Comparison of Pectic Zymograms Produced by Different Clones of *Sclerotinia sclerotiorum* in Culture

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ABSTRACT

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The isozymes of polygalacturonase (PG) and pectinmethylesterase (PME), produced in vitro by 35 isolates belonging to 15 clones of *Sclerotinia sclerotiorum*, were detected by isoelectric focusing on polyacrylamide gels and activity staining of agarose overlays containing the appropriate substrate. Analysis of the PG isozyme banding patterns (zymograms) identified two distinct phenotypes among the isolates from canola, *S. sclerotiorum* zymogram group-1 (SSZG-1) and SSZG-2. Of the 11 clones tested, nine clones had the SSZG-1 phenotype, two clones had the SSZG-2 phenotype, and one clone had both SSZG-1 and -2 phenotypes. Therefore, in this sample, there were fewer PG phenotypes than clonal genotypes. One frequently sampled clone with wide geographic distribution included two isolates of SSZG-1 and eight of SSZG-2. Two isolates, one from cultivated sunflower and one from a wild plant, *Ranunculus ficaria*, showed isozyme patterns different from the isolates obtained from canola. The isolate from sunflower had the SSZG-3 phenotype, and the isolate from *R. ficaria* had the SSZG-4 phenotype. There was no apparent correspondence between isozyme phenotype and either the pathogenicity or aggressiveness of the clones on canola. The isozyme analysis of PME showed no variation among the isolates collected from canola.

Sclerotinia sclerotiorum (Lib.) de Bary attacks a wide range of cultivated and wild plant species. Stem rot of canola (oil seed rape; *Brassica rapa* L. and *B. napus* L.), caused by *S. sclerotiorum*, is an important disease of canola in Western Canada and under conducive conditions can result in severe yield losses. A complete set of cell wall-degrading enzymes, including cellulytic and pectinolytic enzymes that cause maceration of healthy plant tissues and cell death (8), are secreted by pathogenic strains of *S. sclerotiorum* (21). Among the pectinolytic enzymes, various isozymes of endopolygalacturonase, exopolygalacturonase, and pectinmethylesterase (PME) are produced by *S. sclerotiorum* (20,24, 30–33,36). Polygalacturonase (PG), the first hydrolytic enzyme produced by many pathogens, including *S. sclerotiorum*, is considered to be important in the pathogenicity and virulence of soft-rot fungi (7,12,14,15,21). The PG of *S. sclerotiorum* is involved in dissolving pectic components of the middle lamella and primary cell wall, resulting in the typical symptoms of soft (9,12, 20,21) and *Sclerotinia* stem rots (27). Several isozymes of PG are produced by *S. sclerotiorum*, both in culture and in planta (10,12,30).

Zymogram analysis of PG has been used as a phenotypic marker to distinguish fungi and bacteria at the species level (33). The zymograms, which contain isozymes or multiple molecular forms of cell wall-degrading enzymes that share a common substrate but differ in their electrophoretic mobilities, were detected by isoelectric focusing in polyacrylamide gels (33). Enzymes coded by different alleles or separate genetic loci have different electrophoretic mobilities. PG zymogram analyses have been used to distinguish species of *Fusarium* (33), *Sclerotinia* (10), *Erwinia* (29), and *Verticillium* (11). Three species of the genus *Sclerotinia, S. minor, S. sclerotiorum,* and *S. trifoliorum* were compared and distinguished by pectic zymograms, but no intraspecific variation in pectic isozyme pattern was observed (10). An earlier PG enzyme analysis of the isolates within each of the *Sclerotinia* species showed little or no intraspecific variation (37).

Previous studies in our laboratory (16–18) indicate a mainly clonal population structure for *S. sclerotiorum* isolated from canola. Clones are distinguished by the repeated and widespread recovery of genotypes sharing four features: 1) all members of a clone share a unique DNA fingerprint in Southern hybridizations of whole genomic DNA digested with *Bam*HI to a cloned probe, pLK44.20, containing a 4.5-kb repeated dispersed element of nuclear DNA from *S. sclerotiorum* (17,18); 2) based on pairings on defined medium, all members of a clone are mycelially compatible and incompatible with members of other clones (17,18); 3) all members of a clone share one restriction fragment length polymorphism phenotype with a probe carrying the large mitochondrial rRNA gene from *Neurospora crassa* (18); and 4) all members of a clone have or lack a group IC intron within the small mitochondrial rRNA gene (6). It is unlikely that all, or any, of these features are linked, except in clonal lineages. Mycelial compatibility grouping and the unique DNA fingerprint of each clone are routinely used to identify and compare clonal genotypes. In studies on clonal frequencies and their distributions in Canada over a 4-yr period (1989–1992), 659 genotypes were identified among 2,876 isolates. Three categories of clone frequency can be recognized (16–18): 1) 31 clones that have been sampled $\leq$7 times over a wide geographic area, termed "common clones"; 2) 50 clones that have been sampled $\geq$5 but $\leq$2 times; and 3) 578 genotypes that have been sampled once, termed, "rare clones."

The frequency and occurrence of common clones over a wide geographic area is consistent with the hypothesis that such clones may have some selective advantage over clones or genotypes recovered at low frequencies. Greenhouse studies suggest that clones of *S. sclerotiorum* differ significantly in their aggressiveness on a cultivar of canola (Table 1). However, our greenhouse experiments have not demonstrated a correlation between clone frequency and aggressiveness. We are seeking other quantitative phenotypic traits with which to make inter- and intraclonal comparisons with a view toward testing the selective advantage hypothesis. Electrophoretic procedures are attractive because they allow comparison of groups of isolates. Isoelectric focusing

followed by activity staining of substrate overlays and determination of enzyme activity produces qualitative and quantitative phenotypic characters. Comparison of isozymes involved in colonization of the host and the time frame for their production could provide phenotypic characters that are basic indicators of the early phase of host-parasite interaction.

