Extracellular Enzymes Produced by Colletotrichum lindemuthianum and Helminthosporium maydis During Growth on Isolated Bean and Corn Cell Walls

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ABSTRACT

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Helminthosporium maydis, a fungal pathogen of corn (a monocotyledon) grew on isolated cell walls of its host as well as on cell walls isolated from bean (a nonhost dicotyledon). A fungal bean pathogen, *Colletotrichum lindemuthianum,* also grew on cell walls isolated from both bean and corn. Both pathogens sequentially produced extracellular enzymes that degrade pectin, xylan, and cellulose and the exoglycosidases, β-xylosidase and β-glucosidase, during culture on either corn or bean walls. The pectin-degrading enzymes were detected early in culture filtrates from both fungi grown on either cell wall preparation. *Helminthosporium maydis* produced higher levels of laminarinase particularly in corn cell wall cultures. Corn wall cultures of both fungi possessed more xylanase activity than the bean cell wall cultures. Such differential production of certain enzymes may relate to structural differences between the monocotyledon and dicotyledon cell walls.

Plant cells are surrounded by a rigid wall in which several polysaccharides each of defined structure are specifically interconnected (3, 5, 9, 19, 21). Disruption of the plant cell wall is evident during infection by many plant pathogens (1). The extent of disruption may be severe, as in soft rot diseases where individual cells separate (13,18), or more limited, as in penetration of fungal mycelia through a cell wall (20). Plant cell wall degradation results from the action of pathogen-produced enzymes that cleave specific linkages in the wall matrix (1). Sequential secretion of cell wall degrading enzymes has been demonstrated during growth of several fungal pathogens on isolated host cell walls (6, 7, 11, 14).

Production of the cell wall degrading enzymes is not always constitutive. Frequently, induction by low concentrations of the major monomeric component of the enzyme substrate is observed (6). Consequently, the nutritional environment of the pathogen influences the nature of the cell wall-degrading enzymes that it produces.

In this paper, the influence of plant cell origin on the secretion of several polysaccharide-degrading enzymes has been investigated. Cell walls from bean (a dicotyledon) and from corn (a monocotyledon) were chosen because they differ in basic composition (5, 9). The pattern of enzyme secretion on these cell walls was studied for two fungal pathogens: *Colletotrichum lindemuthianum,* the causal agent of bean anthracnose, and *Helminthosporium maydis,* which causes blight on corn. These fungi were selected because the results of previous studies have demonstrated that these fungi produce enzymes capable of plant cell wall degradation (2, 8).

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MATERIALS AND METHODS

Culture of fungi.—*Colletotrichum lindemuthianum* (α race) was maintained as a sporulating culture by monthly transfer to the medium described by Mathur et al (12). The inoculum for liquid culture was obtained as a spore suspension by addition of sterile water to 8-day-old slants.

Cultures of *Helminthosporium maydis* (race T) were isolated from dried infected corn leaves obtained from G. A. Strobel, Montana State University, Bozeman. Inoculum for liquid culture was prepared by transfer to potato-dextrose agar of infected leaves that were surface sterilized by a 30-sec immersion in 5% (v/v) Clorox and then rinsed with sterile distilled water. Growth of the seeded plates for 4 days at room temperature resulted in a dense mycelial mat from which aerial mycelia (approximately 0.5 cm^2) were removed with tweezers and transferred into 10 ml of liquid medium.

The medium for liquid culture of *C. lindemuthianum* and *H. maydis* contained in a liter, 1.5 g KH_2PO_4, 0.24 g $MgSO_4$, 1.44 g KNO_3, 0.05 g $FeCl_3$, 1.12 mg $MnSO_4 \cdot H_2O$, 0.25 mg KI, 0.1 mg $NiCl_2 \cdot 6H_2O$, 0.03 mg $CoCl_2 \cdot 6H_2O$ and 0.5 ml H_2SO_4. Isolated cell walls from bean or corn plants were provided (0.1 g in 10 ml liquid medium) as the carbon source. Ten-ml samples of the media in 50-ml flasks were seeded and these cultures were incubated on a gyratory shaker at 24 C.

Filtrates were obtained from the liquid cultures at various times after inoculation by passage of the suspensions through sintered glass funnels. The filtrates

were assayed for the enzyme activities described below and were stored at 5 C.

Assay of exoglycosidases.—Culture filtrates were assayed for β-xylosidase, α-glucosidase, and β-glucosidase activities by measuring release of *p*-nitrophenol from the appropriate *p*-nitrophenyl derivatives (Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178, USA). Samples (100 μliters) of culture filtrates were incubated at 30 C with 2.0 ml 0.05% (w/v) glycoside derivative dissolved in 50 mM sodium acetate buffer pH 5.2. The reactions were terminated after 30 min incubation by addition of 1.0 ml 1M ammonium hydroxide and the amount of *p*-nitrophenol released by enzyme activities was determined by measuring the increase in absorbance at 400 nm with a Spectronic 20 colorimeter (Bausch and Lomb, 1400 North Goodman Street, Rochester, NY 14602). One unit of glycosidase activity was defined as the amount that produced an absorbance change of 0.01 under the described conditions.

