Effect of Heat-Induced Susceptibility of Tobacco to Black Shank on Protein Content and on Activity of Peroxidases

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ABSTRACT

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When tobacco plants of cultivars Coker 187 (resistant) and Virginia Gold (susceptible) were immersed in a water bath at 50 C for 1 min and inoculated with *Phytophthora parasitica* var. *nicotianae*, disease severity of both cultivars was increased up to 100% compared with nontreated controls. In root tissue, the protein concentration significantly decreased as disease severity increased in both cultivars. Disease susceptibility was not correlated with levels of peroxidase activity in extracts from both cultivars. The heat treatment, however, induced four distinct peroxidase bands in noninoculated and inoculated Coker 187 roots after 1 hr and 10 days, as shown by disc electrophoresis. These bands were not present in gels of nonheated Coker 187 root extracts.

Alterations of environmental conditions of plants before they are inoculated often cause modification in their resistance to pathogens. Reports concerning preinoculation heat treatments, which result in modification of resistance to certain diseases, have been reviewed by Chamberlain and Gerdemann (1) and by Jerome and Müller (6). Moore and Wills (10) reported that the resistance of roots of intact tobacco (*Nicotianae tabacum* L.) plants to *Phytophthora parasitica* Dast. var. *nicotianae* (B. de Haan) Tucker could be inactivated by a hot water (50 C) treatment.

Cellular disruptions in plants caused by specific pathogens often are paralleled by alterations in enzyme composition (14), including peroxidases. Field resistance of potatoes to *Phytophthora infestans* has been associated with peroxidase enzyme activity (4, 11, 16). Tobacco leaf tissue responded to infection by *Colletotrichum destructivum* with synthesis or activation of at least one and possibly two proteins which exhibited peroxidase activity (19). In the wildfire disease of tobacco, peroxidases have been reported to be associated with resistance (3, 7).

This investigation was undertaken to determine the effect of heat treatment, which induces enhanced susceptibility to *P. parasitica* var. *nicotianae*, on the protein content and peroxidase (EC 1. 11. 1.7) activity of flue-cured tobacco.

MATERIALS AND METHODS

Tobacco cultivars Virginia Gold (susceptible to black shank) and Coker 187 (resistant) were used throughout the investigation. Seedlings were germinated in vermiculite, transplanted into modified muffin pans (15), and suspended over pans containing standard Hoagland's nutrient solution.

Plants were treated at the six- to eight-leaf stage by suspending the roots in a water bath containing standard Hoagland's solution at 50 C for 1 min. Inoculum was prepared by culturing *P. parasitica* var. *nicotianae* in 75 ml of potato-dextrose broth dispensed in 250 ml Erlenmeyer flasks for 14 days in the dark at room temperature (10). The broth from each flask was decanted and the mycelium was ground for 10 sec in a Waring Blendor and resuspended in 150 ml of distilled water. One hundred and fifty ml of the aqueous mycelial suspension was added to each pan.

A randomized complete block design was used with four replications of 10 plants each. All data are averages of two repetitions of the experiment and were analyzed by the analysis of variance and Duncan's multiple range test methods. Roots were rated for disease according to degree of browning and maceration at the time of harvest. Disease development of the roots was scored on a 0-3 scale (0 = white roots; 1 = slight browning; 2 = browning, but no apparent maceration; and 3 = both browning and maceration of the roots).

For quantitative assays for protein concentration and peroxidase activity, root tissue was extracted as follows: 2 g of fresh plant and 40 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.5% ascorbic acid (v:v) were ground in an Sorvall Omni-Mixer at 8,000 *g* for 7 min at approximately 4 C. Two, 5-ml samples were placed in pre-weighed bottles, dried at 85 C for 24 hr, cooled in desiccators, and then weighed. The remaining extract was filtered through cheesecloth and centrifuged for 60 min at 10,000 *g* at 4 C before assays were made.

All extracts were assayed for total protein by the method of Lowry, et al. (8). To reduce the amount of phenolic compounds in each sample (18), 10 ml of extract

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was combined with 1 *g* of Polyclar AT (G.A.F. Corporation, New York, NY 10013) and incubated at 5 C. Samples were shaken periodically for a period of 1 hr and then filtered through Whatman No. 2 filter paper. Crystalline bovine serum was used as the standard. Absorption was measured at 750 nm with a Spectronic 20 (Bausch and Lomb) colorimeter.