The overall objective of this study was to use pectic zymogram analysis to screen for inter- and intraclonal variation in *S. sclerotiorum* genotypes isolated from different field populations of canola across Canada (2,500 km). The specific objectives were: 1) to screen and compare clonal genotypes for activity and variation of pectic enzymes; 2) to compare isozyme profiles of strongly aggressive and weakly aggressive genotypes, as well as pathogenic and nonpathogenic mutant genotypes; and 3) to compare canola isolates with two representative isolates from other hosts, namely, cultivated sunflower from Manitoba and a wild, woodland plant, *Ranunculus ficaria* L., collected from Norway.

MATERIALS AND METHODS

Origin of isolates. Descriptions of the isolates of *S. sclerotiorum* used in this study are summarized in Table 1. Clones were identified following previously published protocols (16–18). Twenty-nine isolates of *S. sclerotiorum* were subsampled from 2,876 isolates collected from fields in Canada during 1989–1992. With the exception of the isolate from Norway and the mutant isolates from Nebraska, all isolates were collected from agricultural fields. A pathogenic wild-type isolate, JS-152, and three oxalic acid-deficient mutants derived from JS-152, mutants A2, A4, and B that do not produce disease in the host plants (13), also were included. The isolates were cultured and maintained on potato-dextrose agar medium (Difco Laboratories, Detroit), at 22–23 C for 3 days in the dark.

Screening for aggressive and less aggressive clones. Clones of *S. sclerotiorum* were evaluated for aggressiveness on canola in greenhouse experiments conducted in 1991 and 1993. In 1991, six isolates representing six clones, LMK-223 (clone 1), -198 (clone 2), -205 (clone 3), -213 (clone 7), -221 (clone 8), and -225 (clone 9), were tested on *B. napus* cv. Westar in a two-factorial randomized block design with one replication. Lesion length was measured daily for 14 days after inoculation. In 1993, nine isolates representing seven clones were each tested on six plants of Westar in a two-factorial randomized block design with three replications (Table 2). The experiment was repeated three times in 1993. Lesion lengths were recorded at 3, 7, and 10 days after inoculation. Analysis of variance (ANOVA) of the data was performed with SAS (SAS Institute Inc., Cary, NC).

Production and preparation of extracts for enzyme assays. For pectic enzyme production, isolates of *S. sclerotiorum* were grown on liquid medium (2.0 g of NH_4NO_3, 1.0 g of KH_2PO_4, 0.1 g

TABLE 1. Characteristics of isolates of *Sclerotinia sclerotiorum* used in this study

Isolate[a]	Clone[b]	Origin			Year collected	Aggressiveness[c]	Occurrence[d]	SSZG[e]
		Host	Source	Location				
LMK-200	1	Canola (soil)[f]	Sclerotia	Ontario, Canada	1989	ND[g]	Rare	1
LMK-198	2	Canola (soil)	Sclerotia	Ontario, Canada	1989	Aggressive	Common	1
LMK-211	2	Canola (soil)	Sclerotia	Ontario, Canada	1989	ND	Common	1
F2-61-1P	2	Canola (petal)[h]	Mycelium	Saskatchewan, Canada	1991	ND	Common	1
F2-111-2-5P	2	Canola (petal)	Mycelium	Saskatchewan, Canada	1991	ND	Common	1
F2-24-L1	2	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	Aggressive	Common	1
LMK-205	3	Canola (soil)	Sclerotia	Ontario, Canada	1989	Aggressive	Rare	1
LMK-213	7	Canola (soil)	Sclerotia	Ontario, Canada	1989	Aggressive	Rare	1
LMK-225	9	Canola (soil)	Sclerotia	Ontario, Canada	1989	Less aggressive	Rare	2
F2-21-2P	33	Canola (petal)	Mycelium	Saskatchewan, Canada	1991	ND	Common	1
F2-100-1P	33	Canola (petal)	Mycelium	Saskatchewan, Canada	1991	ND	Common	1
F2-6-L3	33	Canola (lesion)[i]	Mycelium	Saskatchewan, Canada	1991	Aggressive	Common	1
F2-27-L3	33	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	Aggressive	Common	1
F2-2-L3	34	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	Aggressive	Common	2
F2-29-L1	34	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	Aggressive	Common	2
F2-78-L3	34	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Common	2
F2-32-2P	36	Canola (petal)	Mycelium	Saskatchewan, Canada	1991	ND	Common	2
F2-50-4P	36	Canola (petal)	Mycelium	Saskatchewan, Canada	1991	ND	Common	1
F2-70-1P	36	Canola (petal)	Mycelium	Saskatchewan, Canada	1991	ND	Common	2
F2-14-2-L2	36	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Common	2
F2-19-L2	36	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Common	1
F2-55-5-L1	36	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Common	2
F2-58-L1	36	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Common	2
F2-59-L2	36	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Common	2
F2-65-L3	36	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Common	2
F2-115-L2	36	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Common	2
F2-22-L3	275	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Rare	1
F2-62-L1	285	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Rare	1
F2-103-L3	300	Canola (lesion)	Mycelium	Saskatchewan, Canada	1991	ND	Rare	1
S-100-1	S-16	Sunflower	Sclerotia	Manitoba, Canada	1991	Aggressive	ND	3
S4-A2-5	N/A	*Ranunculus ficaria*	Apothecia	Oslo, Norway	1993	Less aggressive	ND	4
JS-152	B-2	Bean	Sclerotia	Nebraska	1980	ND	Common	1
Mutant A2	N/A	Bean	Mycelium	Nebraska	1988	ND	Common	1
Mutant A4	N/A	Bean	Mycelium	Nebraska	1988	ND	Common	1
Mutant B	N/A	Bean	Mycelium	Nebraska	1988	ND	Common	1

