Extracellular Pectic Enzymes Produced by *Colletotrichum graminicola*[1]

JANET ROMANO and RALPH L. NICHOLSON
Department of Botany and Plant Pathology
Purdue University, West Lafayette, Indiana 47907

Abstract

Activities for endo- and exo-polygalacturonase, endo-polymethylgalacturonase and two endo-pectin methyl-trans-eliminases were identified in culture filtrates of *Colletotrichum graminicola*. Extracts of infected maize pith tissue, both susceptible and resistant to *C. graminicola*, exhibited qualitatively identical patterns of enzyme activity. However, activity was markedly less in extracts prepared from infected, resistant maize pith than in extracts from infected susceptible pith.

Synthesis of this broad spectrum of pectic enzymes is suggested to be a significant factor in the successful parasitism of corn by *C. graminicola*.

Introduction

Anthracnose of corn, caused by *Colletotrichum graminicola* (Ces.) Wils. has been considered of little importance in the United States; but in Europe and India the fungus causes substantial crop losses (4). Recently, a severe case of anthracnose caused complete crop failure in commercial sweet corn plantings at three different locations in Benton County, Indiana (13). The extreme pathogenicity of *C. graminicola* to various sweet and dent corn hybrids and inbreds could result in maize anthracnose becoming economically important throughout Indiana and the United States.

Although *C. graminicola* can infect all parts of the plant, the stalk rot phase is probably most important because infection may occur early in the growth of the host. The stalk becomes soft and the whole plant collapses, usually breaking at one or more internodes above the ground (9). Thus, stalk rot alone could cause rapid and extensive crop loss at any time in the growing season.

Pectic substances, which form a portion of the plant cell wall and serve as cementing substances between cells, are known to be degraded by extracellular hydrolytic and trans-eliminative enzymes produced by numerous phytopathogenic fungi and bacteria (2, 14). In addition, the degree of an organism's pathogenicity may at least be partly dependent on the type of extracellular enzymes produced (12).

Because of the importance of the stalk rot phase of corn anthracnose, this study was undertaken to determine the ability of *C. graminicola* to produce extracellular pectolytic enzymes.

Methods and Materials

Culture of the Fungus

Stock cultures of *C. graminicola* (isolate 104) were maintained on oatmeal agar (11) under a constant light source of 300 foot candles at

[1] Supported in part by the National Science Foundation summer research program. Journal Paper No. 5314. Purdue University Agricultural Experiment Station.

24° C. Liquid cultures were grown for 14 days in shake culture followed by 7 days still culture. The medium contained one per cent pectin as the sole carbon source and included the following salts per liter: 5.0 g NH_4NO_3, 0.5 g $MgSO_4$, 0.01 g $ZnSO_4$, 0.015 g $FeCl_3$ and 2.5 g KH_2PO_4. A check for contamination was made by inoculating plates of oatmeal agar, beef peptone agar, and potato dextrose agar (11) with samples from each culture flask.

Host Tissues

One sweet corn hybrid, Jubilee, and two dent corn inbreds, 33-16 and H-59 (susceptible, resistant and susceptible respective to *C. graminicola*) were used as host tissue. Plants, grown in the greenhouse for 5 weeks, were cut at the soil line and living pith tissue was aseptically removed from the first and second extended internodes. The tissue (30 g) was cut to 3-inch lengths, split longitudinally and placed with the cut surface facing up in sterile petri dishes lined with moistened filter paper. The exposed pith was inoculated and incubated in the dark at 24° C for 60 hours. Control uninoculated tissue was similarly treated with 0.5 ml sterile distilled water.

Culture Filtrate and Tissue Extract Preparations

Extracts from liquid cultures were prepared by filtering the culture liquor through cheesecloth, centrifuging (15,000 rpm at 4° C), filtering through a Millipore Filter (0.2 u) and dialyzing the filtrate at 4° C for 12 hours. Toluene (1 ml/l) was used to inhibit bacterial growth. Inoculated pith tissue was homogenized in an equal volume of 0.25 M NaCl. The homogenate was treated as described above. Uninoculated pith tissue treated in the same manner served as a control. Preparations were frozen (—20° C) until used for enzyme assays.

