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Host-Pathogen Interactions

II. PARAMETERS AFFECTING POLYSACCHARIDE-DEGRADING ENZYME SECRETION BY *COLLETOTRICHUM LINDEMUTHIANUM* GROWN IN CULTURE[1]

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ABSTRACT

The effect of a number of physiological variables on the secretion of polysaccharide-degrading enzymes by culture-grown *Colletotrichum lindemuthianum* (Saccardo and Magnus) Scribner was determined. The number of spores used to inoculate cultures grown on isolated bean hypocotyl cell walls affects the time after inoculation at which enzyme secretion occurs, but has no significant effect on the maximal amount of enzyme ultimately secreted. Cell walls isolated from bean leaves, first internodes, or hypocotyls (susceptible to *C. lindemuthianum* infection), when used as carbon source for *C. lindemuthianum* growth, stimulate the fungus to secrete more α-galactosidase than do cell walls isolated from roots (resistant to infection). The concentration of carbon source used for fungal growth determines the final level of enzyme activity in the culture fluid. The level of enzyme secretion is not proportional to fungal growth; rather, enzyme secretion is induced. Maximal α-galactosidase activity in the culture medium is found when the concentration of cell walls used as carbon source is 1% or greater. A higher concentration of cell walls is necessary for maximal α-arabinosidase activity. Galactose, when used as the carbon source, stimulates α-galactosidase secretion but, at comparable concentrations, is less effective in doing so than are cell walls. Polysaccharide-degrading enzymes are secreted by *C. lindemuthianum* at different times during growth of the pathogen on isolated cell walls. Pectinase and α-arabinosidase are secreted first, followed by β-xylosidase and cellulase, then β-glucosidase, and, finally, α-galactosidase.

An important aspect of the role of polysaccharide-degrading enzymes in plant pathogenesis is the control of the production of such enzymes (1). The variety and concentration of mono- and polysaccharides in the environment in which a pathogen is growing affect both quantitatively and qualitatively the array of polysaccharide-degrading enzymes secreted by the pathogen (1).

The bean pathogen, *Colletotrichum lindemuthianum*, secretes several polysaccharide-degrading enzymes when grown in culture in a medium containing, as the sole carbon source, cell wall material isolated from the hypocotyls of young bean plants (4). The secretion of these enzymes is apparently regulated both by the pathogen and by the host plant, since enzyme secretion can be correlated with the strain of the fungus and with the origin of the cell wall material on which the fungus is grown (4). Additional studies have been carried out with this system, and this report describes the effects of several parameters on the production of polysaccharide-degrading enzymes by *C. lindemuthianum* grown in culture.

MATERIALS AND METHODS

Plant Material and Isolation of Cell Walls. Cell walls isolated from the various anatomical parts of the common bean (*Phaseolus vulgaris* L. cv. Red Kidney) were used throughout this study. The growth conditions and method of cell wall preparation are modifications of those described previously (10, 11). Seeds were surface sterilized by immersion for 2 min in a 0.5% sodium hypochlorite (10% Clorox) solution. The sterilized seeds were rinsed thoroughly with distilled water and planted at a depth of 6 mm in a bed of moist vermiculite. Plants were grown in a Percival growth chamber and watered daily with distilled water. A photoperiod regime of 14 hr of maximal light followed by 10 hr of darkness was employed. The temperature was maintained at 28 C during the light period and at 20 C during the dark period. The plants were kept about 1 m from the light. The relative humidity was kept at 50%. Plants were harvested after 8 days of growth. For the final 30 hr preceding harvest, plants were kept in total darkness to ensure that starch would not contaminate the cell wall preparations. The bean plants were subdivided into roots, hypocotyls, first internodes, and primary leaves and frozen and stored in polyethylene bags at −20 C.

Cell walls were prepared by grinding the frozen tissue to a fine powder in liquid nitrogen with the aid of a mortar and pestle. This frozen powder was then ground in a Waring Blendor for 5 min with approximately 2.5 volumes (v/w) of cold 100 mM potassium phosphate buffer, pH 7.0. The insoluble material was collected on a coarse sintered glass funnel by suction filtration. The residue was resuspended in 1 volume of cold buffer in the sintered glass funnel. This suspension was allowed to stand at 2 C for 5 min with occasional stirring before the buffer was removed by suction filtration. This washing procedure was repeated four times, using 1 volume of buffer each time. The five buffer washes were followed by one wash with 1 volume of cold distilled water to remove salts. The residue was suspended in 2.5 volumes of a cold mixture of chloroform and methanol (1:1 v/v) and ground for 5 min in a Waring Blendor. The insoluble material was collected on a sintered glass funnel, washed three times with 1 volume of the chloroform-methanol mixture at room temperature, and then washed three times with 1 volume of acetone at room tempera-

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ture. The residue remaining after the acetone extraction, which constitutes the cell walls used in this study, was air dried and stored at room temperature. Immediately preceding use, the cell walls were placed in a dessicator under reduced pressure for 8 to 12 hr to remove residual water.