[a] Isolates within a clone are mycelially compatible and share a unique fingerprint when *Bam*HI digested and probed with pLK44.20.
[b] Clones were identified by DNA fingerprinting with pLK44.20 and mycelial compatibility grouping (16–18).
[c] Ratings based on greenhouse evaluation of lesion length at 3, 7, and 10 days after inoculation on *Brassica napus* cv. Westar: aggressive (lesion length 95.1–190 mm) and less aggressive (lesion length 60.0–95.0 mm).
[d] Occurrence: common = clones found in a high frequency over a wide geographic area, rare = clones found in a low frequency (once).
[e] *S. sclerotiorum* zymogram group phenotype.
[f] Isolated from sclerotia collected on the soil surface (18).
[g] Not determined.
[h] Isolated from canola petals following the method of Turkington et al (35).
[i] Isolated from excised diseased lesion tissue.

of $MgSO_4$, 0.5 g of yeast extract, 1.0 g of NaOH, and 3.0 g of DL-malic acid per liter of distilled water) supplemented with a carbon source (0.5% w/v). Citrus and apple pectins, sodium polygalacturonic acid (PGA), and canola cell wall extracts were each used as a carbon source. All media were sterilized by autoclaving. Twelve 1-mm plugs of mycelium from actively growing areas of 2-day-old colonies were inoculated in 15 ml of liquid medium supplemented with the carbon source and incubated for 6 days on a shaker incubator at 22–24 C in the dark. The culture fluid was separated from mycelium by filtration. Extracellular supernatant was concentrated approximately 100-fold by freeze-drying. The concentrated material was dialyzed against distilled water at 4 C. The enzyme was desalted with a Bio-gel 6DG column (Bio-Rad Laboratories, Richmond, CA). The resultant samples were assayed for PG and PME activity and stored at −20 C until used for analyses.

Preparation of canola cell walls. Greenhouse-grown, 6-wk-old, flowering canola plants (*B. napus* cv. Westar) were harvested and frozen at −20 C pending further use. Cell wall material from 65 g of frozen canola stems was extracted by the method of Mankarios and Friend (23). The cell wall material was dried in a fume hood for 36 h and stored at −20 C for further use. The cell wall extract supplemented a basal salt medium (5.0 g of KH_2PO_4, 2.5 g of $MgSO_47H_2O$, 10.0 g of KNO_3, and 0.55 g of $FeCl_3$ in 1 L), at pH 5.0, at a rate of 1.5% (w/v). Fifteen-milliliter aliquots of autoclaved cell wall extract medium were inoculated with 12 1-mm plugs of mycelium from actively growing areas of 2-day-old colonies and incubated at 22–24 C for 6 days. The culture filtrates were treated as described in the previous section.

Determination of molecular weight, pH, and temperature optima. Molecular weight of the extracellular proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (19). Ten-microliter samples were loaded on slab gels containing 4% (w/v) stacking gels and 10% (w/v) resolving gels. The gels were stained with Coomassie brilliant blue R (Sigma Chemical Co., St. Louis). PG activity was determined by means of agarose overlays containing 0.1% PGA. PME activity was determined by means of agarose overlays containing 0.1% citrus pectin. Protein concentrations were determined by the method of Bradford (4). PG activity was measured at pH 3, 4, 5, 6, 7, and 8. Thermostability was measured by the residual activity after incubation for 4 h at temperatures ranging from 20 to 60 C.

TABLE 2. Mean lesion lengths of different clones of *Sclerotinia sclerotiorum* on canola (*Brassica napus* cv. Westar) in 1993 greenhouse experiment

Isolate[a]	Clone[b]	Lesion length (cm)[c]			
		Exp. 1[d,e]	Exp. 2[d,f]	Exp. 3[d,g]	Average[h,i]
F2-24-L1	2	91.16	103.41	134.81	109.79
F2-6-L3	33	96.85	86.40	141.82	108.35
F2-27-L3	33	107.37	92.19	134.54	111.37
F2-2-L3	34	118.08	104.81	142.32	121.74
F2-29-L1	34	126.22	112.99	141.63	126.95
F2-75-L2	287	117.84	90.81	138.68	115.78
F2-103-L3	300	112.17	105.22	131.04	116.14
S-100-1	S-16	97.47	105.81	133.53	112.27
S4-A2-5	N/A[j]	61.96	80.43	95.26	79.21
SS4	N/A	88.48	86.09	131.18	101.92

[a] Isolates within a clone are mycelially compatible and share a unique fingerprint when *Bam*HI digested and probed with pLK44.20.
[b] Clones were distinguished by DNA fingerprinting and mycelial compatibility testing.
[c] Averages of lesion lengths recorded 3, 7, and 10 days after inoculation.
[d] Values are the averages of three replications.
[e] The isolate S4-A2-5 is significantly ($P = 0.05$) different from all others, except the isolate belonging to clone 2 and isolate SS4, and clone 34 is significantly ($P = 0.05$) different from the isolate belonging to clone 2 and isolate SS4, according to Scheffe tests.
[f] None of the clones are different ($P = 0.05$), according to Scheffe tests.
[g] The isolate S4-A2-5 is significantly different from all others, which do not differ among themselves ($P = 0.05$), according to Scheffe tests.
[h] Average of three experiments.
[i] The isolate S4-A2-5 is significantly ($P = 0.05$) different from all others, except the isolate belonging to clone 2 and isolate SS4, according to Scheffe tests.
[j] Not available.

