Pectic Enzymes Associated with Black Root Rot of Tobacco

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ABSTRACT

Extracts of healthy and *Thielaviopsis basicola*-infected Vesta 5 (susceptible) and Virginia Gold (resistant) tobacco root tissue were tested for pectic enzyme activity. Pectin methylesterase activity was high in extracts from diseased and healthy root tissues of both varieties, healthy tissue possessing about two-thirds as much pectin methylesterase activity as diseased tissue. Healthy root tissue of both varieties contained low levels of endopolygalacturonase and endopolygalacturonate-*trans*-eliminase activity, and exhibited no macerating activity. Diseased root tissue of both varieties possessed a high level of endopolygalacturonate-*trans*-eliminase activity and moderate endopolygalacturonase activity. Macerating activity of extracts of diseased tissue was high in the pH range 8-9.5, corresponding to the optimum pH range for endopolygalacturonate-*trans*-eliminase activity, and moderate in the pH range 4-5, corresponding to the optimum pH range for endopolygalacturonase activity. The similar activity pattern for enzymes from diseased tissue of Vesta 5 and Virginia Gold indicated that the pectic enzyme activity present in both varieties was similar. The principal difference between the susceptible and resistant varieties was that pectic enzyme activity increased more rapidly and to a higher level after inoculation in Vesta 5 (susceptible) than in Virginia Gold (resistant). Since increases in endopolygalacturonate-*trans*-eliminase activity coincided closely with symptom development, endopolygalacturonate-*trans*-eliminase appeared to be largely responsible for degradation of diseased root tissue during colonization. Phytopathology 60:304-308.

Black root rot disease of tobacco, incited by *Thielaviopsis basicola* (Berk. & Br.) Ferraris, involves extensive maceration of root tissue. The fungus penetrates root epidermal cells and invades cortical tissue both inter- and intracellularly (10). Enzymes that degrade pectic compounds are considered of major importance in the intercellular invasion and maceration of host tissue by pathogenic fungi (16). Pectic compounds are important constituents of the walls of parenchyma cells of tobacco (*Nicotiana tabacum* L.) (2, 4, 15). Furthermore, Silberman (9) showed that the structural carbohydrates of the cell walls of tobacco tissue are susceptible to enzymatic degradation. Photomicrographs of cellulase-, hemicellulase-, and pectinase (polygalacturonase)-treated tobacco tissue indicated that walls of parenchyma cells were readily broken down. The amounts of pectin solubilized by enzyme preparations were functions of enzyme concentration, reaction time, enzyme specificity, and substrate accessibility.

Thornberry (11) reported that culture filtrates of isolates of *T. basicola* pathogenic on tobacco contained pectase (pectin methylesterase) activity, and that the degree of virulence of isolates was correlated with pectase activity. Lumsden & Bateman (7) reported that cultures of *T. basicola* pathogenic on bean (*Phaseolus vulgaris* L.) contained a calcium-stimulated hydrolase more reactive with pectin than with sodium polypectate at pH 4.5, a hydrolase more reactive with sodium polypectate than with pectin at pH 6, and a calcium-stimulated *trans*-eliminase reactive with pectin and sodium polypectate at pH 8.5.

This investigation was undertaken to elucidate the nature and role of the pectic enzymes involved in the black root rot disease of tobacco. Pectic enzymes produced in healthy as well as in diseased tissue of both susceptible and resistant tobacco varieties were determined. Assays were made for kinds and quantities of pectic enzymes present throughout the infection-colonization period of *T. basicola* on tobacco.

Materials and Methods.—*Thielaviopsis basicola* used in this investigation was isolated from tobacco (*N. tabacum*), grown on potato dextrose agar slants at 25 C, and maintained at −20 C.

The tobacco varieties Vesta 5 (susceptible to black root rot) and Virginia Gold (highly resistant) were grown in the greenhouse at an air temperature of about 22 C. Seed was sown in Weblite (expanded shale, Weblite Corp., Roanoke, Virginia). When the plants had reached the four-leaf stage they were transplanted to flats of sterilized or *T. basicola*-infested sand.

Inoculum was prepared by growing *T. basicola* in 75 ml of carrot-glucose broth (200 g carrots, 20 g glucose, 1 liter distilled water) in 250-ml Erlenmeyer flasks for 10 to 12 days at 25 C. The resulting masses of mycelium from two flasks, with added water, were ground in a Waring Blendor, and equal amounts of the aqueous mycelial suspension were mixed with the sand in five flats. The tobacco was immediately transplanted to the infested sand. Two to 3 weeks after transplanting, the roots of both inoculated and noninoculated plants were harvested, rinsed with water, and frozen at −20 C.