Growth of Fungus. Virulent isolates of the α-race of *Colletotrichum lindemuthianum* (Saccardo and Magnus) Scribner were maintained on slants of bean pod agar. The pathogen retains virulence when grown on this medium, whereas it loses virulence when grown on potato dextrose agar (R. W. Goth, personal communication, and unpublished results of this laboratory). The *C. lindemuthianum* slants were grown at room temperature under fluorescent lighting to stimulate sporulation. The virulence of the pathogen was checked before each experiment by the method described previously (4).

Conidiospores to be used as inoculum were obtained by adding 4 ml of sterile distilled water to 7- to 10-day-old slants and agitating with a Vortex mixer. The number of conidia in such water suspensions was determined with the aid of a hemacytometer. The desired inoculum size was obtained by dilution with sterile distilled water.

The secretion of polysaccharide-degrading enzymes by *C. lindemuthianum* was studied by growing the fungus in shake culture. Using sterile technique, the conidial suspension was pipetted into a flask containing a basal salts medium (4), the appropriate carbon source, and penicillin at a concentration of 1 mg/ml, to reduce the risk of bacterial contamination. Streptomycin cannot be used in these cultures because of its deleterious effect on the activity of the polysaccharide-degrading enzymes secreted by the fungus. Penicillin has no apparent effect on the rate of fungal growth, on the amount of enzymes secreted, or on the activities of these enzymes.

Two methods of culturing the fungus were utilized for enzyme production studies. The first method (method I) has been described previously (4). In this method the fungus was grown in 1 liter of medium in a 2800-ml Fernbach flask fitted with a three-way stopcock to permit sampling under aseptic conditions. The cultures were incubated at 23 C on a rotary shaker at a shaking rate of 60 to 70 rpm. A faster rate of shaking results in lower enzyme activities in the culture medium.

It is not practical to grow the fungus in a liter of medium when using high concentrations of cell wall material as the carbon source. For this reason, a second method of culturing the fungus was adopted. In this method (method II) the fungus was grown in 10 ml of medium in a 50-ml Erlenmeyer flask. The same number of spores were introduced into each of a series of flasks containing the same medium. At daily (or longer) intervals, one culture from each of the series was harvested, and the culture fluid from each assayed for enzyme activity. These small cultures were agitated on a rotary shaker at a rate of 150 rpm. In cultures grown for a period of 2 weeks or longer, evaporation has a significant effect on the volume of the culture medium (10% reduction in volume after 2.5 weeks of growth). The volume of the culture fluid from such cultures was restored to 10 ml by the addition of distilled water prior to carrying out enzyme assays. Evaporation was reduced by covering the cotton plugs with Parafilm during the first 4 days of growth of the cultures.

Culture fluid from method I cultures was centrifuged in a clinical centrifuge at 1000*g* to remove cell wall material, fungal mycelia, and spores before being used in enzyme assays. Culture fluid for use in enzyme assays from method II cultures was obtained by filtering the cultures through a coarse sintered glass funnel to remove cell walls and fungal mycelia. The filtered solution was then centrifuged to remove fungal spores. Growth of *C. lindemuthianum* in a given medium results in the production of similar amounts of polysaccharide-degrading enzymes, regardless of which of the two culture methods is used.

Enzyme Assays. The activities of α-arabinosidase, α-galactosidase, β-glucosidase, and β-xylosidase were determined by hydrolysis of the respective *p*-nitrophenyl glycosides. The galactoside, glucoside, and xyloside derivatives were purchased from either Sigma Chemical Company (St. Louis) or Koch-Light Laboratories Ltd. (Colnbrook, Buckinghamshire, England). The *p*-nitrophenyl-α-arabinoside was prepared in this laboratory following the procedure of Fielding and Hough (5). Solutions of the glycosides (1 mg/ml) were made up either in 100 mM potassium phosphate buffer, pH 6.2, or in 100 mM sodium acetate buffer, pH 5.2. In early experiments reactions were carried out at pH 6.2 since this is the pH of the culture medium. It has since been determined that each of these enzymes exhibits a broad pH optimum ranging from pH 4.0 to pH 5.5. For this reason, enzyme activities are now determined at pH 5.2, a pH at which the activity of each enzyme is between 95 and 100% of the activity at the pH optimum. Activities of the enzymes at pH 6.2 are between 80 and 90% of the activities obtained when reactions are carried out at the optimum pH. One milliliter of enzyme solution was added to 1 ml of glycoside solution, and the mixtures were incubated at 27 C for 1 hr. When the activity of the enzyme exceeded 330 nmoles of substrate hydrolyzed per ml of culture medium in 1 hr (2.0 optical density units of *p*-nitrophenol), the medium was diluted with basal salts solution (4), or the enzyme reactions were allowed to proceed for a shorter length of time. The reactions were stopped by adding 1 ml of 1 N NH_4OH containing 2 mM disodium EDTA to the reaction mixture. The addition of the NH_4OH solution raises the pH of the reaction mixture above 9.5 and effectively terminates the reaction while developing the *p*-nitrophenol color. The addition of EDTA prevents the formation of insoluble magnesium hydroxide from the Mg^{2+} ions in the culture medium. The absorbance of the samples at 400 nm was determined in a Gilford Model 2000 spectrophotometer. Control reactions were carried out in which the substrate and enzyme were incubated separately and the absorbance of each was subtracted from that of the complete reaction. Enzyme activities are expressed as the number of μmoles or nmoles of substrate hydrolyzed by 1 ml of the culture fluid in 1 hr at 27 C.

