 1. Colonization of stems of tobacco cultivars Virginia Gold (Δ), North Carolina 88 (□), and North Carolina 1071 (o) by *Phytophthora parasitica* var. *nicotianae*. Brackets represent one standard deviation.

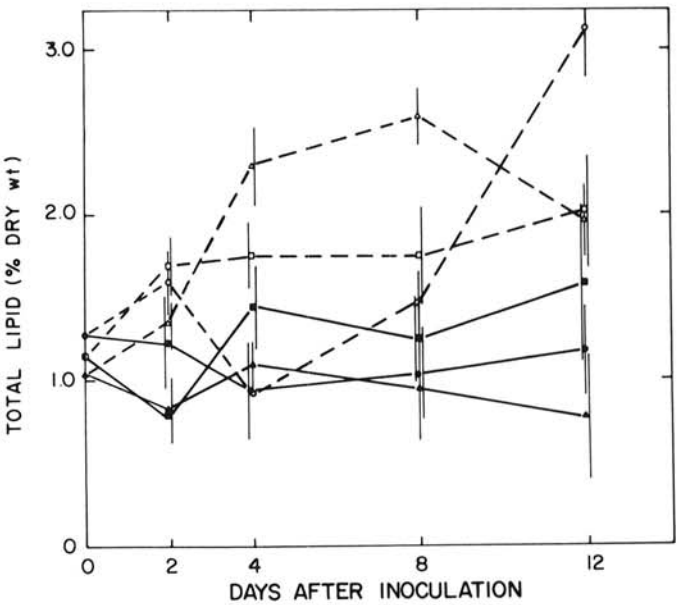

Fig. 2. Changes in the total lipids (percent dry weight) in colonized (broken lines, open symbols) and control (solid lines, solid symbols) stems of tobacco cultivars Virginia Gold (Δ, ▲), North Carolina 88 (□, ■), and North Carolina 1071 (o, ●). Brackets represent one standard deviation.

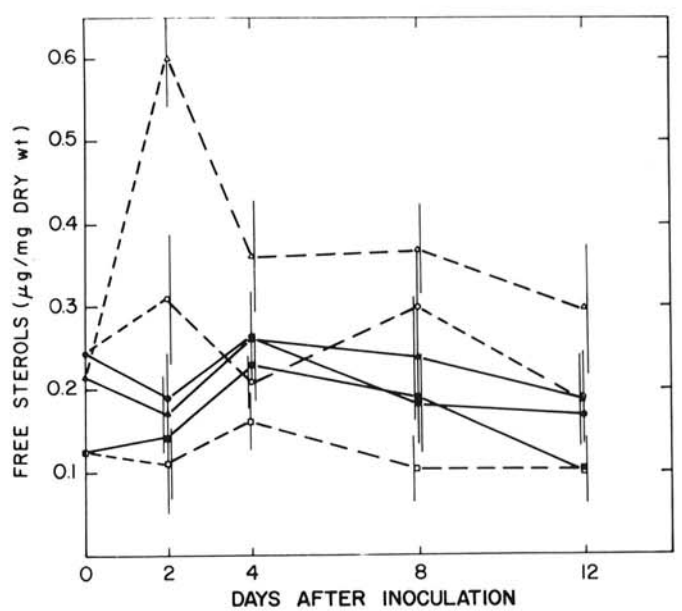

Fig. 3. Changes in the free sterols (micrograms per milligram, dry weight) in colonized (broken lines, open symbols) and control (solid lines, solid symbols) stems of tobacco cultivars Virginia Gold (Δ, ▲), North Carolina 88 (□, ■), and North Carolina 1071 (o, ●). Brackets represent one standard deviation.

significantly higher in diseased Virginia Gold stems than in control stems at day 2 (Fig. 4a). Although differences were not statistically significant, concentrations of these sterols were higher in diseased stems than in control stems 4, 8, and 12 days after inoculation. The reverse was observed for North Carolina 88 stems and those differences also were not statistically significant (Fig. 4b). In resistant North Carolina 1071, sitosterol was consistently, but not significantly, higher in stems of diseased plants while stigmasterol concentrations were not appreciably different from the controls (Fig. 4c).