Assay of polysaccharide-degrading enzymes.—Enzymes which catalyze the depolymerization of xylan, mannan, pectin, and polygalacturonic acid (Sigma), laminarin (ICN Life Science Group, 26201 Miles Road, Cleveland, OH 44128), and CM cellulose (Hercules Inc., 910 Market Street, Wilmington, DE 19899) were assayed by measuring the release of reducing groups from these substrates. Samples (50-200 μliters) of culture filtrates were added to 0.1% solutions of the substrates dissolved in 50 mM sodium acetate pH 5.2 and the mixtures were incubated at 30 C. After 30 min, the method of Nelson (15) was used to measure the amount of reducing groups in the reaction mixtures. The amount of reducing groups in the substrates and culture filtrates also were determined so that the increase in absorbance due to enzyme activity could be calculated. One unit of enzyme activity was defined as the amount which produced 8 μg reducing groups under the described assay conditions.

Determination of neutral sugar composition of bean and corn cell walls.—Bean and corn cell walls were analyzed for neutral sugar composition of the hemicellulose fractions by following the gas-liquid chromatography techniques of Jones and Albersheim (10).

Preparation of plant cell walls.—Cell walls were prepared from 12-day-old Dark Red Kidney bean

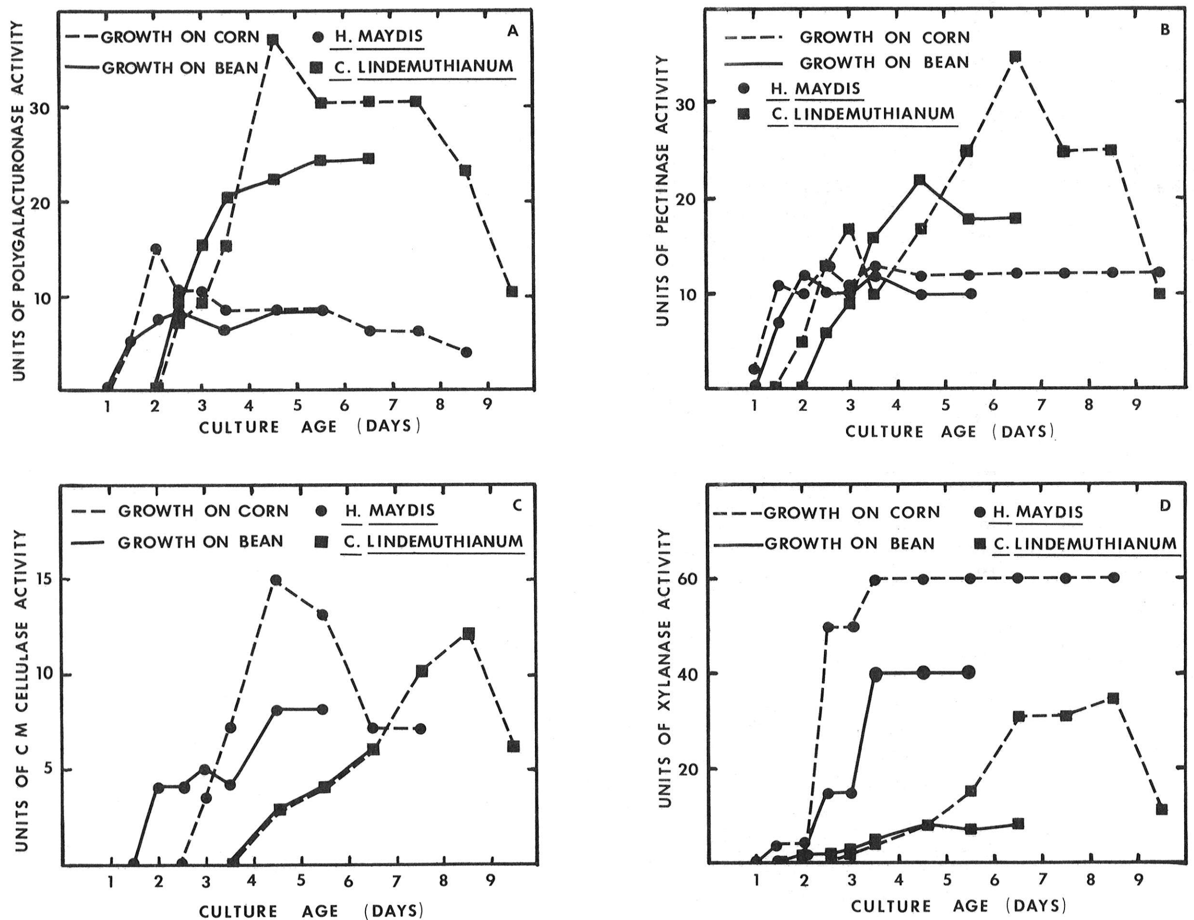

Fig. 1-(A to D). Production of polysaccharide-degrading enzymes by *Colletotrichum lindemuthianum* and *Helminthosporium maydis* when cultured on isolated bean and corn cell walls. Enzyme activities were assayed as described in methods. Comparison of activities of the enzymes which degrade: **A**) polygalacturonic acid; **B**) pectin; **C**) CM cellulose; and **D**) xylan.

