Characterization of Specific Induction, Activity, and Isozyme Polymorphism of Extracellular Cellulases from *Venturia inaequalis* Detected *in Vitro* and on the Host Plant

Andreas Kollar

Biologische Bundesanstalt, Institut für Pflanzenschutz im Obstbau, P.O. Box 1264, D-69216 Dossenheim, Federal Republic of Germany

Specific induction of cellulases (EC 3.2.1.4 and EC 3.2.1.91) in cultures of *Venturia inaequalis* was tested with various cellulosic substrates and their derivatives. The low constitutive amount of extracellular enzymes produced *in vitro* was increased about 100-fold with cellulosic sheets or with solvent-extracted leaves of apple in the medium. Enzyme induction required these materials to be intact. Grinding the substrates did not stimulate cellulase production. Any mechanical disintegration of leaf substrate resulted in a complete loss of inductive capacity. Presumably, topographic features of these substrates induce cellulase production. The yield of enzyme was correlated with mycelial development and was not affected by the addition of further carbon sources. High-pressure liquid chromatography of cellulase degradation products of cellodextrins and of cellulosic sheets indicated an endoglucanase type of cleavage with increasing activity toward substrates with higher degrees of polymerization. β-Glucosidases (EC 3.2.1.21) were mainly associated with fungal hyphae. Isoelectric focusing followed by a zymogram technique revealed a cellulase system of 12 isozymes with isoelectric points in the range of 3.7–5.6. The molecular weights were about 60 kD for at least five enzymes and about 25 kD for the five more prominent isozymes. The cellulase pattern from 19 isolates of *V. inaequalis* were essentially identical, and their differences were restricted mainly to quantitative variability. Cellulases produced *in situ* were isolated from leaf lesions of naturally infected apple trees. The cellulase pattern derived from the host-parasite interaction was qualitatively nearly identical to that of the enzymes produced *in vitro*.

Additional keywords: apple scab, biotrophic interaction, cell wall–degrading enzymes, pathogenesis, resistance.

Venturia inaequalis (Cooke) G. Wint., the causal agent of apple scab, is characterized by a particular type of parasitism. The invading mycelium is limited to a position between the cuticle and the outer epidermal cell walls, where it is associated with the underlying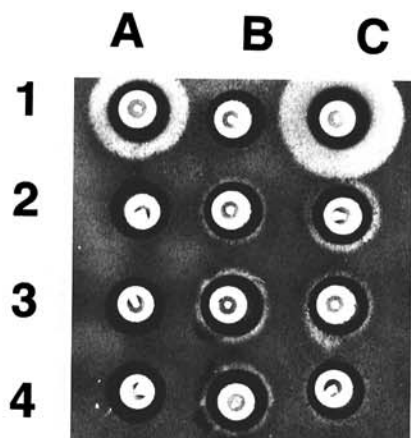 host cells. The fungus derives its nutrition at the host-parasite interface without the development of intracellular haustoria. Degradative enzymes are important for the release of nutrients, the removal of physical barriers, and the induction of plant cell biochemical and physiological reactions. Enzyme-generated products of plant cell wall origin act as elicitors (Ryan 1988) of host defense responses. Extracellular melanoproteins and associated enzymes produced *in vitro* by cultures of *V. inaequalis* are associated with the progress of the apple scab disease (Hignett and Kirkham 1967; Hignett *et al.* 1979; Hignett *et al.* 1984), but the mechanisms by which melanoproteins affect pathogenesis are still subject to speculation. Cutinase isolated from *V. inaequalis* has been identified as a crucial factor in penetration of the plant cuticle and in subcuticular growth of the pathogen; however, the cuticle is not involved in cultivar resistance (Valsangiacomo and Gessler 1988; Köller and Parker 1989; Köller *et al.* 1991; Hellmann and Stösser 1992). Ultrastructural studies of the host-parasite relationship have revealed that the cells of the host and the parasite are closely interlocked and that the plant's upper epidermal layer adjacent to the mycelial cells appears to be extensively degraded (Corlett *et al.* 1976). Leben and Keitt (1948) and Holowczak *et al.* (1962) published the first reports of the ability of *V. inaequalis* to metabolize cell wall components of plants. They demonstrated that *in vitro* the fungus is able to utilize pectin and, in some isolates, cellulose as the sole carbon source. Wagner *et al.* (1988) observed only marginal growth of *V. inaequalis* with cellulose as the sole source of carbon, which was strongly enhanced by a small amount of additional glucose. Very weak activities of cellulases were detected by viscometry versus hydroxyethyl cellulose from a preparation of culture filtrate or mycelium obtained from a defined liquid culture medium supplemented with cellobiose. Most cellulolytic activity was associated with the mycelium. Similarly, Valsangiacomo *et al.* (1989) detected most pectinolytic activity in mycelial extracts. They concluded that plant cell wall–degrading enzymes from *V. inaequalis* are not generally released into the medium and that this is related to the particular type of parasitism exhibited by this pathogen. However, Koller *et al.* (1992) isolated cellulolytic activities by ion exchange chromatography (IEC) of an unspecified amount of culture filtrate. The isolate of *V. inaequalis* was cultivated with a complex medium supplemented with powdered cellulose.

The aim of this study was to add to the knowledge of regulation and properties of cellulases of different *V. inaequalis* isolates, which are thought to be involved in plant resistance or at least to be essential for pathogenicity. The natural substrate for effective induction of enzymes and the detection, isolation, and characterization of enzymes secreted at the site of interaction from affected host plants are described.

RESULTS

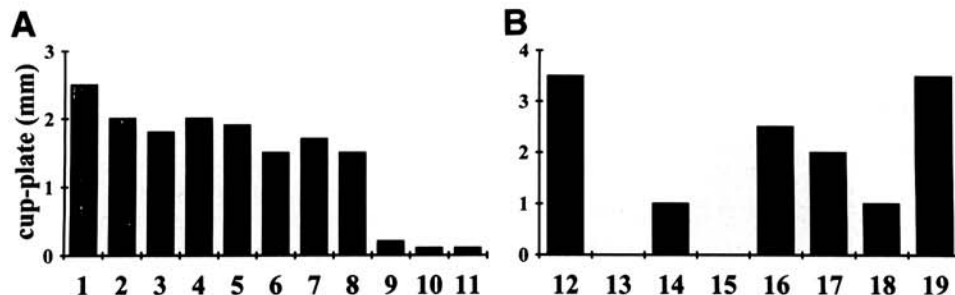### Regulation of cellulases.