To determine the rate and type of enzyme production throughout the infection-colonization period, tobacco roots were harvested at daily intervals for the first 2 weeks after inoculation. Thereafter, up to 26 days after inoculation, they were harvested at 2-day intervals.

Crude enzyme preparations were prepared by grinding one part frozen roots in an Omni-Mixer with one part 2.6 M NaCl (w/v). The crude enzyme preparations were then strained through cheesecloth, centrifuged at 14,000 *g* for 20 min at 0 C, and dialyzed against several hundred volumes of deionized water for 18 hr at 5 C. Protein content of enzyme preparations was determined by measuring the absorbancy of the solutions at 260 and 280 mμ (6).

The type and level of pectic enzymes present in the crude enzyme preparations were determined. Pectin methylesterase activity was determined by measuring the amount of carboxyl groups released from pectin substrate in reaction mixtures. Pectic glycosidase and *trans*-eliminase activities were determined by tests for macerating activity, viscometric and reducing group assays, and spectrophotometric analyses. In assays for pectic enzyme activity, 0.1 M citrate (pH 3, 4, 5, 6, 7) and 0.5 M Tris (pH 8, 9, 9.5) buffers were used. In tests determining the effect of calcium on enzyme activity, the buffers were made 0.0015 M with respect to $CaCl_2$.

To test for pectin methylesterase activity, 1.0 ml of enzyme preparation was added to 8.0 ml of a buffer solution of 1.5% pectin N.F., 0.075 M with respect to NaCl. After 3 hr incubation at 30 C, the reaction mixture was titrated back to its original pH with 0.025 M NaOH. Activity was expressed as specific activity (units/mg protein). One unit equaled 1 μmole carboxyl groups released/min.

Macerating activity of enzyme preparations was determined by measuring loss of coherence of potato tuber tissue. Potato discs were prepared according to the method of Moore & Couch (8). Enzyme preparations were tested for activity by placing 1.0 ml of enzyme preparation with 2.0 ml of buffer in a test tube containing four potato discs. Coherence loss was estimated by prodding the tissue with dissecting needles, and was scored as 0 (no loss), 1 (trace loss), 2 (slight loss), 3 (moderate loss), or 4 (complete loss).

Increase in reducing activity was determined with the dinitrosalicylic acid (DNS) test. The substrate for the test consisted of a solution of 1% sodium polypectate (Sunkist Growers, Ontario, Calif.) or 1% pectin N.F. (Sunkist) prepared in buffer at the desired pH. Reaction mixtures contained 1.0 ml of substrate and 0.5 ml of enzyme preparation and were incubated at 30 C for 20 hr. After the incubation period, reducing activity was determined according to the method of Moore & Couch (8). Results were compared with a galacturonic acid standard curve and were expressed as specific activity (milliunits/mg protein). One milliunit equaled 1 μmole of galacturonic acid equivalents liberated/min $\times 10^3$.

Trans-eliminase activity was determined by a modification of the method of Albersheim & Killias (1). The reaction mixtures were prepared as outlined for the DNS test. After incubation at 30 C for 20 hr, 0.5 ml of reaction mixture was added to 4.5 ml of deionized water and the absorbancy determined at 230 mμ, the absorbancy maximum of the reaction product. Activity was expressed as specific activity (milliunits/mg protein). One milliunit equaled an increase in absorbancy of 1.0/min $\times 10^3$.

RESULTS.—Pectin methylesterase activity of crude enzyme preparations from both healthy and diseased Vesta 5 and Virginia Gold roots was tested in the pH range 3-7, and was found to be highest at pH 7. At pH 7, crude enzyme preparations from healthy Vesta 5 and Virginia Gold had specific activities of 0.26 and 0.27, respectively. Pectin methylesterase activity was, however, higher in diseased root tissue. At pH 7, crude enzyme preparations from diseased Vesta 5 showed a specific activity of 0.39, while preparations from Virginia Gold had an activity of 0.40.

Crude enzyme preparations from healthy root tissue of both tobacco varieties caused some increase in reducing activity of pectic compounds (Table 1). Peaks of activity occurred at pH 5 and 9, with activity being higher on sodium polypectate substrate than on pectin. At pH 5, added calcium did not stimulate activity, but calcium did appear to stimulate activity at pH 9. Calcium stimulation was great with pectin substrate.