Peroxidase activity was measured with a modification of the method described by Weston (18). Extracts were diluted by mixing 1 ml of extract with 49 ml of 0.1 M sodium phosphate buffer (pH 7.0). The reaction mixture contained 1.5 ml of dilute enzyme, 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.0), 0.5 ml of 0.045 M hydrogen peroxide, and 0.5 ml of 0.5% (v:v) *para*-phenylenediamine. Enzyme activity was determined by measuring the increase in absorbancy of the reaction mixture at 485 nm in a Beckman DU-II Spectrophotometer. Peroxidase activity was calculated in terms of specific activity (units/mg protein). One unit of activity was equal to the time required to increase the absorbance of the reaction mixture 0.500 OD units. For controls, samples of the extracts were autoclaved for 30 min at 1.05 kg/cm^2 (15 psi) and 121 C and then assayed for activity.

Qualitative determination of peroxidases was performed as follows: 20 g fresh weight of plant material and 10 ml of 0.05 N tris-HCl buffer (pH 7.4) containing 0.5 M sucrose, 75 mM ascorbic acid, 6.6 mM cysteine hydrochloride, and 14.2 mM mercaptoethanol were combined and ground in a chilled mortar as described by Stavely and Hanson (13). The pH was adjusted to 7.4 with 0.2 N NaOH, and the extracts were filtered through cheesecloth and centrifuged for 60 min at 10,000 *g* at 4 C. The extracts were concentrated with Lyphogel (Gelman Instrument Company, McLean, VA 22101) and immediately assayed. Extracts were subjected to electrophoresis in a 7% gel as described by Davis (2) with 0.05 N tris-glycine buffer (pH 8.3). Stacking gels were prepared at pH 6.7, and separation gels at pH 8.9. Concentrated samples of 0.05 ml were used for peroxidase determinations. Protein concentrations were determined for all samples. The electrophoresis was conducted under refrigeration at 4 ma/tube for a period of 40 to 60 min. The peroxidases were visualized by the method of Macko et al. (9) with guaiacol as the hydrogen donor. Gels were incubated in 0.1 M guaiacol for 30 min followed by rinsing in distilled water and then incubating in 0.01% hydrogen peroxide for 15 min. Gels were recorded immediately after staining because of possible loss of resolution in light. Gels were scanned on a Gilford Model 24 Spectrophotometer equipped with a Model 2310 linear transport mechanism. The peroxidases were determined at 485 nm at a scan speed of 2 cm/min. Final interpretation of samples was performed by comparing spectrometric tracings and visual examinations.

RESULTS

The heated, inoculated roots of Coker 187 and Virginia Gold plants at 10 days were brown and highly macerated. Stem lesions were observed only on stems of nonheated, inoculated and heated, or inoculated Virginia Gold plants. The number of stems with lesion development was higher in those Virginia Gold plants with heated-treated roots than in those which were inoculated but not treated.

In extracts from heated, inoculated root tissue of Coker 187 plants at 10 days (Table 1) there was a significantly lower level of protein compared to those in all other treatments. In the extracts from heated, inoculated roots of Virginia Gold plants, there was a significant decrease in the protein concentration when compared to the extracts of nonheated, inoculated and heated, or noninoculated roots at 10 days. All other treatments were not significantly different from one another.

Extracts of nonheated, noninoculated roots of Coker 187 plants showed a significant increase in peroxidase activity over the controls during the 10-day period. More important, there was no increase in the level of peroxidase activity of extracts from roots of the heated, noninoculated Coker 187 plants over the 10-day period. Extracts of heated, inoculated roots of Coker 187 plants also had significantly lower peroxidase activity when compared to either the nonheated, noninoculated or the nonheated, inoculated treatments at 10 days. Heating alone apparently reduced peroxidase activity or kept it from increasing. However, the root extracts of Virginia Gold plants of the heated, noninoculated treatment showed a significant increase in peroxidase activity over the 10-day period compared to the heated-inoculated treatment.

After electrophoresis, peroxidase (Fig. 1) bands were found in two general areas: R_f 0.15 and 0.30 (lower range)

TABLE 1. Protein content and peroxidase activity in roots of tobacco plants 1 hr and 10 days after the roots were inoculated with *Phytophthora parasitica* var. *nicotianae*

Treatment	Disease[a] index		Protein (mg/g dry wt)		Peroxidase (units/mg protein)	
	C187[b]	VG[b]	C187	VG	C187	VG
1 Hour						
Nonheated-Noninoculated	0	0	8.1 a[c]	7.5 abc	36 b	28 bc
Heated-Noninoculated	0	0	7.8 a	6.8 bc	37 b	37 bc
10 Days						
Nonheated-Noninoculated	0	0	8.8 a	7.8 abc	58 a	39 abc
Nonheated-Inoculated	1	2	7.2 a	8.6 ab	59 a	44 ab
Heated-Noninoculated	1	1	9.0 a	8.8 a	37 b	53 a
Heated-Inoculated	3	3	5.1 b	6.2 c	35 b	22 c