In liquid cultures without effective cellulosic inducers and without a cellulosic substrate, only low levels of constitutive enzyme production were detected in the culture filtrate of isolate V1 (Fig. 1). All the other 18 isolates of the apple scab fungus showed a similar constitutive enzyme production. Serial dilutions of *V. inaequalis* cellulases used as standards in

the cup-plate assay proved that the constitutive concentration of cellulases is at most 1% of the amount detected in induced cultures (data not shown). Isozyme patterns of constitutive cellulases were essentially identical with those of induced enzymes (data not shown). Substrates such as xylan, powdered cellulose, filter paper (Fig. 1), cellodextrins, cellobiose, and carboxymethyl cellulose (CMC) (data not shown) did not enhance the production of cellulases. In addition, there was no induction by other sugars tested (glucose, fructose, arabinose, xylose, galactose, mannose, rhamnose, lactose, maltose, sucrose, and raffinose) or by polyols tested (myo-inositol, sorbitol, and mannitol). Cellulosic sheets (Figs. 1 and 2A) in potato-dextrose broth (PDB) and especially dialysis tubing (Fig. 2A) filled with the medium were effective inducers. Enzyme production in these cellulosic sheet cultures was not affected by the presence of further carbohydrates or all the other noninducing cellulosic substrates (data not shown). Potato broth containing no detectable glucose but containing cellulosic sheets as a major carbon source supported only marginal growth of *V. inaequalis* and low enzyme production. However, this was dramatically changed by the addition of glucose (data not shown). Among the different *V. inaequalis* isolates the level of cellulase production correlated with mycelial growth. Time courses of cellulase, total protein, and glucose concentrations in the medium for isolate V1 are given in Figure 3A. Qualitative and quantitative profiles of isozymes were not changed by the level of production; only the absolute amount of isozymes increased (Fig. 3B). Induction by cellulosic sheets depended on the absolute amount of substrate: a significant increase in enzyme production was evident when 0.5% (w/v) cellulosic sheet was added to the culture medium. Enzyme production increased until the amount of cellulosic sheet accounted for 2%. The size of the cellulosic substrate also had a significant influence on the induction of enzyme production (Fig. 2A). Cellulosic sheets with diameters larger than 200 μm showed an effective induction only. On the other hand, cultures with polyethylene sheets of similar dimensions alone or together with noninducing cellulosic substrates did not enhance enzyme production.

Preparations of leaves subjected to mechanical disruption were not inductive. Figure 2B shows the inducing capacity of solvent-extracted leaves. A preparation of 0.4% (w/v) sol-

Fig. 1. Detection of cellulases with cup-plate assay (20-hr incubation) as zones of hydrolysis indicated by reduced binding of Congo red. Activity of ammonium sulfate precipitations equivalent to about 12 ml of culture filtrate from *Venturia inaequalis* (isolate V1) grown for 45 days in potato-dextrose broth supplemented with different substrates: A1 and C1, cellulosic sheets (A1 is a one-tenth aliquot); A2–A4, water (control); B1, xylan; B2, powdered leaves (solvent-extracted); B3, mature leaves (not solvent-extracted); B4, potato-dextrose broth (control); C2, filter paper; C3, cotton wool; C4, powdered cellulose.


Fig. 2. A, Cellulosic sheet substrates of different sizes and shapes were tested for production of cellulases (zone of hydrolysis in cup-plate assay) in 40 ml-cultures (40 days old, in potato-dextrose broth) with *Venturia inaequalis* isolate V1 and 2% cellulosic substrate. Cellulosic sheet particles were obtained with sieve fractions of milled sheets. Preparations of ammonium sulfate precipitations equivalent to about 12 ml of culture filtrate were used for cup-plate assay. 1, Dialysis tubing containing the medium; 2, cellulosic sheet from the dialysis tubing; 3, cellulosic particles 10 × 10 mm; 4, particles >1.2 mm; 5, particles >0.7–1.2 mm; 6, particles >0.4–0.7 mm; 7, particles >0.3–0.4 mm; 8, particles >0.2–0.3 mm; 9, particles >0.1–0.2 mm; 10, particles >0.06–0.1 mm; 11, particles <0.06 mm. **B,** Apple leaf substrates extracted with chloroform/methanol (1:2, v/v), methanol, and ethanol were tested as inducers for production of cellulases in culture with isolate V1 (see **A**). 12, 2% mature leaves; 13, 2% powdered mature leaves; 14, 1% mature leaves; 15, 0.4% mature leaves; 16, 2% immature leaves; 17, 1% immature leaves; 18, mature and immature leaves, 0.4% each; 19, 2% dialysis tubing containing the medium.

vent-extracted mature leaves was not an effective inducer. A greater reduction or delay of fungal growth was associated with immature leaves at a concentration of about 2% in the culture medium. Autoclaving the leaves did not affect the inducing capacity or the delay of fungal growth. Isozyme patterns and time courses of enzyme production derived from cultures with either solvent-extracted leaves or cellulosic

sheet as inducers were identical. Intact leaves that were not extracted with solvents did not enhance the production of cellulases.

Partial purification and biochemical characterization.