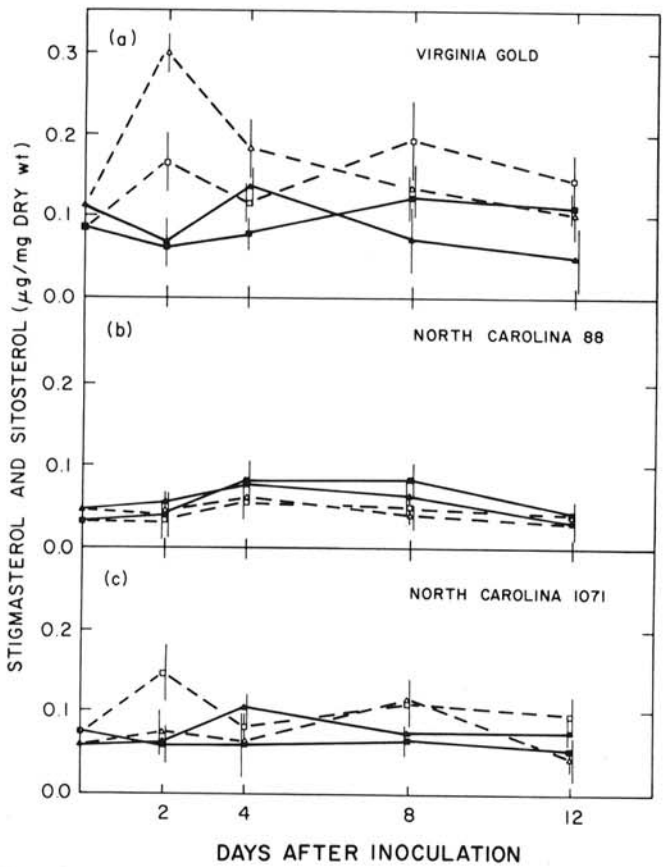

Fig. 4. Changes in stigmasterol (△, ▲) and sitosterol (□, ■) concentrations in colonized (broken lines, open symbols) and control (solid lines, solid symbols) stems of tobacco cultivars Virginia Gold (a), North Carolina 88 (b), and North Carolina 1071 (c). Brackets represent one standard deviation.

DISCUSSION

The day-to-day variation in the free sterol content of control plants within each cultivar or line was similar to that observed by Tso and Cheng (19). Since both light intensity and photoperiod, in addition to age, are important factors in controlling sterol synthesis, one can expect some variation in the sterol content of greenhouse-grown plants over an interval of time (4).

Colonization of the tobacco stem by the race 0 isolate of *P. parasitica* var. *nicotianae* was proportional to the level of resistance of each cultivar and line. Susceptibility could not be correlated with total lipid or free sterol concentrations in the control stems of any cultivar or line. Only relatively early in disease development were increases in lipid concentrations related to lesion development. In susceptible stems, lipid concentration doubled 2 days after inoculation, whereas, in highly resistant stems, increases were not significant until 12 days after inoculation.

Increased concentrations of total lipid have been observed in other host-parasite systems. Lösel (14) reported that during the development of the uredial and telial stages of the rust fungus *Puccinia poarum* on leaves of *Poa pratensis*, lipids accumulated in and around infected tissues at levels more than double those in uninfected leaves. Lipids also accumulated in leaves of *Tussilago farfara* following infection by *P. poarum*. Most of this increase was due to the fungal lipids, which accounted for almost half of the dry weight of mature aerial pustules (15). Cooper and Lösel (1) have reported that mycorrhizal roots of onion, clover, and ryegrass contained significantly more total lipid than uninfected roots. Lipid increases were not associated with diseased leaves in tobacco plants infected with either *Cercospora nicotianae* or *Alternaria alternata* (20).

The possibility that differences in relative concentrations of plant sterols are important in resistant and susceptible reactions in plants has previously been proposed (3,16). They may be particularly true in plants susceptible to pythiaceous fungi, since this group of fungi is incapable of sterol biosynthesis (6). Sterols are required for maximum vegetative growth as well as sexual and asexual reproduction (5–7,9). The maintenance of the sexual stage, which is least active during disease development, is dependent on specific sterol requirements (3,5,6). Competition between sterols of similar structure for active sites in fungal cells that either stimulate or inhibit sexual activity has been hypothesized (2,5,6). Our results indicate that infection affects the total concentration and relative

TABLE 1. Effects of colonization by *Phytophthora parasitica* var. *nicotianae* on the relative percentage of individual free sterols in stems of tobacco cultivars Virginia Gold, North Carolina 88, and North Carolina 1071

	Free sterols (relative percent) postinoculation							
	2 days		4 days		8 days		12 days	
Cultivar and free sterol	Control	Diseased	Control	Diseased	Control	Diseased	Control	Diseased
Virginia Gold								
Cholesterol	11	8	8	8	5	4	6	5
Campesterol	11	14	10	8	11	10	10	10
Stigmasterol	40	50	51	52	30	35	26	36
Sitosterol	38	28	31	32	54	51	57	49
North Carolina 88								
Cholesterol	14	9	11	12	7	8	9	9
Campesterol	22	21	22	18	17	11	13	14
Stigmasterol	36	40	33	37	33	35	32	39
Sitosterol	27	30	34	33	43	46	46	38
North Carolina 1071								
Cholesterol	12	11	13	9	10	9	10	13
Campesterol	20	18	23	22	13	16	15	11
Stigmasterol	35	24	41	32	41	38	44	24
Sitosterol	32	47	23	37	36	37	31	52

abundance of stigmasterol and sitosterol. The significance of this relative to resistance or susceptibility to infection is unknown.