Enzyme Assays

Exo-polygalacturonase (exo-PG) was measured by the reducing group method of Somogyi (10) using Nelson's arsenomolybdate (8) as the color reagent. One per cent (w/v) sodium polypectate in 0.1 M sodium acetate-acetic acid buffer (pH 3.6, 4.0, 4.5, 5.0) served as substrate. Reaction mixtures consisted of 9.5 ml substrate and 0.5 ml culture filtrate. The mixtures were incubated at 30° C and 1 ml samples were removed at 0, 15, 30, 45, and 60 min. Endo-polygalacturonase (endo-PG), endo-polymethylgalacturonase (endo-PMG) and endo-pectin methyl-trans-eliminase (endo-PMTE) were measured by viscosity reduction (1, 3, 6) in Ostwald-Cannon-Fenske No. 300 viscosimeters at 30° C. Activity was expressed as the reciprocal of the time in minutes required for one-half viscosity reduction multiplied by 1,000. Reaction mixtures consisted of 3 ml 1.2% sodium polypectate or pectin, 2 ml water or 0.25 M $CaCl_2$, and 1 ml enzyme preparation Substrates were buffered with 0.05 M tris-HCl (above pH 7) and 0.05 M citric acid—NaOH (below pH 7).

Pectin methyl-trans-eliminase was also determined by the direct spectrophotometric method of Mullen and Bateman (7) by measuring

change in absorption at 230 nm of reaction mixtures consisting of 1 ml 0.1% pectin, 1.8 ml 0.025 M $CaCl_2$ and 0.2 ml filtrate preparation. All assays were carried out in triplicate and autoclaved enzyme preparations served as controls.

Results

Viscosity reduction of sodium polypectate and pectin indicated the presence of extracellular pectic enzymes in culture filtrates of *C. graminicola*.

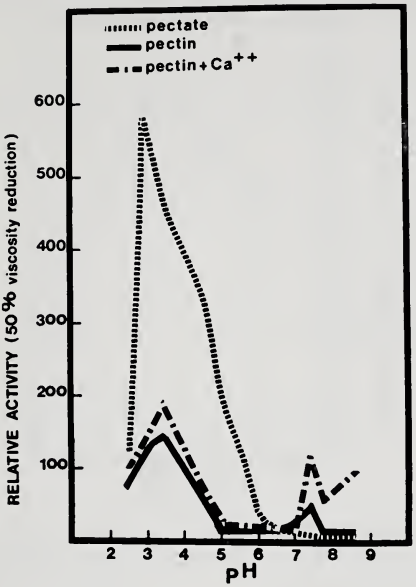

FIGURE 1. *Effect of pH on viscosity reduction of sodium polypectate, pectin and pectin plus calcium by the culture filtrate of* Colletotrichum graminicola.

A single peak for viscosity reduction of sodium polypectate was observed within the pH range studied (pH 2.5-8.6) suggesting the presence of an endo-PG with a pH optimum of about 3.0 (Fig. 1). A slight distortion of the viscosity reduction curve between pH 4 and 5 suggested the presence of a second enzyme capable of degrading sodium polypectate. That this enzyme is probably an exo-PG is suggested by the rapid liberation of reducing groups from sodium polypectate by a component of the filtrate with a pH optimum at 4.5 (Fig. 2).