Fractionated ammonium sulfate precipitation, lyophilization, evaporation at 40° C under vacuum, and IEC did not affect the quality of enzyme preparations, and quantitative losses by these procedures were negligible. Centrifugation of previously frozen culture filtrates and precipitation resulted in a considerable reduction of mucous substances and melanoproteins. Corrected to equivalent CMCase activity (cup-plate, viscometry), IEC increased specific activity to about nine times that in the culture filtrate. The concentration of total protein in culture filtrates was about 50 μg/ml. IEC followed by preparative isoelectric focusing allowed the detection of isoenzymes C1–C12, which otherwise could not be identified, as separated bands, through comparing isozyme banding patterns with total protein patterns (Fig. 4). Isoelectric points (pI) were 3.7 (C1), 3.8 (C2), 4.0 (C3), 4.1 (C4), 4.2 (C5), 4.4 (C6), 4.5 (C7), 4.6 (C8), 4.8 (C9), 5.0 (C10), 5.3 (C11), and 5.6 (C12). Molecular weights of enriched isozyme prepara-
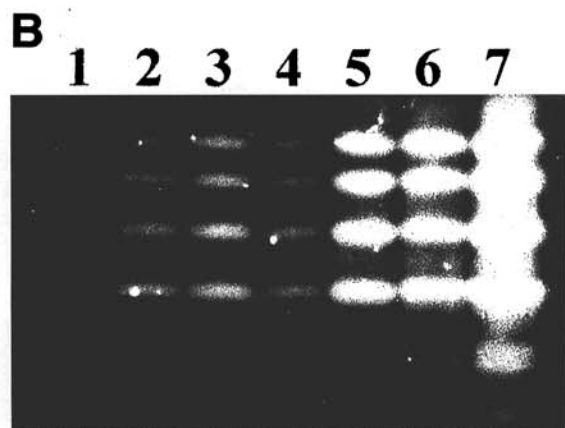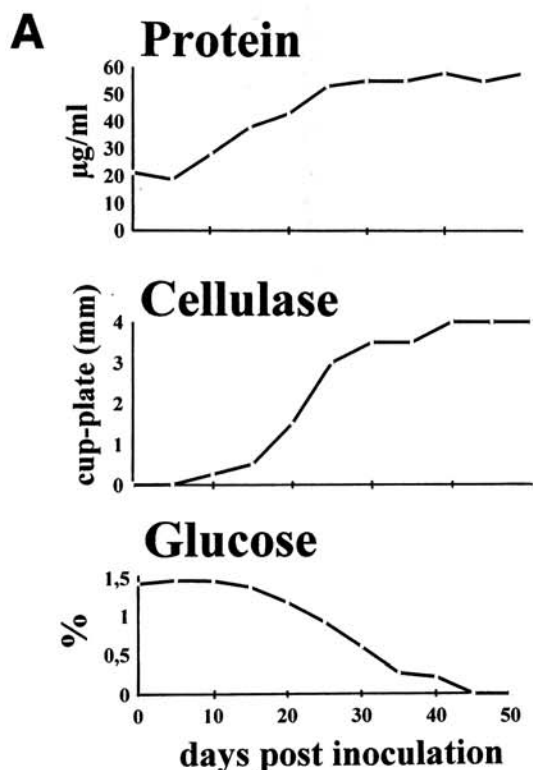
Fig. 3. A, Time course of total protein and glucose concentrations and cellulase activity (zone of hydrolysis in cup-plate assay) analyzed from single dialysis tubing cultures after different periods of incubation. Cultures (40 ml) were inoculated with conidia of *Venturia inaequalis* isolate V1. After 15 days about 50% of the dialysis tubing was covered with mycelia, and maceration of the tubing was evident. Strong maceration and total coverage with mycelia were present on day 25. **B,** Zymogram pattern analysis after different periods of fungal growth. Each lane represents the amount of cellulase preparation of about 0.5 ml of culture filtrate. Lane 1, 20 days; lane 2, 25 days; lane 3, 30 days; lane 4, 35 days; lane 5, 40 days; lane 6, 45 days; lane 7, 50 days.
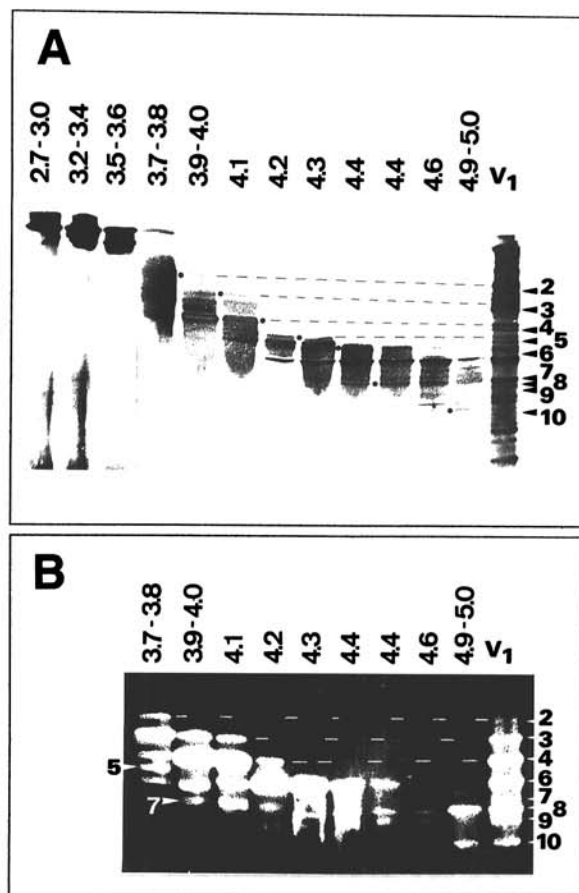
Fig. 4. Analytical isoelectric focusing (IEF) followed by a silver stain (**A**) or by a cellulase zymogram technique (**B**) of the same preparative IEF fractions with the indicated range of pH. Preparative IEF was performed with a crude preparation equivalent to about 1,000 ml of culture filtrate of *Venturia inaequalis* isolate V1. Refocusing was performed twice with fractions revealing cellulase activity in cup-plate assays (Fig. 1). Dashed lines with dots (**A**) indicate the positions of isozymes. Lane V1 was loaded with the unfractionated sample.

tions (Fig. 4) were estimated at 25 kD for C1–C4 and C6 and 60 kD for C5 and C7–C10. Binding to concanavalin A indicated that these cellulases are glycoproteins. IEC preparations of cellulases were stable for at least 15 days at temperatures from 0 to 30° C.

Cellulases were not associated with the mycelium. Ground mycelia or extracts thereof revealed high activities of β-glucosidases only.

Characterization of degradative activity and isozyme polymorphism.

Cellulase activities at different pH values are shown in Figure 5. The pH optimum was pH 4 at 30° C. At pH 5 and 5° C only low activity was detected, but higher temperatures increased enzyme activity. There was no temperature optimum observed up to 45° C. Degradation products of cellodextrins and cellulosic sheets obtained after a short (1-hr) incubation period with enzymes are listed in Table 1. The cleavage pattern was predominantly by endohydrolysis. Cellohexaose and cellopentaose were degraded completely, and 51% of cellotetraose was degraded. Only 15 and 8% of cellotriose and cellobiose were degraded, respectively. The activities of β-
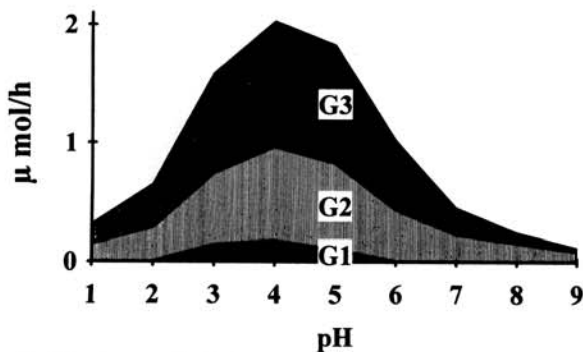
glucosidase and exoglucohydrolase (EC 3.2.1.74) were minute compared to those of cellobiohydrolase (EC 3.2.1.91) and endoglucanase, respectively. The time course of degradation of cellulosic sheets by the cellulases revealed a low steady-state concentration of cellobiose (about 0.5–0.8 µg per 10 µl) beginning after the first day of incubation. Glucose was generated constantly, leading to a linear increase of free glucose, but cellodextrins could not be analyzed. Maceration of the cellulosic sheets was visible after about 6 hr, and the medium became opaque after 24 hr of incubation. After 9 days 80% of the cellulose sheets was liberated as glucose. High-pressure liquid chromatography (HPLC) of degradation products from the cellulase/cellodextrin incubation mixtures supplemented with increasing amounts of cellobiose or glucose did not result in any inhibition of activity. Figure 6 shows that isozyme patterns of cellulases produced by the different *V. inaequalis* isolates were very uniform.

