Cellulase Activity in Isolates of *Verticillium dahliae* Differing in Aggressiveness

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ABSTRACT

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Ten *Verticillium dahliae* isolates from different Galician locations were classified by degree of aggressiveness. High variability exists independent of locality of origin. Two isolates differing in aggressiveness were evaluated for cellulase production on several carbon sources. *V. dahliae* is able to degrade crystalline cellulose (Avicel) and, therefore, has the three enzymes necessary for its hydrolysis. The two isolates behaved similarly in the presence of soluble cellulose, but the most aggressive isolate had greater β -1,4-glucosidase (EC. 3.2.1.21) and endo- β -1,4-glucanase (EC. 3.2.1.4) activity. The less aggressive isolate needed more time to degrade crystalline cellulose. Cellulases may not be the determining factor for aggressiveness and symptom expression; however, they may play a role in penetration.

Verticillium wilt, caused by the soil-borne fungus *Verticillium dahliae* Kleb., limits the production of a broad range of economically important crops including pepper, aubergine, tomato, sunflower, potato, and olive tree (14,28). Host-specific pathogenicity in peppermint (12,17), Brussels sprouts (19), lucerne (15), tomato, and cotton had been reported. In cotton, two races of *V. dahliae* can be differentiated, a defoliant and a nondefoliant type (4). Although some authors indicate the existence of at least two races with different degrees of virulence for the pepper, other reports (40) suggest that pathogenicity is determined by the hosts previously colonized. Inoculation with isolates obtained from various plants showed that only some of them gave rise to severe damage on pepper (20).

Verticillium wilt of pepper causes severe losses in North and Central America (11,20) and Mediterranean countries (3,30,39,41). Because of this economic problem in pepper, it is important to understand pathogenicity, aggressiveness, and genetics of *V. dahliae* isolated from pepper. Molecular genetic techniques that can distinguish between organisms at the DNA level such as random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) (32) can help clarify relationships between isolates.

V. dahliae penetrates the host through the root epidermis or wounds, crossing cortical root tissues to the xylem elements.

In the xylem, the mycelium proliferates by rapid colonization of the whole vascular system (16). The first symptoms of the disease are foliar chlorosis that progresses to necrosis. Other visible symptoms include stunting, epinasty, wilting, leaf abscission, and eventually, browning of the vascular system. In general, wilting is the result of restricted water movement that is caused by complex interactions between toxins, enzymes, and hormones (8).

When *V. dahliae* enters the host plant, the first line of defense that it encounters is the cell wall, composed of three polysaccharides: cellulose, hemicellulose, and pectic substances, and other compounds (35). Plant-pathogenic fungi are known to produce a range of cell wall degrading extracellular enzymes that are vital to infection by facilitating penetration and releasing soluble compounds used as nutrients to support fungal growth (23). Cellulase is a cell wall degrading enzyme complex comprised of three types of enzymes with varying activity in the hydrolysis of the cellulose chain: exo- β -1,4-glucanases (EC. 3.2.1.91) split the β -1,4-glycosidic bonds at the nonreducing end of the cellulase molecule to yield cellobiose; endo- β -1,4-glucanases (EC. 3.2.1.4) attack internal glycosidic bonds; and β -1,4-glucosidases (EC. 3.2.1.21) cleave short cello-oligosaccharides to glucose. All three classes of enzymes are required for the complete depolymerization of native cellulose (9). They may be secreted into the medium, located intracellularly, or bound to the cell wall, with a synergetic action taking place among the three. Cellulose hydrolysis is generally a slow process because of the crystalline nature of the polymers. Cell wall degrading enzymes of plant-pathogenic fungi are considered important pathogenicity factors; however, the relationship of cellulases secreted by *V.*

dahliae and aggressiveness of this pathogen has not been studied previously.

The objective of this work was to examine variation in aggressiveness among isolates of *V. dahliae* obtained from pepper plants in various localities of Galicia and to correlate their aggressiveness with the activity of cell wall degrading cellulases. The ability of the RAPD technique to distinguish pathogenic forms of this fungus also was investigated.