Viscosity reduction of pectin occurred at pH 3.5 and pH 7.4 (Fig. 1). Addition of calcium to reaction mixtures caused a slight increase in activity at pH 3.5. However, activity at pH 7.4 was more than doubled suggesting the presence of a calcium stimulated pectin methyl-trans-eliminase in the alkaline range. A second calcium activiated trans-eliminase is suggested by the increase in activity observed above pH 7.8. Absorption of reaction mixtures at 230 nm (2, 5, 7) further confirms the presence of trans-eliminase activity (Fig. 3). Maximal

absorbency change occurred at pH 8.6 whereas only a slight increase was observed between pH 7.3 and 8.2. Comparison of change in absorption and viscosity reduction data suggests that culture filtrates contained an endo-PMTE, pH optimum near 7.4, and an exo-PMTE, pH optimum near 8.6. Trans-eliminative degradation of pectin could not be detected below pH 7.0 suggesting the observed viscosity reduction in the acid range was the result of hydrolytic cleavage of α 1, 4-glycosidic bonds of pectin by an endo-PMG with a pH optimum near 3.5.

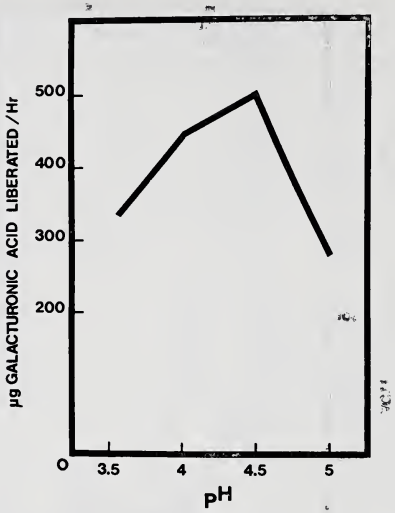

FIGURE 2. *Effect of pH on the liberation of reducing groups (galacturonic acid equivalents) from sodium polypectate by the culture filtrate of* Colletotrichum graminicola.

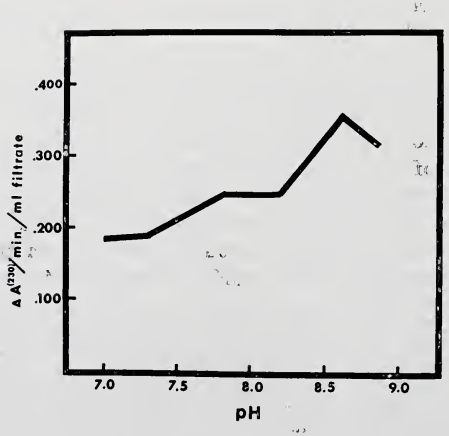

FIGURE 3. *Effect of pH on trans-eliminative degradation of pectin by the culture filtrate of* Colletotrichum graminicola. *Data expressed as change in absorbance at 230 nm/min/ml filtrate.*

Tissue homogenates from infected, susceptible maize pith exhibited qualitatively identical patterns of enzyme activity to those found in culture filtrates. Endo-PG was the predominant enzyme in both tissue and culture filtrate preparations.

Maize lines exhibiting differential resistance and susceptibility to *C. graminicola* (based on leaf inoculations) appeared equally susceptible to pith maceration 60 hours after inoculation with the fungus. However, activity of endo-PG, endo-PMG and endo-PMTE was significantly less in enzyme preparations from the resistant maize than in preparations from susceptible maize lines (Table 1).

TABLE 1. *Viscosity reduction of pectin and sodium polypectate by extracts of* Colletotrichum graminicola *infected resistant and susceptible maize pith tissue.*[1]

Maize Host	Pectin		Sodium Polypectate
	pH 3.5	pH 7.4	pH 3.0
H59 (s)[2]	153.8[3]	47.6	500
Jubilee (s)	135.1	58.8	400
33-16 (r)	47.6	16.1	34.5

[1] Reaction mixtures consisted of 3 ml 1.2% buffered substrate, 2 ml water and 1 ml tissue extract.

[2] (s), susceptible; (r), resistant to *C. graminicola*.

[3] Data represents the average of three replications and is expressed as the reciprocal of time in minutes required for 50% viscosity loss of the substrate multiplied by 1,000.