Isolation and characterization of cellulases secreted on the host plant

The imprints of scabbed leaves and fruits on substrate agar mostly formed a white halo within the area of mycelial growth (Fig. 7), indicating cellulase activity. Isolation of cel-

Fig. 5. pH optimum of cellulases. A cellodextrin mixture (cellohexaose, 9.5%; cellopentaose, 34.6%; cellotetraose, 43.1%; cellotriose, 3.5%; and cellobiose, 0.2%) was digested with cellulase preparations at 30° C and different pH values for 1 hr. Analyses of glucose (G1), cellobiose (G2), and cellotriose (G3) formed by the catabolism of cellulases were done by high-pressure liquid chromatography.

Table 1. Degradation of cellodextrins and cellulose by cellulases of *Venturia inaequalis* to glucose, cellobiose, and cellodextrins (µmol/hr)[a]

Substrate (mol % degraded)	Glc	Glc₂	Glc₃	Glc₄	Glc₅
Sheets of cellulose	0.1–0.6	Trace	...[b]	...	...
Cellohexaose (100)	1.2	11.0	9.1	8.1	...
Cellopentaose (100)	1.8	13.8	12.1	3.1	...
Cellotetraose (51)	1.8	4.8	7.2	...	...
Cellotriose (15)	0.5	2.5	...	1.9[c]	...
Cellobiose (8)	1.3	...	2.2[c]	...	...

[a] Carbohydrates were analyzed by high-pressure liquid chromatography after 1 hr of incubation with substrates and a cellulase preparation equivalent to 125 ml of culture filtrate.
[b] Not detected.
[c] Products indicating a higher degree of polymerization than the substrates used. The retention times were in the range of cellotriose and cellotetraose, respectively. Amounts are expressed as the equivalents of these standards.
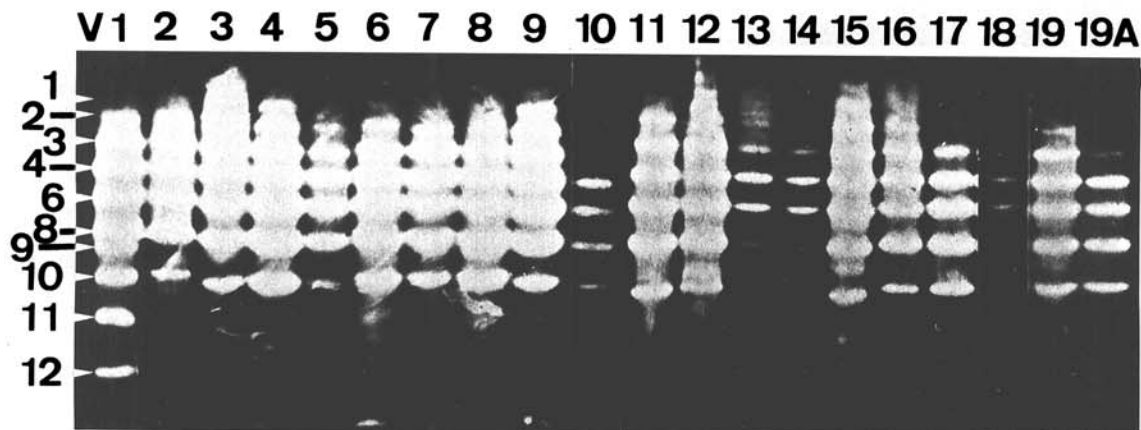

Fig. 6. Cellulase isozyme pattern analysis of *Venturia inaequalis* isolates obtained from the apple cultivar Golden Delicious. The isolates were grown on cellulosic sheet cultures with potato-dextrose medium for 45 days. V1 and V4–V19 are isolates from the orchard of the institute; V2 and V3 were isolated from trees at distances of about 25 and 300 km from the institute, respectively. V19A is an albino mutant of V19. Compared to the other isolates, V10, V13, V14, V18, and V19 showed only sparse mycelial development in cultures used for cellulase production.

lulases from a defined amount of lesions resulted in a total activity per lesion corresponding to about 3 μl of culture filtrate from an induced *in vitro* culture. The isozyme pattern of these isolated cellulases is presented in Figure 8.

DISCUSSION

There is a fundamental problem with the induction of enzymes by substrates which are not water-soluble, because they cannot enter the cells of the pathogen directly. With inducible cellulases it is generally assumed that low-level constitutive enzyme production might be responsible for the liberation of soluble compounds by limited cellulose hydrolysis. These products could induce directly or after a specific chemical modification by the pathogen (Mandels and Reese 1960; Mandels *et al.* 1962). In this study we detected very low constitutive levels of cellulase production in PDB; however, conventional induction with cellulosic substrates was not possible. There was no induction with cellodextrins and cellobiose, which are water-soluble products of cellulose hydrolysis. Analyzing the degradation of cellotriose and cellobiose revealed products with a higher molecular weight than the substrate itself (Table 1). These substances, possibly formed by transglycosylation, might be involved in the induction process. However, experiments with cellodextrins or cellobiose suggest that these derivatives by themselves are not effective inducers.

Cellulase production from *V. inaequalis* was induced only by cellulosic sheets larger than 200 μm in diameter or by intact solvent-extracted leaf material. Effective induction appears to depend on chemotropic and topographic signals, which are present on artificial and natural cellulosic substrates. A common biochemical property of both artificial and natural substrates could be the distinct lower degree of polymerization found in cellulosic sheets and in the primary cell walls of apple leaves. The degree of polymerization of cellulosic glucan chains in cellulosic sheets is about 300; for primary and secondary cell walls of cotton, it is about 20–600 and 11,000, respectively (Timpa 1991).