MATERIALS AND METHODS

Plant material. The pathogenicity and aggressiveness of 10 isolates of *V. dahliae* Kleb., obtained from *Capsicum annuum* L. var. *annuum* cv. Padrón in different localities of Galicia during the summer of 1998, were studied. Pathogenicity experiments were carried out on 2-month-old *Capsicum annuum* var. *annuum* cv. Luesia and Yolo Wonder plants.

Pathogenicity. Luesia and Yolo cultivars, in groups of 10, were inoculated with the different isolates of *V. dahliae* grown on PDA at room temperature for 20 days by a modified method of Melouk (24). Conidial suspensions of each isolate were prepared by washing each plate with sterile water and scraping the cultures with a rubber spatula. Mycelial fragments were removed by filtration through a double layer of sterile cheesecloth, and the inoculum was adjusted to 10^6 conidia ml^{-1} . For inoculation, the roots were cut approximately 1 cm from the apex and suspended in the conidia for 45 min. Another group of 10 plants was treated with sterile water instead of the inoculum as the control group. After inoculation, the plants were transplanted individually in 12-cm-diameter pots with a substrate composed of potting soil and perlite in a 2:1 (vol/vol) ratio and previously sterilized at 120°C for 45 min. The experiments were conducted in a greenhouse at 25°C with a photoperiod of 16-h light and 8-h darkness. Stem pieces of all plants were planted on PDA to check for the presence or absence of the fungus after 15 days. The fungus was identified microscopically by the formation of typical verticillately branched conidiophores.

Disease evaluation. During the 30-day period after inoculation, plants were checked regularly for wilting, chlorosis, or leaf drop produced by each isolate of *V. dahliae*. Disease incidence was determined as percentage of plants displaying symptoms. The percentage of wilted leaves for the severity index on a scale of 0 to 5 was: 0

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= none (healthy plant), 1 = 0 to 20%, 2 = 20 to 40%, 3 = 40 to 60%, 4 = 60 to 80%, and 5 = more than 80% leaves wilted. After 30 days, stems and leaves were separated and the fresh and dry weights were determined.

DNA extraction. For DNA extraction, 1 plug of each *V. dahliae* isolate grown on PDA was transferred to a flask containing 50 ml of Czapek-Dox broth (Duchefa, Haarlem, Netherlands). Cultures were incubated for 9 days at 25°C on an orbital shaker at 50 rpm in darkness. After incubation, the mycelium was recovered by filtration and ground with an autoclaved pestle and mortar. DNA was extracted from 20 mg of ground mycelium (31) and quantified by measuring optical density at 260 nm.

RAPD analysis. Arbitrary random amplification of DNA sequences was performed with a set of 20 ABA primers (each having 10 bases) obtained from Advanced Biotechnologies (Columbia, MA). PCR was carried out in a total volume of 25 µl of reaction mixture. The reaction mixture consisted of 2 mM MgCl₂, 2.5 µl of 10× reaction buffer, 200 µM of each dNTP, 0.5 µM primer, 1.5 units of *Taq* DNA polymerase (Roche Diagnostics, SL, Mannheim, Germany) and 10 ng of fungal DNA. The reaction was performed in a primus 25 PCR-system thermocycler (MWGAG, Biotech, Ebersberg, Germany).

Reaction conditions were: denaturation at 94°C for 30 s followed by 45 cycles of 30 s denaturation at 92°C, annealing for 1 min at 35°C, and extension for 2 min at 72°C. Amplification products were separated electrophoretically in 1.8% agarose gels. Usually, 5 µl of PCR reaction mixture was mixed with 1 µl of loading dye (25% glycerol, 0.025% bromophenol blue). The DNA markers used for electrophoresis were from Roche. When the dye markers approached the bottom of the gel slab, the gel was stained for 20 min with ethidium bromide (0.5 µg/ml), rinsed with water, and visualized with the Dig Doc Photo Documentation System (Bio-Rad Laboratories, Hercules, CA).