Reaction mixtures containing crude enzyme preparations from diseased tobacco caused a large increase in reducing groups liberated from sodium polypectate and pectin substrates (Table 1). Peaks of activity occurred at pH 5 and 9, with specific activity at pH 9 being over twice as high as at pH 5. Activity was

TABLE 1. Increase in reducing activity of sodium polypectate and pectin N.F. substrates incubated at 30 C for 20 hr at various pH levels with crude enzyme preparations from healthy and black root rot-diseased Vesta 5 and Virginia Gold tobacco root tissue

	Healthy tobacco tissue		Diseased tobacco tissue	
pH	Vesta 5	Virginia Gold	Vesta 5	Virginia Gold
		Sodium polypectate		
4	0.65[a]	0.69	1.90	1.40
5	0.72	0.77	3.55	2.64
6	0.35	0.29	2.00	2.48
7	0.07	0.10	1.67	0.88
8	0.41	0.49	5.73	4.38
9	0.61	0.70	7.94	6.83
9.5	0.47	0.52	7.09	5.29
		Sodium polypectate plus $CaCl_2$		
5	0.70	0.75	3.00	2.57
9	0.68	0.77	8.51	6.62
		Pectin N.F.		
4	0.48	0.27	1.37	0.91
5	0.55	0.34	2.86	1.58
6	0.27	0.20	1.90	1.17
7	0.00	0.00	1.30	0.81
8	0.16	0.14	4.42	3.68
9	0.27	0.24	5.94	4.38
9.5	0.22	0.22	4.72	3.45
		Pectin N.F. plus $CaCl_2$		
5	0.52	0.34	2.70	1.61
9	0.87	0.91	6.79	6.63

[a] Expressed as specific activity (milliunits/mg protein). One milliunit = 1 μmole of galacturonic acid equivalents liberated/min $\times 10^3$.

TABLE 2. *Trans*-eliminase activity of crude enzyme preparations from healthy and black root rot-diseased Vesta 5 and Virginia Gold root tissue incubated with sodium polypectate or pectin N.F. substrate at various pH levels for 20 hr at 30 C

	Healthy tobacco tissue		Diseased tobacco tissue	
pH	Vesta 5	Virginia Gold	Vesta 5	Virginia Gold
		Sodium polypectate		
4	0.03[a]	0.03	0.04	0.02
5	0.03	0.02	0.06	0.05
6	0.08	0.07	0.20	0.21
7	0.09	0.09	0.23	0.37
8	0.17	0.14	2.03	1.95
9	0.25	0.26	3.13	2.92
9.5	0.19	0.22	2.82	2.56
		Sodium polypectate plus $CaCl_2$		
5	0.04	0.03	0.11	0.11
9	0.26	0.27	3.44	2.77
		Pectin N.F.		
4	0.01	0.02	0.01	0.00
5	0.02	0.02	0.13	0.06
6	0.04	0.07	0.18	0.16
7	0.05	0.07	0.19	0.26
8	0.10	0.08	1.60	1.61
9	0.15	0.17	2.39	1.81
9.5	0.12	0.14	1.88	1.59
		Pectin N.F. plus $CaCl_2$		
5	0.02	0.02	0.10	0.08
9	0.39	0.30	3.06	2.71

[a] Expressed as specific activity (milliunits/mg protein). One milliunit = increase in absorbancy at 230 mμ of 1.0/min × 10^3.

higher with sodium polypectate substrate than with pectin. Addition of calcium to pectin substrate markedly stimulated activity at pH 9, while addition of calcium to sodium polypectate was, if stimulatory, only slightly so. Addition of calcium to reaction mixtures at pH 5 inhibited activity. Crude enzyme preparations from diseased Vesta 5, the susceptible variety, showed a higher specific activity on both substrates buffered at pH 5 and 9 than did enzyme preparations from Virginia Gold, the resistant variety.

Trans-eliminase activity of crude enzyme preparations from healthy tissue was much lower than the *trans*-eliminase activity of enzyme preparations from diseased tissue (Table 2). *Trans*-eliminase activity was stimulated by calcium, and was highest in the pH range 8 to 9.5, with the peak of activity at pH 9. The peak of activity detected at pH 9 by the DNS test (Table 1) was undoubtedly due to *trans*-eliminase activity. Because tests for *trans*-eliminase activity exhibited insignificant activity at pH 5, the enzyme activity detected by the DNS test at this pH was due to pectic glycosidase activity.