Time course of enzyme secretion. To determine the sequential expression of the PG isozymes, a time-course experiment was carried out with two isolates with the *S. sclerotiorum* zymogram group-1 (SSZG-1) phenotype, LMK-211 (clone 2) and F2-50-4P (clone 36), and two isolates with the SSZG-2 phenotype, LMK-225 (clone 9) and F2-70-1P (clone 36). Twelve 1-mm plugs of mycelium from actively growing areas of 2-day-old colonies were inoculated in 15 ml of liquid medium containing PGA. The cultures were incubated on a shaker incubator at 22–24 C in the dark. The cultures were harvested at 24-h intervals, beginning immediately after inoculation and continuing until day 10. The culture filtrates were treated as described above.

Enzyme assay. Pectinolytic enzyme activity was determined as an increase of reducing-end groups released from various substrates with respect to time. The reducing-end groups were measured by the method of Miller (26) with galacturonic acid as the standard. The enzyme was incubated at 30 C for 30 min in 1 ml of solution with substrate dissolved in 100 mM sodium acetate buffer, pH 5.0. The reaction was stopped by the addition of 3 ml of nitrosalicylic acid reagent. The tubes were boiled in a water bath for 10 min. The enzyme was immediately assayed spectrophotometrically at A_{528nm}. One unit of PG activity was defined as producing 1 μmol of reducing groups per minute on 0.1% PGA in 100 mM of sodium acetate buffer, pH 5.0.

Isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE) and isozyme detection. Isozymes of pectinolytic enzymes in nondenaturing isoelectric focusing gels and in ultrathin layer substrate agarose overlays were detected following the methods of Bertheau et al (1) and Ried and Collmer (28,29). Extracellular proteins of *S. sclerotiorum* were separated by ultrathin IEF-PAGE containing 5% ampholytes (Bio-lyte 3/10, Bio-Rad), pH range 3–10. The gels were prefocused at 2 W for 30 min before the samples were applied. The samples were focused at a constant power of 4 W for 90 min. Marker proteins were from Bio-Rad.

The isozymes of PG and PME in IEF-PAGE gels were detected by a modified activity stain agarose overlay technique described by Ried and Collmer (28). The PG was detected with PGA in the agarose overlay, whereas the PME was detected in a citrus pectin overlay (12,30). Ultrathin (0.4 mm) overlays were cast on agarose gel support film (Bio-Rad). The agarose gels contained 0.1% PGA or citrus pectin, 1.0% (w/v) agarose in 100 mM sodium acetate, pH 5.0. Pectate or PGA overlays were pressed against the native IEF gel and incubated at 45 C for 2 h. Prior to activity staining for PG or PME, the native IEF gel and the agarose overlay were peeled apart and washed in 100 mM sodium acetate, pH 5.0, for 15 min. The native and agarose overlay gels were stained for 30 min in 0.05% ruthenium red. The gels were destained overnight in water and preserved by drying. The ruthenium red stains pectate in the overlay leaving a clear band where pectate has been enzymatically degraded by PG. PME activity is indicated by red bands where free acid groups on the polymer have enhanced the binding of the ruthenium red. There were two replications for each experiment. Protein was freshly extracted from each isolate for each replication.

RESULTS

Molecular weight, pH, and temperature optima. When separated by SDS-PAGE, concentrated samples of extracellular proteins collected from culture media containing each of the different carbon sources revealed bands at 35 and 68 kDa for PG and a band at 45 kDa for PME. PG enzyme showed maximal activity at pH 5.0, and PME showed maximal activity at pH 5.5. Optimum temperature for activities of PG and PME in our samples was 45 C.

Pectic enzymes. Extracellular proteins produced by *S. sclero-*

tiorum on PGA, citrus pectin, apple pectin, and canola cell wall substrates, subjected to analytical IEF-PAGE and activity staining, exhibited PG and PME activities. All of the isolates of *S. sclerotiorum* tested in this study produced multiple forms of PG and PME. The isozymes of PG and PME can be classified into three groups: basic (pH > 8.0), neutral (pH > 7.0–8.0), and acidic (pH < 7.0).

Analysis of isozymes of PME. All of the 35 isolates grown on PGA, citrus pectin, apple pectin, and canola cell wall extracts produced multiple isoforms of PME. Five isoforms of PME were produced by all 35 isolates on PGA, apple pectin and canola cell wall extracts: four acidic isoforms, pI 4.2, 4.4, 4.85, and 5.1, and one basic isoform, pI 8.0. Four isoforms, pI 4.2, 4.4, 4.85, and 5.1, were produced on citrus pectin. No variation in the isoforms of PME was observed among the isolates collected from canola.