Discussion

At least five pectic enzymes were synthesized by *C. graminicola* when the fungus was grown in liquid culture with pectin as the sole carbon source. They included exo- and endo-polygalacturonase and an endo-polymethylgalacturonase which degraded pectic acid and pectin, respectively, by hydrolytic cleavage. In addition, exo- and endo-pectin methyl-trans-eliminases were present. Both are calcium stimulated and specific for pectin as a substrate. Qualitatively identical patterns of enzyme activity were observed in homogenates of infected pith of susceptible maize.

Although pectic enzyme synthesis by plant pathogenic fungi is not uncommon (2, 14), the synthesis of a majority of the known pectic enzymes by *C. graminicola*, both *in vitro* and *in vivo*, suggests that these enzymes play a significant role in the ability of the fungus to cause stalk rot of living maize pith.

In addition, it is important to note that although enzyme activity from infected pith tissue of resistant maize was lower than that observed from infected susceptible maize, both tissues were completely macerated within 60 hours of inoculation. This apparent lack of expected resistance could be the result of the combined effect of the broad spectrum of pectic enzymes produced by the fungus. These data suggest that pith resistance in addition to leaf resistance should be used as criteria for breeding resistance to maize anthracnose.

Literature Cited

1. BATEMAN, D. F. 1966. Hydrolytic and trans-eliminative degradation of pectic substances by extracellular enzymes of *Fusarium solani* f. *phaseoli*. Phytopathology. 56:238-244.
2. ————, and R. L. MILLAR. 1966. Pectic enzymes in tissue degradation. Annu. Rev. Phytopathol. 4:119-146.
3. BELL, T. A., J. L. ETCHELLS, and I. D. JONES. 1955. A method for testing cucumber salt-stock brine for softening activity. U. S. Dep. Agr. ARS-72-5. 15 p.
4. CHOWDHURY, S. C. 1936. A disease of *Zea mays* caused by *Colletotrichum graminicolum* (Ces.) Wils. Indian J. Agr. Sci. 6:833-843.
5. GARIBALDI, A., and D. F. BATEMAN. 1971. Pectic enzymes produced by Erwinia chrysanthemi and their effects on plant tissue. Physiol. Plant Path. 1:25-40.
6. HANCOCK, J. G., R. L. MILLAR, and J. W. LORBEER. 1964. Pectolytic and cellulolytic enzymes produced by *Botrytis alii*, *B. cinerea*, and *B. squamosa* in vitro and in vivo. Phytopathology 54:928-931.
7. MULLEN, J. M., and D. F. BATEMAN. 1971. Production of an endo-polygalacturonate trans-eliminase by a potato dry-rot pathogen, *Fusarium roseum* 'Avenaceum', in culture and in diseased tissue. Physiol. Plant Path. 1:363-373.
8. NELSON, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.
9. PUPIPAT, U., and Y. R. MEHTA. 1969. Stalk rot of maize caused by *Colletotrichum graminicolum*. Indian Phytopathol. 22:346-348.
10. SOMOGYI, M. 1952. Notes on sugar determination. J. Biol. Chem. 195:19-23.
11. TUITE, J. 1969. Plant pathological methods, fungi and bacteria. Burgess Publ. Co., Minneapolis, Minn. 239 p.
12. WALLACE, J., J. KUC, and E. B. WILLIAMS. 1962. Production of extracellular enzymes by four pathogens of apple fruit. Phytopathology 52:1004-1009.
13. WARREN, H. L., R. L. NICHOLSON, A. J. ULLSTRUP, and E. G. SHARVELLE. 1973. Observations of *Colletotrichum graminicola* on sweet corn in Indiana. Plant Dis. Rep. 57:143-144.
14. WOOD, R. K. S. 1960. Pectic and cellulolytic enzymes in plant disease. Annu. Rev. Plant Physiol. 11:299-322.