Growth at different temperatures.

Two isolates, the most and the least aggressive obtained in the pathogenicity test, were grown at 10, 15, 20, 25, 30, and 35°C on PDA medium. Four PDA dishes were seeded centrally with a 3-mm-diameter agar plug from the advancing margins of an actively growing colony of each isolate. The fungal cultures were incubated in the dark, and the diameter of all colonies was measured after 20 days.

Cellulose-degrading enzymes. The two isolates of *V. dahliae* differing in aggressiveness were used to study cellulose degrading enzymes. Assays to detect cellulose degrading enzymes capacity were performed with Cellulose Azure test and with the Red Congo assay.

Enzyme production. The fungus was cultured at room temperature in darkness in a Czapek-Dox liquid medium (25 ml, in 100-ml Erlenmeyer flasks) where glucose was replaced with 0.5% (wt/vol) carboxymethylcellulose (CMC), a soluble cellulose, or 5% Avicel, a crystalline cellulose. Czapek-Dox liquid medium was used to wash spores from 20-day-old PDA plates, and 5 × 10⁶ conidia ml⁻¹ were added to the cellulose substrate media. The cultures were incubated 7 days with shaking, and samples were collected daily. Mycelium was separated from medium by filtration through a glass-fiber filter (Albet FV-A); the liquid medium was dialyzed against distilled water for 18 h at 4°C and held at -80°C until use. Mycelium obtained from the CMC medium was dried at 80°C and weighed. The one obtained from the Avicel medium was weighed and frozen at -80°C.

Enzyme extraction. A modification of de los Rios et al. method (33) was used to extract enzymes. Mycelium from the Avicel medium was placed in liquid nitrogen and later homogenized in cold 0.1 M sodium-phosphate buffer, pH 6.6, 0.2 M NaCl, 10 mM dithiothreitol (10 ml/g fresh weight), and then centrifuged at 26,000 × g_n for 15 min at 2°C. The supernatant was filtered through a glass-fiber filter (Albet

FV-A) and dialyzed against distilled water for 18 h. This filtrate is the *soluble fraction* from cell free extracts of the mycelium. The pellet was resuspended in water (10 ml/g fresh weight), the pH adjusted to 3 with 1 M HCl; NaCl was added up to 1 M, and the suspension was stirred for 30 min. Afterward, the pH was set to 6 with 1 M NaOH; the suspension was stirred for 60 min and centrifuged at 26,000 × g_n for 20 min. The supernatant solubilized from the cell wall, and the incubation medium was filtered and dialyzed the same as the soluble fraction. These fractions are referred to as *cell wall* and *medium fractions*, respectively.

Enzyme assays. Endo-β-1,4-glucanase activity was measured as an increase in reducing groups resulting from the release of glucose from CMC using the copper arsenomolybdate reagents of Nelson, as modified by Somogy (26,38). The reaction mixture contained 1.0 ml of 1.5% CMC in 0.5 ml of acetate buffer (pH 4.8) and 0.5 ml of sample. Absorbance was determined spectrophotometrically at 540 nm. The unit of activity is defined as the amount of enzyme that releases 1 µmol min⁻¹ reducing sugar expressed as glucose equivalent.

β-1,4-Glucosidase activity was determined by measuring the rate of *p*-nitrophenyl released from *p*-nitrophenyl-β-D-glucopyranoside. The standard reaction mixture consisted of 1 ml of 5 mM substrate, 1.8 ml of 0.1 M acetate buffer (pH 4.8), and 0.2 ml of sample. After incubation for 30 min at 50°C, the reaction was stopped with tricine buffer 0.4 M (pH 10.8), and absorbance was determined spectrophotometrically at 420 nm. The unit of activity is defined as the amount of enzyme that releases 1 µmol min⁻¹ of *p*-nitrophenyl.

To determine if the liberation of the *p*-nitrophenyl was due to β-glucosidase activity, gluconolactone, which is a strong inhibitor of this enzyme but has little effect on β-glucanases, was used (10).