Analysis of isozymes of PG. All 35 isolates of *S. sclerotiorum* produced multiple isozymes of PG on PGA, apple pectin, citrus pectin, and cell wall extracts (Fig. 1). The analysis of PG isozymes on PGA-containing medium identified four phenotypes, SSZG-1, -2, -3 and -4 (Table 3). Based on their zymogram patterns, all isolates were categorized in one of four zymogram phenotypes (Table 1). Twenty-two isolates had the SSZG-1 phenotype, 11 isolates had the SSZG-2 phenotype, one isolate had the SSZG-3 phenotype, and one isolate had the SSZG-4 phenotype (Table 1). The PG isozyme patterns produced by the isolates on citrus and apple pectins were similar to the patterns from PGA substrate.

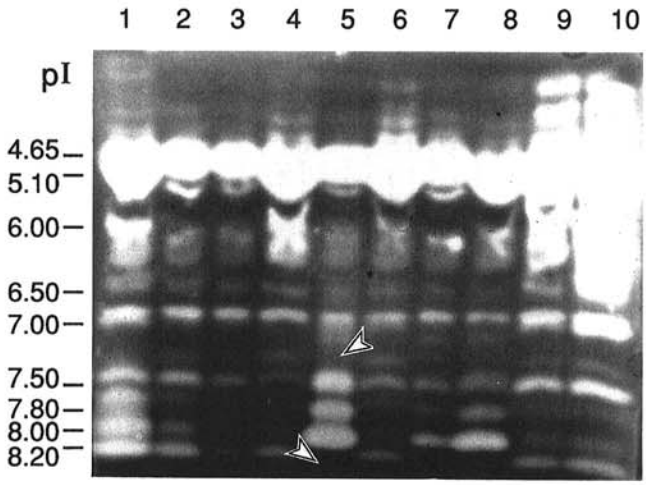

Fig. 1. Isoelectric focusing (IEF) profiles of extracellular polygalacturonase (PG) produced by different isolates of *Sclerotinia sclerotiorum* on medium supplemented with 1.0% polygalacturonic acid. Samples (5 μl) were separated by broad-range analytical IEF, and PG activity was detected by the activity stain overlay technique. IEF markers are indicated on the left. Lane 1, clone 2 (isolate LMK-211); lane 2, clone 2 (isolate LMK-198); lane 3, clone 3 (isolate LMK-205); lane 4, clone 7 (isolate LMK-213); lane 5, clone 9 (isolate LMK-225); lane 6, clone 36 (isolate F2-50-4P); lane 7, clone 36 (isolate F2-70-1P); lane 8, clone 36 (isolate F2-32-2P); lane 9, clone 33 (isolate F2-100-1P); and lane 10, clone 33 (isolate F2-111-4-2P). The absence of two isozymes is indicated by arrows in lane 5. Lanes 1–4, 6, 9, and 10 had the *S. sclerotiorum* zymogram group-1 (SSZG-1) phenotype. Lanes 5, 7, and 8 had the SSZG-2 phenotype.

All of the isolates of the SSZG-1 phenotype, when grown on canola cell wall extracts, produced an extra isozyme at pI 6.55. There was no change in the number of isozymes among isolates of the SSZG-2 phenotype when grown on canola cell wall extracts. (Fig. 2). All the isolates produced twofold more enzyme on cell wall extracts as compared to the amount of enzyme produced on PGA.

Variation of PG zymograms within clones. Of the four common clones tested, isolates of only one clone (clone 36) showed intraclonal variation (Table 1). Of the 10 isolates of clone 36 tested, all from the same field, two, F2-50-4P and F2-19-L2, had the SSZG-1 phenotype, and eight had the SSZG-2 phenotype.

Comparison of PG zymograms of isolates collected from different hosts. The zymogram profiles of isolates from canola, sunflower, and *R. ficaria* are shown in Figure 2. The isolates from canola had either the SSZG-1 or -2 phenotype, the isolates from bean had the SSZG-1 phenotype, the isolate from sunflower had the SSZG-3 phenotype, and the isolate from *R. ficaria* had the SSZG-4 phenotype (Table 1).

Variation of PG zymograms among clones collected from canola from Ontario and Saskatchewan. Of six isolates collected from Ontario, five isolates had the SSZG-1 phenotype, and the remaining one had the SSZG-2 phenotype (Tables 1 and 3; Fig. 1). Of 23 isolates collected from Saskatchewan, 13 isolates had the SSZG-1 phenotype, and 10 had the SSZG-2 phenotype (Table 3; Figs. 1 and 2).