Pectinase and cellulase activities were determined by measuring the reduction in viscosity of 1% solutions (w/v) of citrus pectin (Sunkist Growers Association) and carboxymethyl cellulose (Hercules Powder Co., Type 7MP), respectively, with the aid of a Wells-Brookfield microviscometer. These substrates were dissolved in 100 mM sodium acetate buffer, pH 5.2. In the pectinase assays, 1 ml of culture fluid enzyme was added to 3 ml of substrate, and the reactions were incubated at 27 C for 10 min, at which time the viscosity was determined. Cellulase assays were carried out in the same manner except that 4 ml of substrate and 20 μl of enzyme solution were used. Control reactions were carried out in which the substrates were incubated in the presence of buffer rather than enzyme solution. The relative activities of pectinase and cellulase are expressed as the square of the per cent loss in viscosity after a 10 min reaction time (2).

RESULTS

Effect of Inoculum Size on Levels of α-Galactosidase Secretion. *C. lindemuthianum*, when grown in culture using isolated cell walls as the sole carbon source, secretes α-galactosidase into the culture medium. An experiment was carried out to determine the effect of initial inoculum size on the eventual maximal level of culture medium α-galactosidase activity. A spore suspension, containing 5×10^6 spores/ml, was diluted serially. One-milliliter aliquots containing either 5×10^6, 1×10^6, 5×10^5, 1×10^5, 1×10^4, or 1×10^3 spores were used to inoculate 1-liter cultures containing, as the sole carbon source, 500 mg of cell wall material isolated from Red Kidney bean hypocotyls. The cultures were

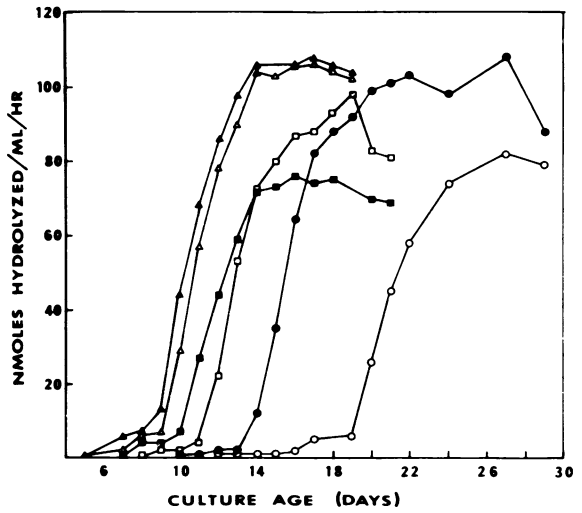

FIG. 1. The activity of α-galactosidase (ability to hydrolyze *p*-nitrophenyl-α-D-galactoside) in the culture medium of *C. lindemuthianum* grown on isolated cell walls has been studied as a function of culture age in cultures inoculated with different numbers of *C. lindemuthianum* spores. Spores (suspended in 1 ml of sterile distilled water) were introduced into 1 liter of medium containing as the sole carbon source 500 mg of cell wall material isolated from the hypocotyls of 8-day-old Red Kidney beans. Cultures were grown according to method I (see "Materials and Methods"). The numbers of spores used in inoculating the cultures were 1×10^3 (○), 1×10^4 (●), 1×10^5 (□), 5×10^5 (■), 1×10^6 (△), and 5×10^6 (▲).

grown according to method I. The amount of α-galactosidase activity in the culture medium was measured at daily intervals. The enzyme reactions were carried out at pH 6.2. It may be seen from the results of this experiment, which are shown in Figure 1, that the number of spores used to initiate a culture affects the time after inoculation at which α-galactosidase secretion occurs, but the inoculum size has no significant effect on the amount of enzyme ultimately secreted.

There is no practical way in which fungal growth can be measured in such cultures. The medium contains no organic molecules other than the cell wall material, so that a radiolabeling technique is not feasible. And it is not possible to measure the mass of fungal mycelia since there is insoluble bean cell wall material present in the cultures. In all cultures, α-galactosidase activity was first measurable in the culture medium 2 or 3 days after fungal mycelia became visible. Cultures inoculated with fewer spores were very much slower to exhibit mycelial growth than were cultures inoculated with greater numbers of spores, although the total mycelial growth eventually reached approximately the same levels in all flasks. A plot of the data in Figure 1 as the log of α-galactosidase activity *versus* the log of time yields a family of straight lines with similar slopes, indicating that the rate of α-galactosidase secretion (and probably the rate of growth) achieved by all of the cultures is the same. It is most likely, therefore, that the effect of inoculum size on enzyme secretion is a reflection of a lag which occurs before logarithmic growth is initiated; the duration of this lag varies inversely with respect to the number of spores used to inoculate a culture.