Enzyme preparations from diseased root tissue showed high macerating activity in the pH range 8-9.5 (with highest activity at pH 9) and lower activity at pH 4-5 (Table 3). Macerating activity of enzyme preparations from both Vesta 5 and Virginia Gold exhibited similar patterns, with the activity of enzyme preparations from Vesta 5 being somewhat higher. Levels of pH for maximum macerating activity coincided with those for maximum pectic glycosidase and *trans*-eliminase activity, indicating that maceration was due to the activity of those enzymes. Tests of enzyme preparations from healthy Vesta 5 and Virginia Gold root tissue revealed no macerating activity.

The pectic glycosidases and *trans*-eliminases found in Vesta 5 and Virginia Gold healthy and diseased root tissue were further characterized by viscosity tests and enzyme purification (12, 13, 14). At the time of 50% reduction in viscosity of sodium polypectate or pectin substrates by the pectic glycosidases or *trans*-eliminases, it was found (DNS test) that no more than 2.8% degradation of the substrate had occurred. This small amount of degradation at the time of 50% reduction in viscosity indicated that random cleavage of the substrate had occurred. Both the pectic glycosidases and the *trans*-eliminases were thus of the endo- type. To determine substrate specificity, the pectic glycosidases and *trans*-eliminases present in diseased Vesta 5 and Virginia Gold tobacco roots were partially purified by differential enzyme inactivation and precipitation. The partially purified enzymes were specific for polygalacturonic acid, and could degrade pectin extensively only in the presence of pectin methylesterase. The pectic glycosidases present in enzyme

TABLE 3. Loss of coherence of potato (*Solanum tuberosum*) discs incubated at 30 C with crude enzyme preparations from black root rot-diseased Vesta 5 and Virginia Gold tobacco root tissue

	Vesta 5[a]					Virginia Gold[b]				
	Incubation period, hr					Incubation period, hr				
pH	1.5	3	6	12	24	1.5	3	6	12	24
3	0[c]	0	0	0	2	0	0	0	0	0
4	0	0	0	0	3	0	0	0	0	2
5	0	0	0	0	3	0	0	0	0	3
6	0	0	0	0	1	0	0	0	0	1
7	0	0	0	0	0	0	0	0	0	0
8	1	2	3+	4	4	0	1	3	4	4
9	1	3+	4	4	4	1	2	4	4	4
9.5	1	2	3+	4	4	1	2	3+	4	4

[a] Crude enzyme preparation contained 5.1 mg protein/ml.
[b] Crude enzyme preparation contained 4.9 mg protein/ml.
[c] Coherence loss based on a severity scale of 4 (complete loss) to 0 (no loss).

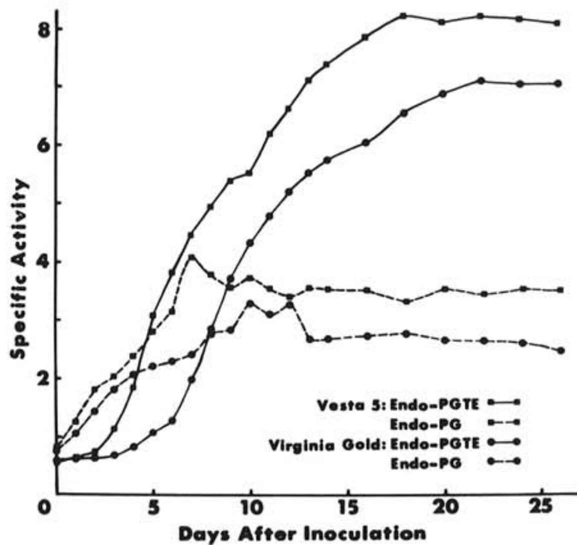

Fig. 1. Endopolygalacturonase (endo-PG) and endopolygalacturonate-*trans*-eliminase (endo-PGTE) activity development during the infection-colonization period of *Thielaviopsis basicola* on Vesta 5 (resistant) and Virginia Gold (susceptible) tobacco roots. Specific activity = milliunits/mg protein. One milliunit of endo-PG = 1 μmole galacturonic acid equivalents liberated from sodium polypectate substrate buffered at pH 5/min $\times 10^3$. One milliunit of endo-PGTE = 1 μmole galacturonic acid equivalents liberated from sodium polypectate substrate buffered at pH 9/min $\times 10^3$.

preparations were thus designated endopolygalacturonases and the *trans*-eliminases present were designated endopolygalacturonate-*trans*-eliminases.