Total cellulase activity was determined by adding to a strip of Whatman paper 1.5 ml of 0.1 mM acetate buffer (pH 4.8) and 0.5 ml of sample. It was incubated at 50°C for 60 min, and the reducing sugars were calculated by the Somogy-Nelson method.

Protein determination. Protein was determined by the Bradford method (7) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the standard. Each sample was measured in duplicate.

Statistical analysis. The data were studied by one-way analysis of variance (ANOVA) and Duncan's test ($P \leq 0.05$). The statistical analysis was performed with the Statgraphics Plus 4.0 software for Windows.

RESULTS

Pathogenicity and aggressiveness. All isolates from Galicia used in this study had been previously identified as *V. dahliae*

Table 1. Fresh and dry weights (FW and DW) of two pepper cultivars inoculated with 10 isolates of *Verticillium dahliae*

Isolate	Host				Geographical origin
	Yolo Wonder		Luesia		
	DW ^z	FW ^z	DW ^z	FW ^z	
53	56.67 a	42.86 a	51.69 ab	45.17 ab	Cambados
17	59.31 ab	46.71 a	61.63 bcd	55.36 c	Padrón
50	60.34 ab	46.38 a	51.45 ab	40.80 a	Cambados
33	60.37 ab	49.34 ab	54.43 abc	45.83 ab	Padrón
3	61.47 ab	50.03 ab	48.04 a	44.24 ab	Cambados
5	62.44 ab	49.60 ab	58.06 abcd	50.11 bc	Cambados
7	63.04 ab	49.65 ab	63.34 cd	56.10 c	O Rosal
19	63.67 ab	48.41 ab	57.16 abcd	49.29 abc	Padrón
100	66.12 ab	48.98 ab	65.43 de	56.22 c	Padrón
18	69.32 b	56.03 b	74.29 e	64.76 d	Padrón

^z Data are expressed in percentage of weight of plants inoculated with *V. dahliae* compared with a control inoculated with sterile water. Data are means of two independent experiments. Values followed by different letters were significantly different as determined by analysis of variance (ANOVA) using Duncan's test ($P \leq 0.05$).

based on morphological characteristics, including production of verticillate conidiophores and microsclerotia. All 10 *V. dahliae* isolates caused disease symptoms in cultivars Yolo Wonder and Luesia of *Capsicum annuum* var. *annuum*. The more relevant symptoms were foliar wilting, defoliation, moderate chlorosis, stunting, and vascular browning. The presence of these symptoms, together with reisolation of the 10 isolates from the inoculated plants according to Koch's postulates, confirmed that all isolates were pepper pathogens.

The reduction in plant fresh weight and dry weight caused by all 10 isolates on the two pepper cultivars permitted arranging the different isolates on a scale representing aggressiveness (Table 1). The isolates varied from the highly aggressive isolate UDC53Vd to the mildly aggressive isolate UDC18Vd.

Disease incidence. Inoculated plants of the two different cultivars developed the disease symptoms slowly when infected with the less aggressive isolate and rapidly with the highly aggressive isolate. In the Yolo Wonder cultivar, symptoms started to develop 9 to 10 days after inoculation; whereas in the Luesia cultivar, symptoms did not appear until 15 days after inocula-

tion. At the end of the experiment, disease incidence was 100% in all cases.

Disease severity. The leaf symptoms (withered leaves) determined the severity index presented in Figure 1. Thirty days after inoculation, more than 60% (severity index 4.1 ± 0.5) of the leaves of Yolo Wonder plants inoculated with the more aggressive isolate showed wilting symptoms, whereas only 40 to 60% (severity index 3.2 ± 0.6) of the leaves wilted where inoculated with the weaker isolate. In the Luesia cultivar, the behavior was similar, but the severity index was lower in plants inoculated with both isolates.