Effect of the Type of Cell Walls Used as Carbon Source on α-Galactosidase Secretion. Another parameter which affects enzyme secretion by culture-grown *C. lindemuthianum* is the type of cell wall material used as the carbon source. A study of the ability of cell walls isolated from the different morphological parts of bean plants to support fungal growth and to stimulate α-galactosidase secretion was undertaken. One-liter cultures containing 500 mg of cell wall material as the carbon source were inoculated with 1×10^6 spores and grown according to method I. The cell wall material in each culture was isolated from either leaves, first internodes, hypocotyls, or roots of 8-day-old Red Kidney beans. The activity of α-galactosidase in the culture medium was measured over a 21-day period. The enzyme activity was determined at pH 6.2. The results are shown in Figure 2. Cell walls isolated from leaves, first internodes, and hypocotyls stimulate α-galactosidase secretion significantly, but only a small amount of α-galactosidase activity is found in the medium of *C. lindemuthianum* cultures grown on root cell walls. The rate and extent of mycelial growth is similar regardless of which of the four types of cell walls the fungus is grown on.

Two other factors which might cause variation in the ability of cell walls to stimulate enzyme secretion are the age of the tissue from which the cell walls were obtained and the method of cell wall isolation. Cell walls isolated from hypocotyls of 4-, 6-, 8-, and 10-day-old bean plants, when used as the carbon source in *C. lindemuthianum* cultures, all stimulate α-galactosidase secretion to approximately the same extent. (Bean hypocotyls are susceptible to *C. lindemuthianum* infection until they are about two weeks old.) Cell walls isolated from plant material which had been chopped with a razor blade (11) and cell walls isolated from plant material ground in liquid nitrogen stimulate enzyme secretion to a similar extent. This is true even though the cell walls prepared in liquid nitrogen are a much finer (less fibrous) and apparently cleaner preparation. Homogenization of plant material in buffers of different concentrations (from 10 mM–500 mM) also has no significant effect on the ability of cell walls isolated from these differently extracted tissues to stimulate enzyme secretion; this is true even though some polysaccharides are extracted from cell walls by high buffer concentration.

It has been reported by two of us (4) that the ability of bean hypocotyl cell walls to stimulate secretion of α-galactosidase by *C. lindemuthianum* is correlated with the susceptibility to *C. lindemuthianum* infection of the bean variety from which the cell walls were isolated. We have more recently obtained results

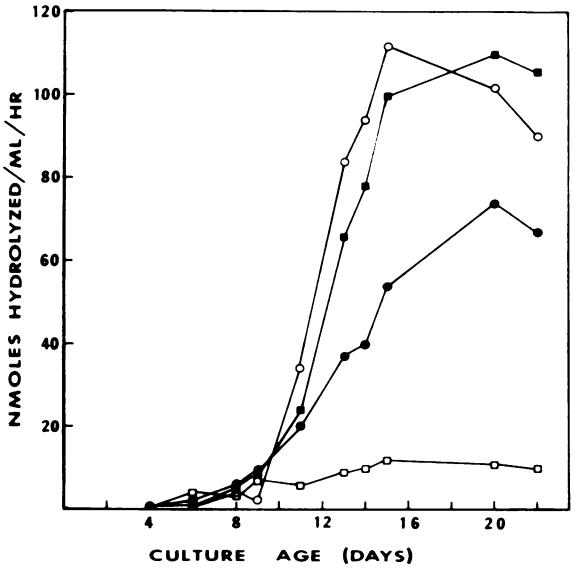

FIG. 2. The activity of α-galactosidase (ability to hydrolyze *p*-nitrophenyl-α-D-galactoside) in the medium of *C. lindemuthianum* cultures was determined as a function of culture age for fungal cultures in which the cell walls used as carbon source were isolated from different morphological parts of 8-day-old Red Kidney beans. One-liter cultures containing 500 mg of cell wall material obtained from leaves (●), first internodes (■), hypocotyls (○), and roots (□) were inoculated with 1×10^6 spores and grown according to method I (see "Materials and Methods").

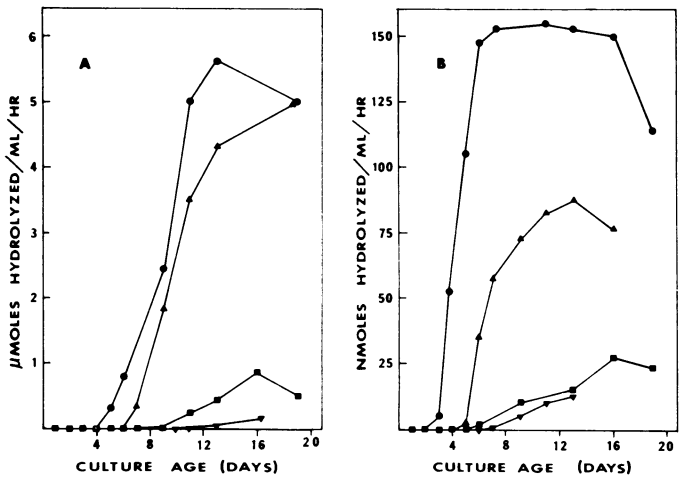

FIG. 3. The activity of α-galactosidase (A) and α-arabinosidase (B) (ability to hydrolyze *p*-nitrophenyl-α-D-galactoside and *p*-nitrophenyl-α-L-arabinoside, respectively) in the medium of *C. lindemuthianum* cultures was determined as a function of culture age for cultures in which the amount of cell wall material used as the carbon source varied. Each of a series of 10-ml cultures containing 0.1% (▼), 0.3% (■), 1.0% (▲), and 2.0% (●) cell walls isolated from the hypocotyls of 8-day-old Red Kidney beans was inoculated with 1 × 10^4 spores and the cultures grown according to method II (see "Materials and Methods").