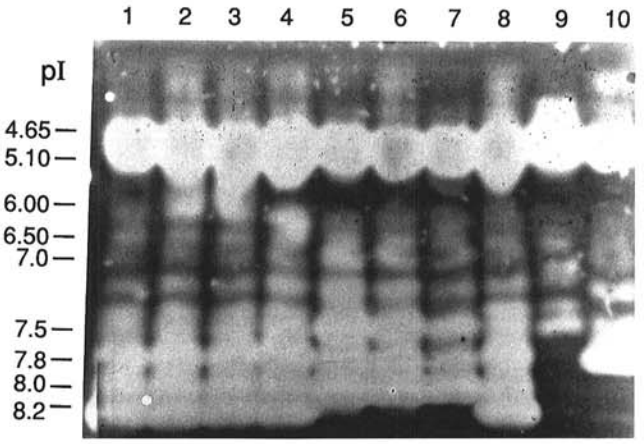

Fig. 2. Isoelectric focusing (IEF) profiles of extracellular polygalacturonase (PG) produced by different isolates of *Sclerotinia sclerotiorum* on medium supplemented with 1.5% canola cell wall extracts. Samples (2.5 μl) were separated by broad-range analytical IEF. PG activity was detected by the activity stain overlay technique. IEF markers are indicated on the left. Lanes 1–8, isolates collected from canola in Canada; lane 9, isolate collected from sunflower in Canada; and lane 10, isolate collected from *Ranunculus ficaria* in Norway. Lane 1, clone 2 (isolate F2-61-1P); lane 2, clone 2 (isolate F2-24-L1); lane 3, clone 33 (isolate F2-27-L3); lane 4, clone 33 (isolate F2-6-L3); lane 5, clone 34 (isolate F2-29-L3); lane 6, clone 34 (isolate F2-78-L3); lane 7, clone 34 (isolate F2-2-L3); lane 8, clone 300 (isolate F2-103-L3); lane 9, clone S-16 (isolate S-100-1); and lane 10, clone N-1 (isolate S4-A2-5). Lanes 1–4 and 8 had the *S. sclerotiorum* zymogram group-1 (SSZG-1) phenotype. Lanes 5–7 had the SSZG-2 phenotype. Lane 9 had the SSZG-3 phenotype. Lane 10 had the SSZG-4 phenotype.

TABLE 3. Extra cellular polygalacturonase (PG) isozymes produced by *Sclerotinia sclerotiorum* on liquid medium with polygalacturonic acid as a carbon source

SSZG[a]	Isozymes of PG at pI[b]											
	4.20	4.40	4.85	5.10	5.25	6.90	7.05	7.20	7.45	7.7	8.00	8.30
1	+	+	+	+	+	+	−	+	+	+	+	+
2	+	+	+	+	+	+	+	−	+	+	+	−
3	+	+	+	+	+	+	−	+	+	tr	tr	tr
4	+	+	+	+	+	+	+	+	+	+	tr	tr

[a] *S. sclerotiorum* zymogram group phenotype.
[b] Isoelectric point: + = presence; − = absence; and tr = trace activity of PG isozymes.

Variation of PG zymograms among aggressive and less aggressive clones. Although detailed results of the greenhouse experiments on aggressiveness will be published in another publication (D. Errampalli, L. J. Brunner, and L. M. Kohn. *unpublished data*), the basic results are as follows. Although the clones of *S. sclerotiorum* differed significantly in their aggressiveness on canola, there was no correlation between clone frequency and aggressiveness. We classified each isolate in one of two categories based on average lesion length on canola: aggressive (lesion length 95.1–190.0 mm) and less aggressive (lesion length 60.0–95.0 mm; Table 1). In 1991, the isolates LMK-223 (clone 1), -198 (clone 2), -205 (clone 3), -213 (clone 7), -221 (clone 8), and -225 (clone 9) produced average lesion lengths of 65.90, 187.50, 154.10, 174.00, 95.80, and 66.50 mm, respectively. The ANOVA identified a significant difference between the clones ($P = 0.01$). Based on the average lesion length, isolates representing clones 1 and 9 were categorized as less aggressive. Average lesion lengths produced by isolates of clones 2 and 7 were significantly different from isolates of clones 1 and 9, and the other comparisons were not significantly different ($P = 0.05$) using the Scheffe test.

Results of the experiments carried out in 1993 are presented in Table 2. Based on the average lesion lengths produced by the each of the isolates, isolate S4-A2-5 was categorized as less aggressive, while all other isolates were categorized as aggressive (Table 1). The ANOVA identified a significant difference among the clones ($P = 0.001$) and among the experiments ($P = 0.001$). Although a significant difference existed among the experiments, the absence of a significant clone-experiment interaction ($P = 0.0593$) indicated that the clones performed consistently within each of the experiments. The absence of significant difference between the average lesion lengths produced by the isolates representing clones 33 and 34 indicate that there was no observed difference in aggressiveness between the isolates within each of the clones. There was also no significant difference in lesion lengths produced by isolates representing rare clones, clones 287 and 300, and common clones, clones 2, 33, and 34. This indicated a lack of correlation between clone frequency and aggressiveness. There was no significant difference between one isolate collected from sunflower and other isolates collected from canola. The one isolate (S4-A2-5) from the wild (noncultivated) population of *R. ficaria* was significantly different from all other isolate pairs, indicating that the isolate from *R. ficaria* produced lesions at a slower rate than other isolates, except for one isolate representing clone 2 and one isolate, SS4, used in routine canola resistance screening. Strain SS4, originally isolated from bean, has been maintained in culture for many years.

Zymogram analysis of isolates rated as aggressive and less aggressive are presented in Table 1. Eight of the isolates in five clones rated as aggressive and one isolate in one clone rated as less aggressive had the SSZG-1 phenotype. Two of the isolates in one clone rated as aggressive and one isolate in one clone rated less aggressive had the SSZG-2 phenotype.

Variation of PG zymograms among wild-type and mutant isolates. Eleven isozymes of PG were produced by a wild-type isolate, JS-152, collected from field bean (*Phaseolus vulgaris* L.) and three nonpathogenic oxalic acid-deficient mutants, A2, A4, and B, derived from JS-152. A higher level of enzyme activity was produced by the three mutants than by the wild type (data not shown), in agreement with a previous report (13). JS-152 and all three mutants had the SSZG-1 phenotype (Table 3).