Commonly, most cellulases are affected by end-product inhibition, catabolite repression, or failure of induction when the substrate is not present as the sole carbon source. Such effects were not found with *V. inaequalis* cellulases. Inhibition of the induction process with unextracted apple leaves should provide the basis for future investigations concerning natural compounds regulating cellulase production.

The high cellulolytic activities detected in cultures supplemented with effective inducers in this study indicate that Wagner *et al.* (1988) and Koller *et al.* (1992) evidently investigated the constitutive level or at most a slightly enhanced level of cellulases of the fungal strain used. Furthermore, IEC enrichment of cellulases (Koller *et al.* 1992) could not be comprehensive, because the pH for separation was not suited to the enzymes with low pI. Accordingly, the pH optimum was about 5, with a pronounced decrease of enzyme activity at higher acidity levels. This study revealed an optimum at pH 4, with a functional range of pH 1–7. Valsangiacomo *et al.* (1989) suggested that pathogens (including *V. inaequalis*) which cause no maceration of host tissues and are not able to penetrate cell walls in the course of disease development produce only low amounts of cell wall–degrading enzymes associated with fungal hyphae. This investigation indicated that a specific induction was necessary for the production of high levels of cellulases, which were released into the medium *in vitro* and also onto cell walls of diseased apple leaves. Specific chemotropic and topographic signals regulating cellulase production may be the result of coevolution of host plant and
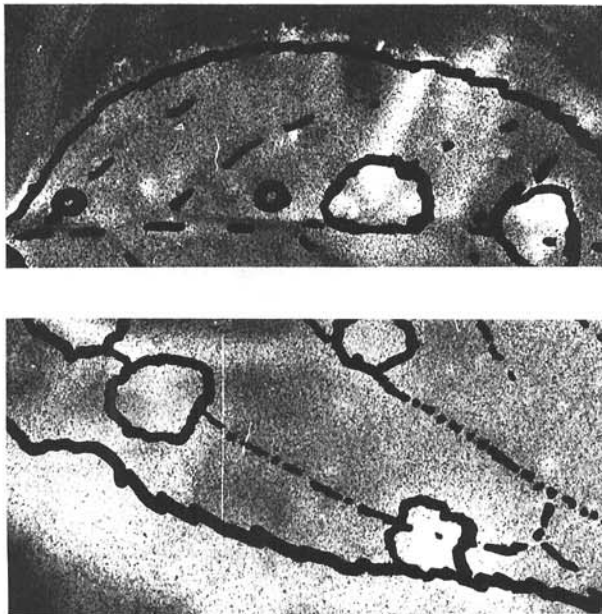
Fig. 7. Leaves from the apple cultivar Golden Delicious that were naturally infected with *Venturia inaequalis* (primary infections) were imprinted on agar with a carboxymethyl cellulose substrate for 20 hr. The lesions and leaves were outlined on the back of the dish. After the leaves were removed, the plates were incubated for 20 hr and stained with Congo red for detection of cellulases.
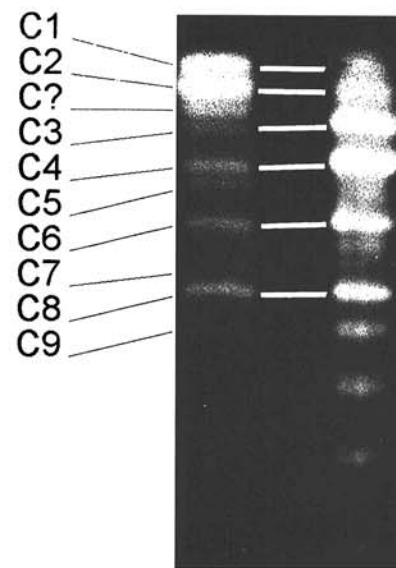
Fig. 8. Cellulase isozyme pattern analysis of enzymes isolated from leaf lesions of apple trees that were naturally infected with *Venturia inaequalis* (left lane) and isozymes produced *in vitro* by *V. inaequalis* isolate V1 (right lane). Nonnecrotic lesions from leaves of the cultivar Golden Delicious were scraped off and subjected to ion exchange chromatography. An aliquot of the preparation, corresponding to tissue from about 30 lesions, was used for isoelectric focusing.

parasite. During the biotrophic phase of parasitism a strong selection pressure may favor the development of an effective host-adapted cellulase system.

Generally, data from *in vitro* cultures with synthetic substrates should be interpreted with caution, because metabolism could be quite different in host-parasite interactions *in vivo*. This study revealed that solvent-extracted apple leaves could induce cellulase production *in vitro* only if the native structure of the leaves was preserved. The lack of induction by intact unextracted leaves *in vitro* may be caused by leaf substances of the dead tissues, which during culture may alter their compartmentation and chemistry after chemical sterilization or autoclaving. The initiation of cellulase production by the fungal pathogen on living epidermal cell walls is probably controlled by topographic and biochemical signals. The isolated activity of cellulases from lesions of scabbed leaves was determined as an activity developed per lesion equivalent to about 3 µl of culture filtrate from an induced culture. Considering the minute amounts of mycelial mass on leaf lesions in contrast to the huge amounts of mycelia obtainable with *in vitro* culture, together with losses during the preparation procedure, the production of cellulases *in situ* should be at least as high as *in vitro*. Furthermore, cellulases of host-parasite interaction were isolated from sporulating lesions of leaves from the orchard. In this case the cuticle was ruptured by emerging annellophores exposing the host-parasite interface to meteorological and microbial events, which could have interfered with cellulases secreted onto host cell walls. The high biochemical stability of cellulases revealed *in vitro* may be favorable for their specific activity *in situ*.

Isozyme patterns from cellulases isolated from the host-parasite interface were somewhat different from those of isozymes produced *in vitro*. One new band was detected in the zymograms, and C1 and C2 (pI 3.7 and 3.8) were more predominant within the pattern of isozymes (C3–C9). This mainly quantitative change may have several causes. As shown for *Armillaria*, differences in pectinolytic isozyme patterns are caused by the cultivation media (Wahlström *et al.* 1991). Beaulieu *et al.* (1993) proposed that for bacterial pectinases some enzymes could be produced in early stages of disease but are lost during late phases of infection. They also suggested a differential production of plant-inducible pectinases within host plants. Cellulase patterns derived from cellulosic sheets and apple leaf cultures did not reveal any differences. However, other biochemical factors in biotrophic growth, which may have been removed during solvent extraction in our experiments, could have changed the quantitative and qualitative profiles of the cellulases. The pattern obtained could be an indicator of a fine adjustment of the parasite to the host cultivar, Golden Delicious. Otherwise the isolation procedure could have led to a selective enrichment of several isozymes, or the availability of enzymes could have been different, because of an uneven loss of isozymes through host and environmental conditions.