which contradict this finding. Cell walls isolated from Red Kidney bean hypocotyls are most effective in stimulating α-galactosidase secretion by the α, β, and γ strains of the fungus, cell walls from Pinto bean hypocotyls have an intermedite ability to stimulate α-galactosidase secretion by all three races of the fungus, whereas cell walls isolated from Small White bean hypocotyls are least effective in stimulating enzyme secretion by the three races. Thus, while stimulation of α-galactosidase secretion by all three races of *C. lindemuthianum* can be correlated with the variety of bean from which cell walls are isolated, there is no correlation between ability to stimulate enzyme secretion and susceptibility to *C. lindemuthianum* infection (4). Our earlier results probably reflected uncontrolled variation in the rate of growth of the bean seedlings from which the cell walls were prepared (controlled environment chambers were unavailable to us at that time).

Effect of Carbon Source Concentration on the Levels of α-Galactosidase and α-Arabinosidase Secretion by *C. lindemuthianum*. Experiments were carried out to determine the effect of carbon source concentration on the levels of enzyme activity secreted by *C. lindemuthianum*. The cultures were grown according to method II. Figure 3 shows the results of an experiment in which hypocotyl cell walls isolated from 8-day-old Red Kidney beans were used as the sole carbon source. Series of 10-ml cultures were grown in which the cell wall concentrations were either 0.1, 0.3, 1.0, or 2.0% (w/v). The activities in the culture medium of α-galactosidase and α-arabinosidase were determined over a 19-day period. Enzyme assays were carried out at pH 5.2. α-Galactosidase secretion (Fig. 3A) is stimulated maximally when the concentration of cell walls in the medium is 1%; raising the concentration to 2% does not result in a significant increase in the maximal amount of enzyme secreted. The effect of cell wall concentration on α-arabinosidase is different (Fig. 3B). More α-arabinosidase secretion is observed in cultures containing 2% cell walls than in cultures containing 1% cell walls. It is not feasible to grow *C. lindemuthianum* in medium containing more than 2% cell walls (the suspension becomes so viscous that it will not aerate by shaking). Therefore, it was not possible to determine whether maximal stimulation of α-arabinosidase secretion is obtained with 2% cell walls or if a higher concentration of cell walls would result in the secretion of still larger amounts of this enzyme.

C. lindemuthianum was also grown in culture utilizing D-galactose as the sole carbon source. Flasks containing various concentrations of galactose in 25 ml of medium were inoculated with 2.5 × 10^4 spores. The cultures were harvested after 15 days by filtering through weighed Millipore filters (0.45 μ pore size). The filter discs were dried in an oven at 100 C and the dry weight of fungal mycelia determined. Since high concentrations of galactose inhibit α-galactosidase, the medium from all cultures in which the concentration of galactose used as carbon source exceeded 0.2% was diluted with basal salts solution such that the final galactose concentration was equal to 0.2%. The mycelia-free culture fluid from each flask was then assayed for α-galactosidase activity at pH 6.2. Figure 4 shows the effect of culture medium galactose concentration on both the amount of culture medium α-galactosidase activity and the dry weight of the mycelia. The increase in α-galactosidase activity is a logarithmic function of the galactose concentration while mycelial mass is increased only slightly by galactose concentrations greater than 2%.

Other monosaccharides also have the ability to stimulate polysaccharide-degrading enzyme secretion by *C. lindemuthianum*. L-Arabinose, when used as carbon source, stimulates α-arabinosidase secretion when present in concentrations up to 3%. At arabinose concentrations of greater than 3%, the secretion of α-arabinosidase is suppressed, and at arabinose concentrations of greater than 6%, growth of the fungus is inhibited as well. When D-xylose is used as the carbon source, significant amounts of both β-xylosidase and β-glucosidase activity are found in the culture medium. These results were obtained by growing the fungus in