Time-course study of PG isozymes. A time-course study was run on the PG isozyme profiles of two isolates with the SSZG-1 phenotype, LMK-211 (clone 2) and F2-50-4P (clone 36), and two isolates with the SSZG-2 phenotype, LMK-225 (clone 9), and F2-70-1P (clone 36). Figure 3 shows representative PG isozyme profiles of the isolate LMK-225 over a 10-day period. This isolate, and the other isolates tested, produced two acidic isozymes at pI 4.85 and 5.1 after 24 h in culture. The remaining isozymes appeared in 2-day-old culture. Figure 4 shows the rate of PG production by the four isolates. PG activity produced by the four isolates was detected 24 h after inoculation and reached a plateau 6 days after inoculation. The isolate LMK-225 (clone 9) produced twice the amount of PG activity of isolate LMK-211 (clone 2). The two isolates of clone 36 produced similar amounts of PG. After inoculation, increases in the number of PG isozymes were not necessarily associated with increases in total enzyme activity. Between day one and day two, eight additional PG isozymes were produced by LMK-225 (Fig. 3). However, the 10 isozymes on day two did not yield a correspondingly higher total activity than did the two isozymes produced on day one (Fig. 4).

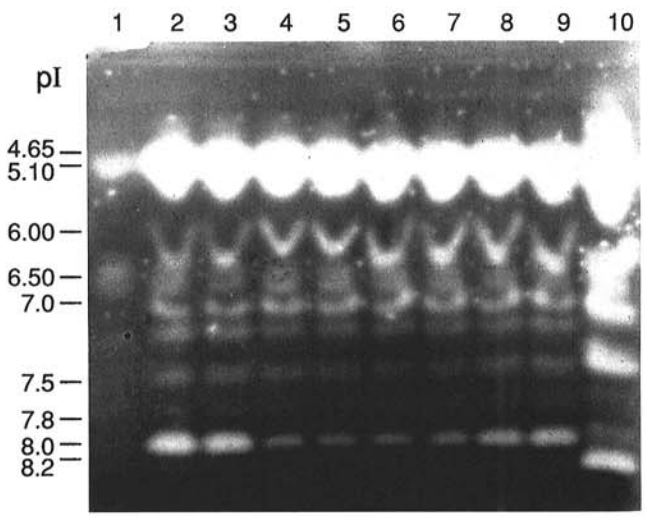

Fig. 3. A time-course study showing sequential expression of isozymes produced by clone 9 (isolate LMK-225). Isoelectric focusing (IEF) profiles of extracellular polygalacturonase (PG) produced by two isolates of *Sclerotinia sclerotiorum* on medium supplemented with 1.0% polygalacturonic acid. Samples (5 μl) were separated by broad-range analytical IEF. PG activity was detected by the activity stain overlay technique. IEF markers are indicated on the left. Lanes 1–9, clone 9 (isolate LMK-225), 1–9 days in culture, respectively; and lane 10, clone 36 (isolate F2-50-4P) after 6 days in culture. Clone 9 (isolate LMK-225) had the *S. sclerotiorum* zymogram group-2 (SSZG-2) phenotype, and clone 36 (F2-50-4P) had the SSZG-1 phenotype.

DISCUSSION

In this study, zymogram analysis of PG distinguished two phenotypes, SSZG-1 and -2, among 11 clonal genotypes of *S. sclerotiorum* collected from canola. Repeatable zymograms with consistent patterns were produced by all isolates. Nine clones

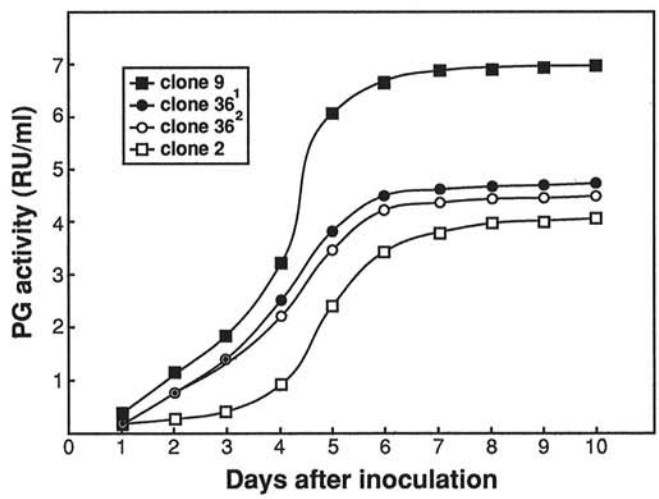

Fig. 4. Polygalacturonase (PG) activity produced by four isolates of *Sclerotinia sclerotiorum*. over a period of 10 days on medium supplemented with 1.0% polygalacturonic acid. Clones 9 (isolate LMK-225) and 36^1 (isolate F2-70-1P) had the *S. sclerotiorum* zymogram group-2 (SSZG-2) phenotype, and clones 2 (isolate LMK-211) and 36^2 (isolate F2-50-4P) had the SSZG-1 phenotype.

had the SSZG-1 phenotype, two clones had the SSZG-2 phenotype, and one clone had both SSZG-1 and -2 phenotypes. Therefore, there are fewer phenotypes than there are clonal genotypes for canola isolates sampled across Canada. Similar results were reported from pectic zymogram analysis of several anastamosis groups of *Rhizoctonia solani* (22).