Isozyme patterns of different *V. inaequalis* isolates showed considerable uniformity for the most active cellulases (C2, C3, C4, C6, C8, and C10). Variation was mainly restricted to quantitative differences revealing more or less strong bands for each isozyme. *V. inaequalis* isolates with weak mycelial development *in vitro* showed reduced cellulolytic activity, but the amount of cellulase was similar to that of faster-growing organisms when the time for growth was restricted to give comparable mycelial growth. Generally, isozyme uniformity is suggested to be a feature of obligate parasites, because the host plant may impose particularly stringent biochemical requirements on a fungal pathogen and exert strong selection against any variation. Saprophytic nonpathogens are highly variable, and facultative pathogens lie between these extremes (Clark *et al.* 1989; Newton 1991). The facultative pathogen *V. inaequalis* revealed a specific production of cellulases during its biotrophic phase, and the low isozyme variability may indicate some common features of obligate parasites. This complex 12-morph cellulolytic system should be associated more strongly with pathogenesis than enzymes of the common pathways are, because it may be correlated with the virulence of the fungus.

MATERIALS AND METHODS

Media, growth conditions, and fungal strains.

Monoconidial isolates of *V. inaequalis* were all obtained from leaf and fruit lesions of the cultivar Golden Delicious and maintained on potato-dextrose agar (Difco). V1 and V4–V19 were isolated from an orchard next to the institute. V4 and V5 were obtained from fruit lesions. V2 and V3 were isolated from leaves of two trees grown at distances of about 25 and 300 km from the institute, respectively. V19A is an albino mutant derived from V19 after a spontaneous sector mutation. Production of cellulases was achieved by liquid static 250-ml culture (PDB, Difco) supplemented with 5-g cellulosic sheets (Cellophane) or by the use of dialysis tubing (Serva), 29 mm in diameter, made of regenerated cellulose. The dialysis tubing was filled with 250 ml of PDB, and both ends were closed in a knot. The tubing was placed in a 500-ml Erlenmeyer flask prior to autoclaving. Both cellulose materials were heated with an excess of double-distilled water to about 100° C and washed twice with water prior to use in the culture. Cultures were maintained at 20° C for 40–45 days. Inoculation was performed with mycelial plugs or mostly with conidia from muslin wick cultures (Puttoo and Basu Chaudhary 1988) of the respective fungal isolates. Culture experiments concerning the regulation of cellulases were performed with 40-ml cultures. For media with either no carbohydrate or a specified carbohydrate concentration, potato broth was prepared by heating 200 g of peeled potatoes to 120° C for 10 min in 1 L of double-distilled water. After infusion overnight, the liquid was filtered through Miracloth and frozen at −20° C. Before use the medium was thawed, centrifuged (15,000 *g* at 0° C) and tested (by HPLC; see below) for possible contamination with carbohydrates. Sugars and polyols were tested at concentrations of 1 mM, 10 mM, 0.1 M, and 0.5 M, and the cellodextrin mixture (CIII, Merck: G_2, 0.2%; G_3, 3.5%; G_4, 43.1%; G_5, 34.6%; G_6, 9.5%) at 0.025, 0.05, 0.1, and 0.5%. Xylan, cellulose (Avicel, Merck), filter paper (Whatman No. 1), cotton wool, cellophane, polyethylene sheets (sterilized with 70% ethanol), and CMC were used at concentrations of 0.1, 0.25, 0.5, 1, 2, and 2.5% (w/v). All components were tested as the single constituent for induction and in combination with cellulosic sheets for repression. Cellulosic sheet cultures were also supplemented with the other cellulosic substrates. Particles of cellulosic sheets were obtained by milling cellophane in liquid nitrogen and

subsequent fractionating by sieves of a defined mesh size. Immature, just-unfolded leaves and circular disks (15 mm in diameter) of mature, fully expanded leaves of the cultivar Golden Delicious were extracted in an excess of chloroform/methanol (1:2, v/v) by slight shaking. The solvent was replaced after 2, 5, and 20 hr. Two additional 30-min washes with methanol and ethanol, respectively, were followed by removal of the residual solvent under vacuum. For culture experiments the plant material was sterilized with 70% ethanol or by autoclaving together with PDB. Equivalent amounts of leaf material (number of disks or immature leaves) of non-solvent-extracted leaves were also used in culture experiments. Cell walls were isolated according to English et al. (1971) or by solvent extraction (see above) of leaves milled in the first solvent used. The powdered leaf material was incorporated into the culture medium as the extracted leaves were. All cultures were inoculated with isolate V1; the culture experiments were repeated at least twice with two replications, under the conditions described for cellulase production.

Cellulase and β-glucosidase assays.

Cup-plate diffusion assay was performed with 0.05 M citric acid, 0.1 M Na_2HPO_4 (pH 5), 1% CMC-Na-salt (Serva) (dp 500–520, degree of separation approximately 0.7%), and 0.8% agarose heated to boil under constant stirring. After cooling to 50° C, 40-ml aliquots were poured into standard petri dishes (9 cm in diameter). A siliconized cup former was dipped in the agarose medium, creating cups of about 80-μl size. These cups were filled with 80 μl of test solutions and incubated for about 20 hr at ambient temperature. To obtain higher sensitivities, incubation was extended up to 4 days with 0.02% NaN_3 as a preservative in the samples. For visualization of hydrolysis the plates were first flooded with a 0.1% aqueous solution of Congo red for at least 30 min, followed by an additional 30-min incubation with 5 M NaCl. Standard viscometric analyses were done with reaction mixtures of 5 ml of CMC solution as used in the cup-plate assay (without agarose) and 2 ml of enzyme solution in a Cannon-Fenske viscometer maintained at 30° C in a water bath. Viscometer efflux times were taken according to the enzyme concentration, with 20 hr as the longest incubation time. β-Glucosidase activity was monitored by continuous recording of the increase of absorbance at 400 nm. The enzyme solution consisted of either 2.5 ml of crude culture filtrate or 1 ml of mycelial extract (prepared as described below) and 1.5 ml of water. Reaction was started with 0.5 ml of 5 mM p-nitrophenyl-β-D-glucopyranoside.

Preparations from culture filtrates and mycelium.