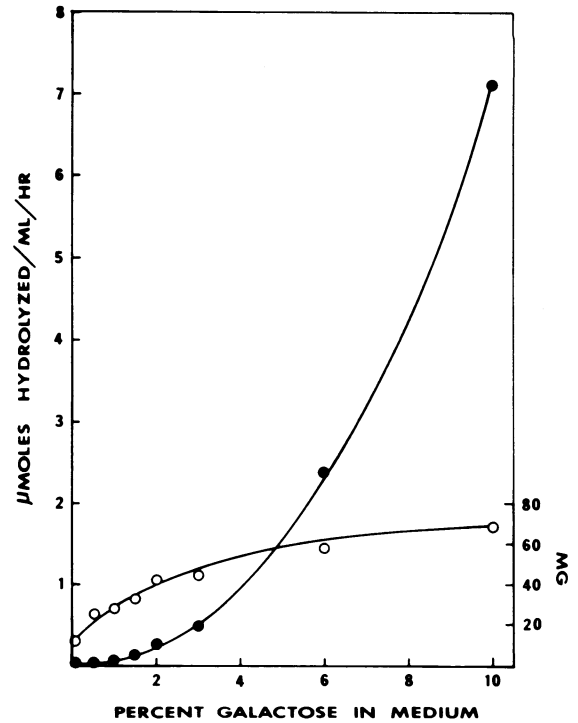

FIG. 4. The activity of α-galactosidase (ability to hydrolyze *p*-nitrophenyl-α-D-galactoside) in the medium of *C. lindemuthianum* cultures and the dry weight of mycelia were determined as a function of the concentration of galactose used as the sole carbon source. Twenty-five-milliliter cultures, each containing a different galactose concentration, were inoculated with 2.5 × 10^4 spores and grown for 15 days. The cultures were harvested and the activity of α-galactosidase in the culture fluid (●) and the dry weight of the mycelia (○) determined.

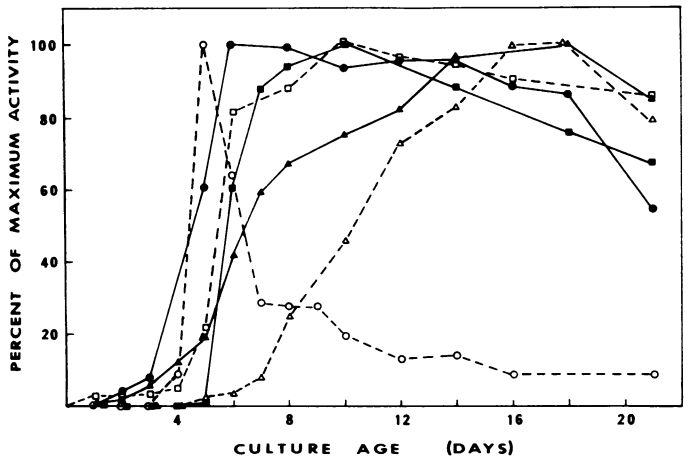

FIG. 5. The activity of polysaccharide-degrading enzymes in the medium of *C. lindemuthianum* cultures grown in a medium containing 1% Red Kidney bean hypocotyl cell walls as the sole carbon source was determined as a function of culture age. Each of a series of 10-ml cultures was inoculated with 1×10^4 spores and grown according to method II (see "Materials and Methods"). The enzyme activities detected are pectinase (○), α-arabinosidase (●), β-xylosidase (□), cellulase (■), β-glucosidase (▲), and α-galactosidase (△). Enzyme assay procedures are described in "Materials and Methods."

culture medium in which the xylose was present at a concentration of 2%; the effect of different concentrations of xylose on the secretion of these enzymes was not determined.

Sequential Nature of Polysaccharide-Degrading Enzyme Secretion by *C. lindemuthianum*. The culture age at which *C. lindemuthianum* secretes particular polysaccharide-degrading enzymes into its medium was examined in experiments in which the fungus was grown according to method II in a medium containing 1% Red Kidney bean hypocotyl cell walls as the carbon source. The activity in the culture medium of the polysaccharide-degrading enzymes was assayed over a 21-day period. All enzyme reactions were carried out at pH 5.2. The results of one such experiment are shown in Figure 5. It is evident from such results that enzyme secretion occurs in a sequential manner during the growth of the fungus. The first activities detectable in the medium are those of pectinase and of α-arabinosidase. The next enzymes to be secreted are β-xylosidase and cellulase, followed by β-glucosidase, and, finally, by α-galactosidase. This pattern of sequential induction has been observed in several experiments and, although the culture age of half maximal activity for a particular enzyme may vary by as much as a day, the order in which the enzymes are secreted remains the same. The maximal activity detected in the culture medium for each of the polysaccharide-degrading enzymes assayed in this experiment (Fig. 5) was determined. One milliliter of fluid from 5-day-old cultures caused a 20% reduction in the viscosity of a 1% pectin solution in 9.5 min, and 1 ml of fluid from 10-day-old cultures caused a 20% reduction in the viscosity of a 1% carboxymethyl cellulose solution in 0.04 min. One milliliter of culture fluid released, at pH 5.2, the following amounts of *p*-nitrophenol in 60 min: 180 nmoles from *p*-nitrophenyl-α-L-arabinoside on day 6, 170 nmoles from *p*-nitrophenyl-β-D-xyloside on day 10, 500 nmoles from *p*-nitrophenyl-β-D-glucoside on day 18, and 11.4 μmoles from *p*-nitrophenyl-α-D-galactoside on day 18.