RAPD-PCR analysis of *V. dahliae* isolates. Out of 20 10-mer RAPD primers tested on 10 *V. dahliae* isolates, only five gave results that were considered suitable for further investigation. The other 15 primers gave no bands, poor patterns, or nonreproducible results. Among these five selected primers, only two (ABA 13 and ABA 18) generated simple banding patterns that revealed DNA polymorphisms among *V. dahliae* isolates. Representative RAPD patterns of all 10 *V. dahliae* isolates, using primer ABA 13 and primer ABA 18, are presented in Figure 2. Amplification of genomic DNA with primer ABA 13 revealed polymorphism among isolates

from different localities of Galicia. Isolates from Cambados had a similar pattern, whereas those of Padrón and O Rosal all had the same pattern, but different from that of other isolates. When DNA was amplified with primer ABA 18, polymorphic banding patterns among the different isolates were not observed and only isolates UDC50Vd and UDC19Vd presented a different banding pattern.

Mycelial growth at different temperatures. Two selected isolates with different degrees of aggressiveness were subjected to a range of temperatures between 4 and 35°C. Both isolates grew similarly, with the best growth occurring at 20 to 25°C (Fig. 3).

Cellulose-degrading enzymes. The two isolates of *V. dahliae* differing in aggressiveness used to study cellulose-degrading enzymes showed cellulolytic activity. In the Red Congo assay, a hydrolysis halo was observed, with the same diameter for the two isolates. This halo corresponds to the area where cellulose was hydrolyzed.

Enzymatic activity. CMC as a carbon source. The growth of isolates in CMC was based on mycelium dry weight (Fig. 4A) and by protein secreted into the medium (Fig. 4B). Protein increased over 7 days, with this increase always being larger with the more aggressive isolate; however, dry weight only changed slightly (Fig. 4A and B).

β -1,4-Glucosidase activity increased throughout the 7 days with both isolates, but more with the more aggressive one (Fig. 5A). Endo- β -1,4-glucanase activity was detected in both isolates from the first day and increased until the fourth day (Fig. 5B). The behavior of the two isolates was similar. The inability to degrade cellulose paper suggests that exo- β -1,4-glucanase was not present when CMC was used as a carbon source.

Avicel as a carbon source. Avicel, a crystalline cellulose, made it possible to obtain the complete cellulase complex in

Fig. 1. Severity index of pepper cv. Luesia (A) and cv. Yolo Wonder (B) inoculated with *Verticillium dahliae* isolates UDC53Vd (●) and UDC18Vd (■). Data represent the mean $\pm$ SD of two independent experiments. * Indicates significant differences at $P \leq 0.05$.

Fig. 2. Random amplified polymorphic DNA profiles of *Verticillium dahliae* DNA amplification with primers A, ABA 13 and B, ABA18. Lanes: M, molecular weight standards; 1, UDC03Vd; 2, UDC05Vd; 3, UDC50Vd; 4, UDC53Vd; 5, UDC07Vd; 6, UDC17Vd; 7, UDC18Vd; 8, UDC19Vd; 9, UDC33Vd; 10, UDC100Vd; 11, control.

Fig. 3. Growth of *Verticillium dahliae* isolates UDC53Vd (●) and UDC18Vd (■) at different temperatures after 20 days of growth. Data represent the mean $\pm$ SD of three independent experiments.

the culture medium, in the soluble fraction, and in the fraction bound to the cell wall. The behavior of the two isolates differed in degradation of Avicel. The less aggressive isolate needed more time to degrade Avicel than the more aggressive one. For this reason, samples were collected on the seventh day for the more aggressive, but on the fourteenth day for the less aggressive isolate (Table 2).

In both isolates, the protein content, as well as the activity of endo- β -1,4-glucanase, β -1,4-glucosidase, and total cellulase, were higher in the culture me-

dium than in the soluble and cell wall fractions, respectively. The capacity to degrade cellulose paper confirmed the existence of the three enzymes of the cellulase complex if Avicel was used as a carbon source.