hypocotyls and from 12-day-old leaf shoots from corn by following the procedures of English et al (7). To remove starch grains, a stirred suspension of the walls in 50 mM potassium phosphate buffer pH 7 was incubated with 1,000 units of amylase (Type IIA, Sigma) per gram dry wt of plant cell walls for 24 hr at room temperature. The treated plant walls were judged to be starch-free by failure to give a blue coloration with potassium iodide/iodine solution. These starch-free cell walls were extensively washed with water to remove the buffer and starch degradation products, and then with acetone before being air-dried.

RESULTS

Composition of bean and corn cell walls.—Analysis of isolated bean and corn walls showed that the same sugars were present but in differing amounts (Table 1).

Enzyme production by Colletotrichum lindemuthianum and Helminthosporium maydis.—Both fungi, when grown on either bean or corn cell walls, produced enzymes that degraded polygalacturonic acid (Fig. 1-A), pectin (Fig. 1-B), CM-cellulose (Fig. 1-C), and xylan (Fig. 1-D) Xylanase production by *C. lindemuthianum* was greater in corn cell wall cultures. Mannanase was detected only in late corn-grown *C. lindemuthianum* cultures and not at all in *H. maydis* cultures (Fig. 2-C). Although both fungi produced α- and β-glucosidase and xylosidase, the amounts varied with culture conditions [Fig. 2-(A to C)]. Greater levels of α-glucosidase were observed in the corn

TABLE 1. Analysis of the neutral sugars of the hemicellulose fractions of bean and corn cell walls

Neutral sugar[a]	Wall source: Bean	Corn
Rhamnose	6	1
Fucose	2	0
Arabinose	6	12
Xylose	50	64
Mannose	6	1
Galactose	20	2
Glucose (noncellulosic)	10	19

[a]Expressed as percent of dry weight of wall materials prepared from bean hypocotyls or corn stems as described in methods.