DISCUSSION

Secretion of α-galactosidase has previously been correlated with the ability of *C. lindemuthianum* to infect bean plants. Virulent isolates of this pathogen secrete significant amounts of α-galactosidase when grown in culture using bean hypocotyl cell walls as the sole carbon source, while avirulent isolates of the fungus secrete very little of this enzyme when grown under the same conditions (4). The pattern of α-galactosidase secretion by *C. lindemuthianum*., when this fungus is cultured on cell walls isolated from different morphological parts of the bean plant (Fig. 2), constitutes additional evidence that this enzyme is important in pathogenesis. Leaves, first internodes, and hypocotyls are tissues susceptible to infection by *C. lindemuthianum*, and cell walls isolated from these tissues stimulate this fungus to secrete relatively large amounts of α-galactosidase. Roots are resistant to infection by *C. lindemuthianum*, and cell walls isolated from this tissue fail to induce high levels of α-galactosidase.

The amount of α-galactosidase secreted by *C. lindemuthianum* cultured on different types of cell walls (Fig. 2) is not proportional to the amount of galactose in the cell wall polysaccharides. The cell walls isolated from leaf, first internode, and hypocotyl tissue of 8-day-old Red Kidney beans contain approximately the same amount of acid-hydrolyzable galactose (10–13% of the dry weight), whereas the cell wall material isolated from the roots of such plants contains approximately 5% acid-hydrolyzable galactose (11). Whereas there is only a 2- to 3-fold difference in the amount of hydrolyzable galactose recoverable from the cell walls of these different tissues, there is a 10-fold difference in their ability to stimulate α-galactosidase secretion. The differential ability to stimulate α-galactosidase secretion may reflect differences in the structures of the galactose-containing cell wall polymers. The structure of the galactose-containing wall polysaccharides will determine which galactose-containing oligosaccharides and the amount of galactose itself, which can be released from the cell wall during pathogen growth. Such oligosaccharides or galactose in conjunction with other sugars are likely to be the inducers of α-galactosidase secretion.

The amount of each of the polysaccharide-degrading enzymes secreted by *C. lindemuthianum* is not proportional to the growth of the fungus. If this were the case, the patterns of secretion of α-galactosidase and α-arabinosidase (Fig. 3) would be similar. The fact that the ratio of activities of two different enzymes present in the same culture medium varies with the concentration of the cell walls used as carbon source demonstrates induction of enzyme secretion. The α-galactosidase inducer is apparently present in saturating levels when the concentration of 8-day old hypocotyl cell walls in the medium is 1% or more. The α-arabinosidase inducer, on the other hand, is not present at saturating levels until the concentration of the cell walls is greater than 1%. The maximal detectable culture medium activities of α-galactosidase and of α-arabinosidase differ by almost 40-fold. This may result from a difference in the amount of these enzymes present in the culture medium or from a difference in the efficiency with which the enzymes hydrolyze their model substrates.

The results summarized in Figure 4 demonstrate that galactose is an inducer of α-galactosidase secretion. Although galactose induces secretion of α-galactosidase, it is unlikely that this sugar is, by itself, the primary inducer of α-galactosidase secretion when the fungus is growing on cell walls. Medium containing 1% cell walls stimulates α-galactosidase secretion much better than medium containing 1% galactose. This is true even though only 10 to 13% of the dry weight of cell walls is galactose. Since medium containing 1% cell walls is about 0.1% with respect to galactosyl residues, this monosaccharide cannot be solely responsible for the induction of α-galactosidase secretion. The inducer of α-galactosidase secretion is more likely to be either a galactose-containing fragment or a mixture of monosaccharides released from the bean cell walls by the action of *C. lindemuthianum* enzymes. It has not been determined whether the inducer is a galactose containing di- or trisaccharide, because the only oligosaccharides available commercially which contain α-galactosyl residues are raffinose

and stachyose. These sugars both contain glucose which we have found suppresses α-galactosidase secretion by *C. lindemuthianum*.

C. lindemuthianum grows more rapidly in cultures containing cell walls as the sole carbon source than it does in cultures containing a single monosaccharide as the carbon source. This may be due to the protein in cell walls or it may reflect synergistic enhancement of fungal growth due to the presence of a number of different sugars. This synergistic effect has been demonstrated with *Ophiostoma multiannulatum* where several monosaccharides have been shown to stimulate growth of this fungus on D-galactose (8). *Ophiostoma multiannulatum* is unable to grow in a medium in which galactose is the sole carbon source. D-Xylose in the medium permits this fungus to utilize galactose; this is true even when xylose is present in very low concentrations as compared to the concentration of galactose. L-Arabinose also stimulates growth of *O. multiannulatum* on galactose, but in order for arabinose to do so, this pentose must be present in concentrations four times greater than the concentration of galactose. The fungus can also utilize galactose for growth when this sugar is present in mixtures with other hexoses. A similar phenomenon has been observed in studies of the growth of the watermold *Allomyces macrogynus* on a number of monosaccharides (9). These observations demonstrate that, in these systems at least, mixtures of sugars, and the proportions of various sugars in these mixtures, can regulate fungal growth. It is possible that *C. lindemuthianum* possesses such a system.