The principal difference between the susceptible and the resistant tobacco varieties was in the rate and degree of pectic enzyme development after inoculation (Fig. 1). Pectic enzyme activity increased more rapidly and to a higher level in Vesta 5 after inoculation than in Virginia Gold. Pectin methylesterase activity in Vesta 5 began to increase immediately after inoculation, and reached a maximum level (specific activity 0.43) the 3rd day after inoculation. With Virginia Gold, pectin methylesterase specific activity increased up to the fourth day (specific activity 0.44) after inoculation, and then leveled off. In Vesta 5 root tissue, endopolygalacturonase activity increased rapidly after inoculation until the 7th day (specific activity 4.09), after which activity slightly decreased and leveled off. In Virginia Gold, endopolygalacturonase activity increased to about the 10th day (specific activity 3.28), after which it decreased somewhat and leveled off. In both tobacco varieties, maximum polygalacturonase activity occurred at the time the first symptoms of root rot appeared. In Vesta 5, endopolygalacturonate-*trans*-eliminase specific activity increased slowly the first 2 days following inoculation, began to increase more rapidly by the 3rd day after inoculation, and increased up to about the 18th day (specific activity 8.18), after which time activity leveled off. With Virginia Gold, activity did not begin to increase rapidly until 6 days after inoculation, and increased until about the 22nd day after inoculation (specific activity 7.05). Increase in *trans*-eliminase specific activity appeared to be closely correlated with the increase of the root rot in both tobacco varieties.

Discussion.—It appears that three pectic enzymes play a role in the enzymatic degradation and maceration of black root rot-diseased tobacco roots: a pectin methylesterase (EC 3.1.1.11) most active at pH 7; an endopolygalacturonase (EC 3.2.1.15) with an activity optimum at pH 5; and a calcium-stimulated endopolygalacturonate-*trans*-eliminase (EC 4.2.99.3) with optimum activity at pH 9. In both highly resistant Virginia Gold and susceptible Vesta 5, the activity of the pectic enzymes found in diseased tissue was markedly higher than the activity found in healthy tissue. Extracts from diseased root tissue of both varieties exhibited macerating activity on potato tuber tissue in the pH ranges optimum for endopolygalacturonase and endopolygalacturonate-*trans*-eliminase. Pectic enzyme activity of extracts from healthy tissue, however, was too low to bring about the maceration of potato tuber tissue.

The pectic enzymes present in Vesta 5 and in Virginia Gold were similar. The main difference between the susceptible and the resistant varieties was in the speed of pectic enzyme increase after infection by *T. basicola*. Also, as shown by tests for increase of reducing activity and by *trans*-eliminase and macerating tests, activity of endopolygalacturonase and endopolygalacturonate-*trans*-eliminase increased to a higher level in diseased Vesta 5 than in diseased Virginia Gold. The differences in the rate and degree of enzyme increase coincided with the differences in susceptibility of the tobacco varieties.

The reason for the faster and greater increase of pectic enzymes in the susceptible variety is not clear. Possibly, this variety provides a more suitable nutritional substrate for the growth of the fungus. Possibly, as Conant (3) reported, the more resistant variety provides mechanical barriers to the growth of the fungus. More likely, as first suggested by Jewett (5), chemical factors such as pectic enzyme inhibitors are involved.

The study of pectic enzyme activity after inoculation indicates that increase in endopolygalacturonase activity stopped at the time of appearance of root rot symptoms. However, increase in endopolygalacturonate-*trans*-eliminase activity continued and coincided closely with symptom development, a result also reported by Lumsden & Bateman (7) for bean inoculated with *T. basicola*. The quantities of pectin and low-methoxyl pectinates decreased to a much greater extent in diseased root tissue than did the quantities of pectic acid and insoluble pectates (12). Thus, pectin methylesterase activity on pectin and low-methoxyl pectinates is apparently significant, allowing more extensive degradation of these compounds by endopolygalacturonate-*trans*-eliminase and endopolygalacturonase. It seems, then, that the activity of endopolygalacturonase in conjunction with pectin methylesterase is important in

penetration and in early stages of colonization of the tobacco root tissue. The activity of endopolygalacturonate-*trans*-eliminase in conjunction with pectin methylesterase, however, is largely responsible for the extensive degradation of root tissue during colonization.

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