DISCUSSION

Pathogenicity assays allowed us to demonstrate that all *V. dahliae* isolates tested were pathogenic to pepper. It does not mean that they are pathogenic to other species since host specialization among strains has been demonstrated in some cases (22,25). Variation in pathogenicity of

the 10 isolates tested was observed; however, this difference could be due to their host of origin. Although all the isolates were from pepper cv. Padrón, in some cases such as with the least aggressive isolate, the pepper was grown in greenhouses after tomatoes had been cultivated. Thus, its host of origin may have been tomato. It is reported that tomato isolates did not cause disease symptoms in paprika (41). Other authors (20,25,40) suggested that the pathogenicity of isolates of *V. dahliae* is determined by the previous cropping history.

The isolates tested caused wilting, defoliation, vascular browning, and stunting. The decreased size, together with defoliation, accounts for the lower dry weight of inoculated plants. Differences in dry weight of inoculated plants permitted classifying the different isolates according to their aggressiveness. On the other hand, disease incidence indicated by the presence of foliar symptoms was variable and did not always reflect the overall severity of the disease. The aggressiveness scale, together with the incidence of disease and the severity index, confirmed that isolate UDC53Vd was more aggressive and that isolate UDC18Vd was the least aggressive. Stunting has been used as an indication of disease severity (25,36), although other works have described this parameter as an unreliable indication of pathogenicity (5,27). Nevertheless, other authors (6) indicated that disease scoring for symptoms such as wilting and defoliation, in combination with growth parameters similar to this study, is more meaningful to evaluate pathogenicity.

Difference in the degree of aggressiveness among the different isolates suggests there is high variability among the sampling populations independent of their original location. RAPD patterns were not associated with pathogenicity in *V. dahliae*, indicating that RAPD fragments are neutral and independent of this phenotypic trait. Only differences found among isolates from different localities might allow one to distinguish between geographical races as observed in other pathogens (1,21).

Fig. 4. Growth of *Verticillium dahliae* isolates UDC53Vd (●) and UDC18Vd (■) in Czapek-carboxymethylcellulose medium. **A**, Mycelia dry weight; **B**, protein content. Data represent the mean $\pm$ SD of three independent experiments. * Indicates significant differences at $P \leq 0.05$.

Fig. 5. Time course of endo- β -1,4-glucanase activity (**A**) and β -1,4-glucosidase activity (**B**) in Czapek-carboxymethylcellulose medium by both *Verticillium dahliae* isolates, UDC53Vd (●) and UDC18Vd (■). Data represent the mean $\pm$ SD of two independent experiments. * Indicates significant differences at $P \leq 0.05$.

Table 2. Cellulase activity, endo- β -1,4-glucanase activity, β -1,4-glycosidase activity, and protein content in the medium, and soluble and cell wall mycelial fractions of two *Verticillium dahliae* isolates, UDC53Vd after 7 days growth and UDC18Vd after 14 days^w

	Isolate	Medium fraction	Soluble fraction	Cell wall fraction
Cellulase activity ^x	UDC53Vd	0.772 $\pm$ 0.141 (78.14%)	0.110 $\pm$ 0.009 (11.13%)	0.106 $\pm$ 0.004 (10.72%)
	UDC18Vd	0.602 $\pm$ 0.410 (73.95%)	0.107 $\pm$ 0.009 (13.51%)	0.102 $\pm$ 0.004 (12.53%)
Endo- β -1,4-glucanase activity ^x	UDC53Vd	4.487 $\pm$ 0.617 (95.49%)	0.087 $\pm$ 0.008 (1.91%)	0.122 $\pm$ 0.015 (2.59%)
	UDC18Vd	2.880 $\pm$ 1.784 (94.21%)	0.076 $\pm$ 0.015 (2.49%)	0.101 $\pm$ 0.017 (3.30%)
β -1,4-Glycosidase activity ^y	UDC53Vd	0.461 $\pm$ 0.089 (88.82%)	0.043 $\pm$ 0.005 (8.28%)	0.015 $\pm$ 0.004 (2.89%)
	UDC18Vd	0.213 $\pm$ 0.094 (78.90%)	0.047 $\pm$ 0.004 (17.41%)	0.010 $\pm$ 0.002 (3.70%)
Protein content ^z	UDC53Vd	0.825 $\pm$ 0.095 (64.40%)	0.414 $\pm$ 0.013 (32.32%)	0.042 $\pm$ 0.003 (3.27%)
	UDC18Vd	0.724 $\pm$ 0.425 (69.51%)	0.300 $\pm$ 0.052 (28.80%)	0.018 $\pm$ 0.004 (1.69%)