Hendrix (8) concluded that there was no difference in the uptake and metabolism of cholesterol and sitosterol by *P. parasitica* var. *nicotianae*. He also reported that cholesterol, stigmasterol, and sitosterol stimulated the linear growth of this organism. The amount of free sterol incorporated into an agar medium was not correlated with the ability of that sterol to stimulate vegetative growth of *P. infestans* (13). All four of the demethylated sterols tested stimulated the growth of *P. infestans* significantly more than the control without added sterol (12).

A number of investigators have reported that free sterols, steryl esters, steryl glycosides, or total sterols increased in diseased tissues of higher plants (1,10,11,14,15). They concluded that the sterol content of different varieties or cultivars was not related to susceptibility or resistance. This investigation has shown that a significant increase (70%) in free sterols occurred in the colonized stems of the highly susceptible cultivar relatively early in colonization. Since free sterols are required for reproduction and for stimulation of vegetative growth in pythiaceous fungi, the excessively high level of free sterols 2 days after inoculation may influence the rapid rate of disease development in the highly susceptible plants.

LITERATURE CITED

1. Cooper, K. M., and Lösel, D. M. 1978. Lipid physiology of vesicular-arbuscular mycorrhiza. I. Composition of lipids in roots of onion, clover and ryegrass infected with *Glomus mosseae*. New Phytol. 80:143-151.
2. Elliott, C. G. 1968. Competition and synergism between cholesterol and cholestanol in oospore formation in *Phytophthora cactorum*. J. Gen. Microbiol. 51:137-143.
3. Elliott, C. G., and Knights, B. A. 1969. Interactions between steroids in the growth of *Phytophthora*. J. Sci. Food Agric. 20:406-408.
4. Grunwald, C. 1978. Function of sterols. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 284:544-558.
5. Hendrix, J. W. 1964. Sterol induction of reproduction and stimulation of growth of *Pythium* and *Phytophthora*. Science 144:1028-1029.
6. Hendrix, J. W. 1970. Sterols in growth and reproduction of fungi. Annu. Rev. Phytopathol. 8:111-130.
7. Hendrix, J. W. 1974. Lipids of phytopathogenic fungi. Annu. Rep. Ky. Agric. Exp. Stn., Lexington 87:87.
8. Hendrix, J. W. 1975. Differential uptake and metabolism of sitosterol and cholesterol by *Achyla, Pythium* and *Phytophthora* species. Can. J. Microbiol. 21:735-737.
9. Hendrix, J. W., Guttman, S.-M., and Wightman, D. L. 1969. Cation and sterol effects on growth of *Phytophthora parasitica* var. *nicotianae*. Phytopathology 59:1620-1624.
10. Hoppe, H. H., and Heitefuss, R. 1975. Permeability and membrane lipid metabolism of *Phaseolus vulgaris* infected with *Uromyces phaseoli*. V. Sterols in healthy and rust-infected bean leaves resistant and susceptible to *Uromyces phaseoli*. Physiol. Plant Pathol. 5:273-281.
11. Jennings, P. H., Zscheile, F. P., Jr., and Brannaman, B. L. 1970. Sterol changes in maize leaves infected with *Helminthosporium carbonum*. Plant Physiol. 45:634-635.
12. Langcake, P. 1974. Sterols in potato leaves and their effects on growth and sporulation of *Phytophthora infestans*. Trans. Br. Mycol. Soc. 63:573-586.
13. Langcake, P. 1974. Uptake of sterols by *Phytophthora infestans*, their intracellular distribution and metabolism. Trans. Br. Mycol. Soc. 64:55-65.
14. Lösel, D. M. 1978. Lipid metabolism of leaves of *Poa pratensis* during infection by *Puccinia poarum*. New Phytol. 80:167-174.
15. Lösel, D. M., and Lewis, D. M. 1974. Lipid metabolism in leaves of *Tussilago farfara* during infection by *Puccinia poarum*. New Phytol. 73:1157-1169.
16. Mellano, H. M., Munnecke, D. E., and Sims, J. J. 1970. Relationship of pectic enzyme activity and presence of sterols to pathogenicity of *Pythium ultimum* on roots of *Antirrhinum majus*. Phytopathology 60:943-950.
17. Stössel, P., and Hohl, H. R. 1981. Sterols in *Phytophthora infestans* and their roles in the parasitic interactions with *Solanum tuberosum*. Ber Schweiz. Bot. Ges. 90:118-128.
18. Trevathan, L. E., Moore, L. D., and Orcutt, D. M. 1979. Symptom expression and free sterol and fatty acid composition of flue-cured tobacco plants exposed to ozone. Phytopathology 69:582-585.
19. Tso, T. C., and Cheng, A. L. S. 1971. Metabolism of cholesterol-4-^{14}C in *Nicotiana* plants. Phytochemistry 10:2133-2137.
20. Wassef, M. K., and Hendrix, J. W. 1975. Effect of leaf disease on tobacco lipids. Fed. Proc. 34:656.
21. Wills, W. H., and Moore, L. D. 1971. Response of some cultivars and lines of tobacco to stem inoculation with *Phytophthora parasitica* var. *nicotianae*. Tob. Sci. 15:51-53.