Culture filtrates were used either without concentration or concentrated 20 times by vacuum evaporation at 40° C for routine viscometry, cup-plate assay, and β-glucosidase test. Prior to ammonium sulfate precipitation the culture filtrates were frozen at −20° C and then, after thawing to 0° C, were centrifuged at 27,000 g for 25 min. Aliquots of 250 ml were evaporated or mostly lyophilized to dryness. Water (8 ml) was added to freeze-dried aliquots, and saturated ammonium sulfate solution was added to a saturation of 50%. After incubation at 0° C for 20 min, precipitations were removed by centrifugation (27,000 g at 0° C), and further ammonium sulfate solution was added to the supernatant to obtain a final

saturation of 80%. After a further incubation and centrifugation, cellulase was obtained by resuspending the pellet in 1 ml of water. For electrophoresis of isozymes, samples were desalted by gel filtration on a Sephadex G-25 column (Pharmacia), 280 × 15 mm i.d., with water as the eluent. The fractions of cellulases were lyophilized and reconstituted with 1 ml of water. Prior to IEC, cellulase preparations were desalted in the same way, with 50 mM Tris and 0.1 M NaCl, pH 10, as the eluent for buffer exchange. Fractions of cellulases equivalent to 750 ml of culture filtrate were loaded on a QAE Sephadex A-50 column (Pharmacia), 100 × 15 mm i.d., equilibrated with the same buffer. Cellulases were recovered by step elution with buffer containing 1 M NaCl, and each respective fraction was loaded on the gel filtration column (see above) with water as the eluent. Cellulase fractions were freeze-dried and reconstituted to an appropriate volume for analytical and preparative IEF or activity experiments. Mycelia obtained from 40-ml cultures were homogenized at 0° C (Potter homogenizer) with 2 ml of water or buffer (as in the cup-plate assay), and these mycelial suspensions were used for the reaction mixtures in viscometry. Mycelia were homogenized in 20 ml of water or buffer and centrifuged (27,000 g at 4° C) for 30 min. A 1-ml sample of the supernatant was used for β-glucosidase assay. Experiments with mycelium and extracts, respectively, were repeated four times with fungal isolates V1, V2, and V3. Binding of cellulases to Concanavalin A–Sepharose (Pharmacia) was performed as recommended by the manufacturer with ammonium sulfate precipitations after buffer exchange by gel filtration.

Protein content was estimated according to Bradford (1976), with bovine gamma globulin used as the standard (Bio-Rad, Standard I).

Substrate assays in vitro and HPLC analysis.

Reaction mixtures consisted of 0.5 ml, containing 2 mg of cellulose derivative, an IEC preparation of cellulase equivalent to 125 ml of culture filtrate, and a final concentration of 0.02% NaN_3 and the buffer used for the cup-plate assay (see above). Time course experiments were run with mixtures of 6 ml, using the same concentrations per 0.5 ml. Inhibition of cellulase activity was tested in the 0.5-ml reaction mixtures containing 2 mg of the cellodextrin mixture combined with various amounts of glucose or cellobiose (20, 15, 10, 8, 6, 4, 2, 1, 0.5, 0.25, and 0.1 mg, respectively). Reactions were incubated at 30° C for 1 hr and cleaned up as described by Kollar and Seemüller (1990), on an Aminex HPX-87C (Bio-Rad) column with a flow rate of 0.5 ml of water per minute at 85° C and refractive index (RI) detection. Sugar fractions were dissolved in 0.5 ml of water, and 10-μl samples were used for HPLC analyses.

pH and temperature optimum and stability of cellulases.

All reaction mixtures consisted of 2 mg of cellodextrin mixture III, an IEC preparation of cellulase equivalent to 125 ml of culture filtrate, and the buffers described below in a total volume of 0.5 ml. Samples were incubated at 30° C (pH optimum) or at various temperatures at pH 5 (5–45° C, at intervals of 5° C) for 1 hr, and the degradation products were analyzed by HPLC as described above. Reactions were buffered to pH 1–7 with 0.05 M citric acid and 0.1 M Na_2HPO_4

adjusted with HCl or NaOH, or to pH 8 and 9 with 10 mM Tris-HCl. The stability of enzymes from IEC preparations was tested in 100 μl of water (including 0.02% NaN$_3$) at 0, 5, 15, and 30° C with incubation times of 1 hr and 1–15 days. Thereafter the enzyme aliquots were used to complete the reaction mixtures (pH 5, as described above), and HPLC analyses were performed after incubation at 30° C for 1 hr. All experiments were repeated once.

Analytical and preparative IEF and isozyme pattern analysis.

Proteins were separated according to their pI by IEF on ultrathin layers of polyacrylamide gel (Servalyt Precotes 3-6, Serva), 125 × 125 mm and 150 μm thick, at 4° C (Isobox HE 950, Hoefer) as recommended by the manufacturer. After prefocusing at 500 V, samples were loaded with applicator strip (Serva), slot 7 × 1 mm, at the cathode. Gels were stained with silver according to the method of Hempelmann and Kaminsky (1986) for total protein. Standards for pI estimation were the Test Mix 9 (Serva) and the Low pI Calibration Kit (Pharmacia). For cellulase zymograms enzymes were transferred to CMC-agarose as prepared for cup-plate assay. Substrate agarose was poured into dishes in a layer at least 0.5 cm thick. Usually IEF gels were first blotted for 10 min and then blotted further on a second substrate agarose layer for 60 min. After removal of the gel, the substrate agar was incubated overnight at ambient temperature. Isozyme banding pattern was visualized with Congo red as described for cup-plate assay. For preparative isoelectric focusing (Rotofor, Bio-Rad) desalted ammonium sulfate or IEC preparations equivalent to 500–1,000 ml of culture filtrate were mixed with water and ampholytes (Servalyt 3-10 or 2-11, Serva) to a concentration of 2% in a final volume of about 55 ml. The sample was focused at 4° C for 5 hr at 12 W of constant power. Cellulase fractions detected by cup-plate assay were pooled and refocused twice. The voltage increased from about 500 V initially to 800 V after the first run, to 1,000 V after the second run, and to 1,600 V after the third run. For analytical IEF the respective preparative fractions were desalted routinely by gel filtration and lyophilized.

Determination of molecular weight.