^w Enzyme activities represent the mean $\pm$ SD of three independent experiments.

^x Expressed in μ mol glucose $\text{min}^{-1} \text{g}^{-1}$ fresh weight.

^y Expressed in μ mol *p*-nitrophenyl $\text{min}^{-1} \text{g}^{-1}$ fresh weight.

^z Expressed in mg protein g^{-1} fresh weight.

Temperature has a significant influence on the growth of the different isolates of *V. dahliae*, but all reach a maximum growth rate between 20 and 25°C. Slow growth at 30°C confirmed that these isolates were *V. dahliae*, since isolates of *V. albo-atrum* are not capable of growing at this temperature (37).

These *V. dahliae* isolates produced cellulases capable of degrading soluble and crystalline cellulose to establish that they possess the three enzymatic activities necessary for cellulose hydrolysis (8). All the enzymes were primarily extracellular. This enzymatic system allows *V. dahliae* to grow in media where cellulose is a source of carbon and may contribute to degradation of the cell wall with great importance in penetration of the host (9,29).

The two isolates behaved similarly in the presence of soluble cellulose, and both were able to degrade it; however, the more aggressive isolate had greater β -1,4-glucosidase and endo- β -1,4-glucanase activity. In *V. albo-atrum*, haploid isolates highly pathogenic to cotton had 10 times more β -1,4-glucosidase activity than diploid ones, which are less pathogenic (18). In the necrotrophic carrot pathogen *Mycocentrospora acerina*, endo- β -1,4-glucanase activity was higher in the more aggressive isolates (22). The behavior of these isolates differed when trying to degrade a source of crystalline cellulose since the more aggressive isolate secreted the enzyme into the culture medium earlier than the less aggressive isolate. The fact that the less aggressive isolate needs more time to degrade Avicel may be important in pathogenesis, since a reduction in activity on native cellulose might suggest that the fungus has a diminished capacity to colonize the host, leading to a delay in the appearance of symptoms and a milder expression of the disease. These results can suggest that *V. dahliae* produces the cellulases required for contributing to degradation of pepper cell walls. Moreover, the rate and magnitude of the synthesis of cellulases should play an important role in aggressiveness of *V. dahliae* since it has been established previously for interactions in several pathogenic situations (2).

Cellulases may not be the determining factor for aggressiveness and, consequently, the cause of symptoms; however, they may play an important role in the penetration process (42). Bacteria cellulases have been correlated with aggressiveness since induced mutants of *Clavibacter michiganensis* that do not possess the plasmid pCS1 containing the gene for cellulases were avirulent. Cellulase activity, together with other enzymes or the synthesis of toxins by the pathogen, may be responsible for aggressiveness of the pathogen. Plant pathologists have generally felt that cellulases are not particularly important in pathogenesis, since extensive

cellulose degradation typically occurs only late in infection, if at all (8); however, when the major endoglucanase genes of the phytopathogenic bacteria *Pseudomonas solanacearum* and *Xanthomonas campestris* pv. *campestris* were disrupted, virulence decreased (13,34). Although the relation between enzymes and pathogenicity has not been established to date, they are considered to be possible aggressive factors since both host colonization and subsequent survival may depend on the ability of the pathogen to degrade components of the cell wall (9).

Studies with mutants of *V. dahliae* deficient in cellulases would help determine the importance of these enzymes in infection and therefore any relationship with aggressiveness of the pathogen.

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