The order in which the polysaccharide-degrading enzymes are secreted when *C. lindemuthianum* is grown on cell walls may reflect the order in which these enzymes must work to degrade the cell walls. Karr and Albersheim (7) have found that the action of a "wall modifying enzyme" is necessary before other polysaccharide-degrading enzymes can degrade cell wall polysaccharides. The sequential induction of polysaccharide-degrading enzymes has also been observed in another system. Barker *et al.* (3) have found that when *Rhodopseudomonas palustris* is grown in a medium containing orosomucoid, several enzymes are secreted which degrade the polysaccharide portion of this glycoprotein. The enzymes are secreted sequentially, and the sequence in which they are secreted reflects the availability of the glycosidic linkages which the enzymes are capable of cleaving.

Hayashi and Lin (6) have established that product induction is an important regulatory mechanism in the utilization of some carbohydrates by *E. coli*. The glycerol transport system in this bacterium is induced by L-α-glycerophosphate, which is the product of glycerol kinase, one of the essential enzymes in this transport system. Product induction apparently controls the sequential production of polysaccharide-degrading enzymes in both the *R. palustris* system and the *C. lindemuthianum* system. The induction of a particular enzyme in the *R. palustris* system does not occur until the sugar to be cleaved by that enzyme is present as terminal residues on the polysaccharide (3). This observation can be explained by product induction, since the small amount of enzyme secreted by noninduced cells could release enough sugar residues to induce that enzyme. In the *C. lindemuthianum* system, the secretion of α-galactosidase, α-arabinosidase, and β-xylosidase can be induced by that monosaccharide which is produced by the action of each enzyme on its model substrate.

The sequential manner in which polysaccharide-degrading enzymes are secreted when *C. lindemuthianum* is grown on cell walls may also have important implications concerning the ability of the fungus to grow so rapidly on this carbon source. The level of activity of the enzymes secreted by the fungus will affect the relative amounts of sugars available to the fungus. If the relative abundance of available sugars regulates growth rate, as has been shown to be true for *O. multiannulatum* (8), the sequential order of secretion and the relative activities of different polysaccharide-degrading enzymes may thus provide *C. lindemuthianum* and other plant pathogens with the proper environment for rapid growth in susceptible hosts. If a plant presents to a pathogen a mixture of sugars or polysaccharides which leads to the insufficient secretion or wrong timing of secretion of polysaccharide-degrading enzymes by the pathogen, abortive development of the pathogen may result. This would render the plant resistant to infection by that pathogen. It is possible that this situation could be mimicked in a susceptible host plant by applying externally sugars or sugar analogues to interfere with the normal induction of specific polysaccharide-degrading enzymes by the pathogen. This interference could inhibit normal development of the pathogen, giving the host time to develop resistance to infection. Indeed, nonpoisonous chemicals which interfere with the secretion of polysaccharide-degrading enzymes offer exciting possibilities for plant pest control.

LITERATURE CITED

1. ALBERSHEIM, P., T. M. JONES, AND P. D. ENGLISH. 1969. Biochemistry of the cell wall in relation to infective processes. Annu. Rev. Phytopathol. 7: 171–194.
2. ALMIN, K. E. AND K.-E. ERIKSSON. 1967. Enzymatic degradation of polymers I. Viscometric method for the determination of enzymic activity. Biochim. Biophys. Acta 139: 238–247.
3. BARKER, S. A., G. I. PARDOE, M. STACEY, AND J. W. HOPTON. 1963. Sequential enzyme induction: A new approach to the structure of complex mucoproteins. Nature 197: 231–233.
4. ENGLISH, P. D. AND P. ALBERSHEIM. 1969. Host-pathogen interactions. I. A correlation between α-galactosidase secretion and virulence. Plant Physiol. 44: 217–224.
5. FIELDING, A. H. AND L. HOUGH. 1965. The synthesis of *p*-nitrophenyl-α-L-arabinofuranoside. Carbohyd. Res. 1: 327–329.
6. HAYASHI, S. AND E. C. C. LIN. 1965. Product induction of glycerol kinase in *Escherichia coli*. J. Mol. Biol. 14: 515–521.
7. KARR, A. L. AND P. ALBERSHEIM. 1970. Polysaccharide degrading enzymes are unable to attack plant cell walls without prior action by a "wall modifying enzyme." Plant Physiol. 46: 69–80.
8. LINDBERG, M. 1969. Stimulating effect of pentoses on the utilization of galactose in *Ophiostoma multiannulatum*. Physiol. Plant. 22: 1273–1281.
9. MACHLIS, L. 1957. Effect of certain organic acids on the utilization of mannose and fructose by the filamentous watermold, *Allomyces macrogynus*. J. Bact. 73: 627–631.
10. NEVINS, D. J., P. D. ENGLISH, AND P. ALBERSHEIM. 1967. The specific nature of plant cell wall polysaccharides. Plant Physiol. 42: 900–906.
11. NEVINS, D. J., P. D. ENGLISH, AND P. ALBERSHEIM. 1968. Changes in cell wall polysaccharides associated with growth. Plant Physiol. 43: 914–922.