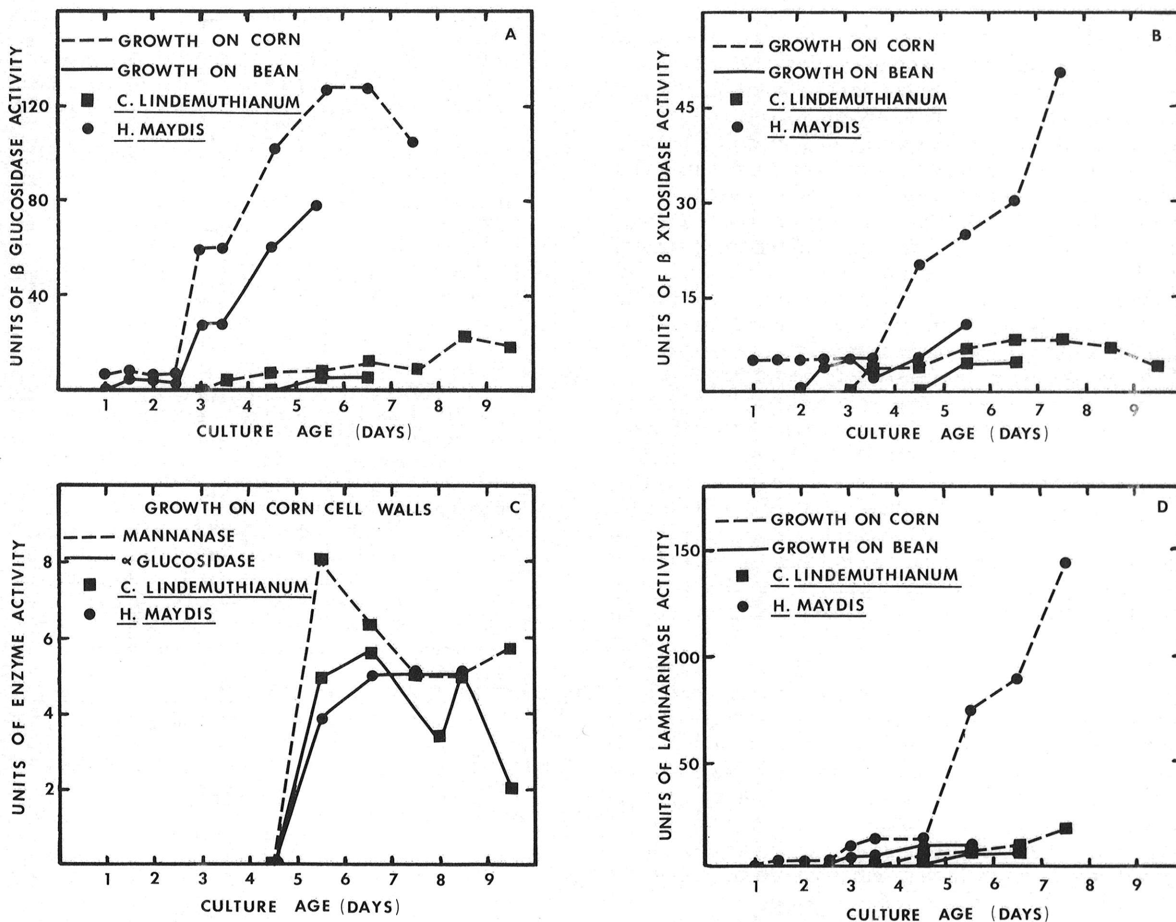

Fig. 2-(**A to D**). Production of extracellular enzymes by *Colletotrichum lindemuthianum* and *Helminthosporium maydis* cultured on isolated bean and corn cell walls. Enzyme activities were assayed as described in methods. Comparison of activities of: **A**) β-glucosidase; **B**) β-xylosidase; **C**) α-glucosidase and mannanase; and **D**) laminarinase.

cell wall cultures of both fungi, and xylosidase production was only significantly observed with *H. maydis* grown on corn cell walls.
walls.

Both fungi demonstrated a sequential production of the cell wall degrading enzymes, and in corn as well as bean cultures the pectin-degrading enzymes were the first to be detected. For *H. maydis* the production of the pectin-degrading enzymes preceded the appearance of CM cellulase by about 1 day, xylanase and β-glucosidase by about 1.5 days, and β-xylosidase by about 2 days. Similarly, with *C. lindemuthianum* the lag in appearance of pectin-degrading enzymes was about 1 day for β-glucosidase, 2 days for CM cellulase, and 3 days for xylanase.

An enzyme that degraded laminarin was detected in significant amounts only in late cultures of *H. maydis* grown on corn cell walls (Fig. 2-D).

DISCUSSION

Colletotrichum lindemuthianum and *H. maydis* both grew on isolated bean or corn cell walls. Thus, a pathogen of a monocotyledon utilized nonhost dicotyledon cell walls for growth, and a pathogen of a dicotyledon utilized nonhost monocotyledon cell walls. During growth, these pathogens produced extracellular enzymes that degraded plant cell wall polymers, and exoglycosidases which may function to further reduce the size of the polysaccharides fragments released by endoenzymes.

Most of the enzymes assayed were produced by both pathogens whether grown on corn or bean walls. This agrees with the observations that monocotyledon and dicotyledon cell walls possess some polysaccharides with similar structures (5, 9). The delayed initiation of enzyme production by *C. lindemuthianum* by about 1 day, compared to *H. maydis,* may be due to the need for the inoculum of *Colletotrichum* spores to germinate. Skipp and Deverall (17) have demonstrated that germination of *C. lindemuthianum* spores requires about 24 hr.

The sequential production of cell wall degrading enzymes demonstrates that pathogen utilization of monocotyledon and dicotyledon cell walls is similar. These observations confirm previous studies with other fungal pathogens grown on host dicotyledon cell walls (6, 11, 14). The detection of pectin-degrading enzymes in early cultures suggests that pectin hydrolysis may be an essential activity for monocotyledon wall degradation, just as it is believed to be in dicotyledon wall breakdown (8).

Although many components are present in common, bean and corn walls do have structural differences (5, 9). One variation is that xylan in arabinoxylan is a component of primary monocotyledon cell walls (5) whereas xylans are secondary additions in dicotyledons (16). This change in the structural role of xylans may account for the greater production of xylanase and β-xylosidase observed during growth of *H. maydis* and *C. lindemuthianum* on corn compared to bean cell walls. Similarly, the presence of a β-1,3-glucan in corn (4) but not bean walls may explain the greater production of laminarinase by *H. maydis* in corn wall cultures. Overall, however, the structural and compositional differences did not prevent utilization of monocotyledon walls by a pathogen of a dicotyledon, or of dicotyledon walls by a pathogen of a monocotyledon. Consequently, although the ability to degrade plant cell walls may be an essential requirement for virulence with certain pathogens, the degradative ability alone is unlikely to be the primary factor in determining the host range specificity.

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