Gel filtration chromatography was performed with a Sephadex G-75 SF gel bed (Pharmacia), 550 × 10 mm, in 0.05 M Tris and 0.1 M NaCl, pH 10. Molecular weight standards (Serva) consisted of bovine serum albumin (66,000), ovalbumin (45,000), and chymotrypsinogen A (25,000). Desalted and lyophilized fractions of preparative IEF were dissolved in 200 μl of buffer and fractionated to 1-ml samples by gel filtration. All samples were tested for cellulase activity with a cup-plate assay, and samples with peaks of activity were pooled, desalted, and lyophilized for analyses with analytical IEF. Molecular weights from peaks of activity in the gel filtration were correlated with enriched isozyme bands obtained with analytical IEF.

Isolation and detection of cellulases from infected apple leaves.

About 400 single nonnecrotic apple scab lesions from leaves removed from the orchard were used for one experiment. Naturally infected tissues of the cultivar Golden Deli-

cious were used. The lesions were covered with a layer of buffer (0.05 M Tris and 0.1 M NaCl, pH 10) and scraped off with a scalpel. Care was taken to remove only the epidermis together with mycelium. Suspensions of about 30–40 lesions were transferred immediately to 10 ml of the same buffer at 0° C, and each 10-ml aliquot was loaded on an IEC column as described before. The column was eluted with the buffer described until unbound substances were removed, and the cellulases were recovered with buffer containing 1 M NaCl. Desalting and lyophilization were done as described, and the cellulases were dissolved in 200 μl of water for cup-plate assay and IEF. Detection of cellulolytic activity on scabbed leaves was done by blotting leaves on CMC-substrate agar (cup-plate, zymogram) for 20 hr and then removing the leaves and incubating the medium for an additional 20 hr. Congo red was used to detect activity, as described for the cup-plate assay.

LITERATURE CITED

Beaulieu, C., Boccara, M., and Van Gijsegem, F. 1993. Pathogenic behavior of pectinase-defective *Erwinia chrysanthemi* mutants on different plants. Mol. Plant-Microbe Interact. 6:197-202.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Clark, J., Butters, J., Brent, K. J., and Hollomon, D. W. 1989. Isozyme uniformity in *Erysiphe graminis* f. sp. *hordei*. Mycol. Res. 92:404-409.

Corlett, M., Chong, J., and Kokko, E. G. 1976. The ultrastructure of the *Spilocaea* state of *Venturia inaequalis* in vivo. Can. J. Microbiol. 22:1144-1152.

English, P. D., Byrne Jurale, J., and Albersheim, P. 1971. Host-pathogen interactions. II. Parameters affecting polysaccharide-degrading enzyme secretion by *Colletotrichum lindemuthianum* grown in culture. Plant Physiol. 47:1-6.

Hellmann, M., and Stösser, R. 1992. Development of cuticle thickness and epidermal cells of apple leaves. Gartenbauwissenschaft 57(5):223-227.

Hempelmann, E., and Kaminsky, R. 1986. Long term stability of colors after silver staining. Electrophoresis 7:481.

Hignett, R. C., and Kirkham, D. S. 1967. The role of extracellular melanoproteins of *Venturia inaequalis* in host susceptibility. J. Gen. Microbiol. 48:269-275.

Hignett, R. C., Roberts, A. L., and Carder, J. H. 1979. The properties of extracellular enzymes of *Venturia inaequalis* and their association with loss of virulence of the fungus in culture. J. Gen. Microbiol. 110:67-75.

Hignett, R. C., Roberts, A. L., and Carder, J. H. 1984. Melanoprotein and virulence determinants of *Venturia inaequalis*. Physiol. Plant Pathol. 24:321-330.

Holowczak, J., Kuć, J., Williams, E. B. 1962. Metabolism *in vitro* of phloridzin and other compounds by *Venturia inaequalis*. Phytopathology 52:1019-1023.

Kollar, A., and Seemüller, E. 1990. Chemical composition of phloem exudate of mycoplasma-infected apple trees. J. Phytopathol. 128:99-111.

Koller, B., Müller, M., Valsangiacomo, C., and Gessler, C. 1992. Cell wall degrading enzymes and inhibitors involved in the interaction between *Venturia inaequalis* and *Malus domestica*. Acta Phytopathol. Entomol. Hung. 27(1-4):353-359.

Köller, W., and Parker, D. M. 1989. Purification and characterization of cutinase from *Venturia inaequalis*. Phytopathology 79:278-283.

Köller, W., Parker, D. M., and Becker, C. M. 1991. Role of cutinase in the penetration of apple leaves by *Venturia inaequalis*. Phytopathology 81:1375-1379.

Leben, C., and Keitt, G. W. 1948. *Venturia inaequalis* (Cke.) Wint. V. The influence of carbon and nitrogen sources and vitamins on growth *in vitro*. Am. J. Bot. 35:337-343.

Mandels, M., Parrish, F. W., and Reese, E. T. 1962. Sophorose as an inducer of cellulase in *Trichoderma viride*. J. Bacteriol. 83:400-408.

Mandels, M., and Reese, E. T. 1960. Induction of cellulase in fungi by cellobiose. J. Bacteriol. 79:816-826.

Newton, A. C. 1991. Isozyme variability in isolates of some facultative phytopathogenic fungi. J. Phytopathol. 131:199-204.

Puttoo, B. L., and Basu Chaudhary, K. C. 1988. A muslin wick culture technique for mass production of conidia of *Venturia inaequalis*. J. Phytopathol. 121:373-375.

Ryan, C. A. 1988. Oligosaccharides as recognition signals for the expression of defensive genes in plants. Biochemistry 27:8879-8883.

Timpa, J. D. 1991. Application of universal calibration in gel permeation chromatography for molecular weight determinations of plant cell wall polymers: Cotton fiber. J. Agric. Food Chem. 39:270-275.

Valsangiacomo, C., and Gessler, C. 1988. Role of the cuticular membrane in ontogenetic and Vf-resistance of apple leaves against *Venturia inaequalis*. Phytopathology 78:1066-1069.

Valsangiacomo, C., Wagner, K., Stadler, B., Ruckstuhl, M., Manini-Gessler, P., Michel, M., and Gessler, C. 1989. Aspects of host resistance and pathogenesis in the interaction between *Venturia inaequalis* and apple leaves. Pages 191-204 in: Integrated Control of Pome Fruit Diseases. Vol. 2. IOBC/WPRS Bull. 12/6.

Wagner, K., Hitz-Germann, L., Seng, J. M., and Gessler, C. 1988. Cellulolytic ability of the scab fungus, *Venturia inaequalis*. J. Phytopathol. 123:217-221.

Wahlström, K., Karlsson, J. O., Holdenrieder, O., and Stenlid, J. 1991. Pectinolytic activity and isozymes in European *Armillaria* species. Can. J. Bot. 69:2732-2739.