[a]Severity scale: 0= healthy to 3 = complete rot.
[b]Abbreviations: C187 = Coker 187; VG = Virginia Gold.
[c]Values followed by the same letter are not significantly different from each other at the 5% level.

and R_f 0.50 and 0.95 (higher range). Four additional peroxidase bands were present in the gels of root extracts of heated, noninoculated Coker 187 plants at 1 hr (Fig. 1-I), and in the gels of root extracts of heated-inoculated Coker 187 plants at 10 days (Fig. 1-L). The gels of extracts from nonheated, inoculated Coker 187 roots did not contain these extra bands (Fig. 1-K). No such variations appeared in the root extracts of Virginia Gold plants except that three faint bands were present in the higher R_f range of the extracts from heated, noninoculated and nonheated, inoculated roots at 10 days (Fig. 1-D, E).

Mycelial extracts of *P. parasitica* var. *nicotianae* had no peroxidase activity.

DISCUSSION

This investigation confirmed that upon heat treatment the roots of resistant Coker 187 plants become susceptible to attack by *P. parasitica* var. *nicotianae*. Heat treatment of the roots of susceptible Virginia Gold plants also resulted in increased disease development.

The heat treatment used in the present experiment probably did not result in the inactivation of the oxidases. It has been reported by Sisler and Johnson (12) that treatment at 70 C for 1 min resulted in only a 5% inactivation of *ortho*-diphenol oxidase activity in tobacco leaves.

In contrast to our data, Yu and Hampton (19) reported that leaf tissue of Kentucky 26 tobacco responded to infection by *C. destructivum* with the synthesis or activation of at least one and possibly two proteins with peroxidase activity. Our results, however, are similar to those reported by Ho and Weaver (5) who studied the peroxidase isozymes of roots of soybean cultivars susceptible and resistant to *Phytophthora megasperma* var. *sojae*. Though peroxidase isozymes changed with age, they were essentially similar in both healthy resistant and susceptible cultivars. We also could not demonstrate a direct correlation between peroxidase activity, either quantitatively or qualitatively, and the susceptibility of the roots of tobacco to black shank disease development.

The report of Veech (17) concerning black shank and peroxidase is similar to ours although he did not determine specific peroxidase activity. The following facts were in agreement: (i) prior to inoculation, the peroxidase activity of the resistant and susceptible cultivars was similar; (ii) with severe disease development in either cultivar there was a decrease in peroxidase activity; and (iii) the gel patterns of the extracts from roots of inoculated plants of both cultivars were similar. In contrast, our study did show that peroxidase activity was higher in roots of both the resistant and susceptible plants with low levels of disease development.

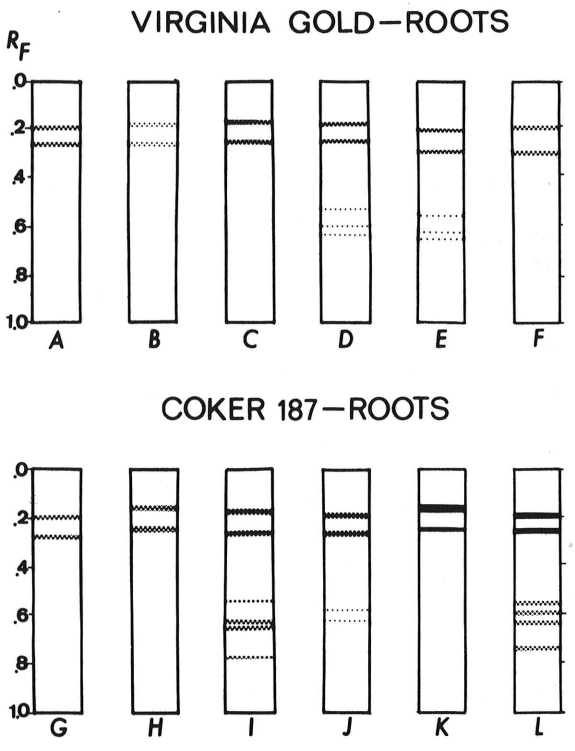

Fig. 1-(A to L). Peroxidase zymograms of roots of Virginia Gold (A-F) and Coker 187 (G-L) plants with the following treatments: **A)** nonheated, noninoculated–1 hr, **B)** nonheated, noninoculated–10 days, **C)** heated, noninoculated–1 hr, **D)** heated, noninoculated–10 days, **E)** nonheated, inoculated–10 days, **F)** heated, inoculated–10 days, **G)** nonheated, noninoculated –1 hr, **H)** nonheated, noninoculated–10 days, **I)** heated, noninoculated–1 hr, **J)** heated, noninoculated–10 days, **K)** nonheated, inoculated–10 days, **L)** heated, inoculated–10 days.

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