Although many isoforms of PG were observed for each isolate, only two phenotypes were identified among the canola isolates. Given the amount of variation in individual bands, many zymogram phenotypes might have been expected. Even within what has been demonstrated with independent markers to be a genetic clone (clone 36), both phenotypes were observed. This raises the question of whether what we have determined to be clones are actually clones and, if so, how the observation of two zymogram phenotypes among members of one clone could be consistent with the concept of clonality. Two lines of reasoning support our current designation of clones, which may harbor some intraclonal variability. First, during 4 yr of sampling a total of 2,876 isolates, many clones have been sampled repeatedly, from year-to-year and in widely separated geographic locations. By our interpretation, clones are distinguished by the repeated and widespread recovery of genotypes sharing the four features discussed earlier, including two features based on the mitochondrial genome: one feature based on a repeated, dispersed, retrotransposon-like fingerprinting element (16,18), and one feature, mycelial compatibility, probably based on one or more nuclear genes (17,18). Alternatively, we could weight the presence of two zymogram phenotypes within one of four clonal genotypes tested, differentiated by two isozymes, as indicating that zymogram groups are truer indicators of clonality. We feel that the evidence is far stronger for the former interpretation of clonality based on the repeated association of four independent features.

The second line of reasoning supporting our interpretation of clonality is that the observation of two zymogram phenotypes within one clone is consistent with somaclonal variability, which has been reported in this species (2). Although the vernacular and genetic uses of the term clone imply that members of a clone are identical, mutations occur in mitotic or selfed meiotic, clonal lineages, resulting in treelike evolutionary patterns (25); members of a clone cannot be expected to be identical, especially over many generations.

The enzyme activity produced by different clones of the pathogen in pure substrate cultures with PGA, apple pectin, citrus pectin, and canola cell wall extracts in this study demonstrates the ability of the pathogen to produce both PG and PME. The isozyme patterns produced on canola cell wall extracts may bear a closer comparison to the disease situation. Also, induction of higher enzyme activity (twofold) and a higher number of isozymes observed on cell wall extracts compared to other polymer substrates suggests that the PG may be more efficient in degrading cell wall substrates.

All the isolates of *S. sclerotiorum* tested produced multiple forms of PME. Previous work with a limited number of isolates of *S. sclerotiorum* showed the production of PME (10,30). Another pectic enzyme analysis, different from the isozyme analysis used here, detected production of PME in some, but not all, of the isolates tested (27). The detection of isozymes of PME in all the isolates tested in our study can be attributed to the sensitivity of the pectate agarose overlay technique (28). No variation in PME isozymes was observed among the isolates tested. Nonregulatory enzymes such as esterases may vary and probably will detect a high level of intraspecific variation (3). In contrast, lack of variation in the isozyme patterns of PME produced by 35 isolates from canola, bean, sunflower, and *R. ficaria* suggests that this enzyme does not vary in *S. sclerotiorum*.

Multiple forms of PG were produced by all the *S. sclerotiorum* isolates tested. This is consistent with previous reports on PG produced in planta or in culture filtrates by isolates of *S. sclerotiorum* from other hosts (10,12,24,30). The isoform at pI 8.3 produced by all the isolates in SSZG-1 may be similar to pI 8.35 (12) and pI 8.3 observed in Italian isolates of *S. sclerotiorum* (24). Although Waksman et al (36) found that this isozyme was unstable, we were able to detect pI 8.3 in all the isolates in SSZG-1. Favaron and Marciano (12) showed that in *S. sclerotiorum* this isozyme (pI 8.35) was produced on cell wall and cell wall-related substrates but not on PGA. Our results, however, show that this isozyme (pI 8.3) was produced on PGA and citrus and apple pectins, as well as cell wall extracts, indicating that substrates other than cell wall and its components can induce this isozyme.

The isolates from sunflower and *R. ficaria* produced phenotypes other than SSZG-1 and -2. A conservative hypothesis, which could be tested, is that the clonal population on canola in Canada shows less variation than the species, *S. sclerotiorum*, shows as a whole. The variation in isozyme patterns shows no relationship to geographic origin of isolates collected from canola.

The specific role of cell wall-degrading enzymes has yet to be demonstrated, either in determining disease or in modifying the course of disease development. Some previous reports have suggested that PG, alone or in association with PME, can cause tissue maceration (14,21,24). Other reports have concluded that PG is not required in pathogenicity (7,34). Our data suggest two things. First, our data, in agreement with previous reports based on both in vitro and in vivo studies (24,27), indicate no relationship between isozymes of PG and PME and aggressiveness of isolates. Second, the lack of variation in zymogram patterns of the pathogenic wild-type isolate and the nonpathogenic oxalic acid-deficient mutant isolates indicates that PG and PME are not the sole determinants of pathogenesis or disease development.

The time-course study of PG isozymes produced by the isolates with SSZG-1 and -2 phenotypes shows a consistent sequence in the production of PG isozymes in culture with PGA substrate. A similar sequence of isozymes produced in culture filtrates by isolates of *S. sclerotiorum* from other hosts has been reported in the literature (10,12,24,30,32,36). Detection of two isozymes of PG, pI 4.85 and 5.1, after 1 day in culture and additional isozymes of PG after 2 days in culture suggested that *S. sclerotiorum* uses this isozyme complex as a catabolic pathway to depolymerize complex pectic polysaccharides.

What does this study tell us about phenotypic variability among clones of *S. sclerotiorum* on canola? Only two phenotypes were distinguished, and no relationship between phenotype and aggressiveness was observed. The detection of only two phenotypes among 29 isolates from 2 yr of sampling on Canadian canola does not rule out the possibility that a comparison of a larger subsample could reveal additional phenotypes, but it does suggest that additional phenotypes are infrequent among canola isolates. Isolates are being screened for other phenotypic traits, such as apothecial production, ascospore germinability, and tolerance of seed-treatment fungicides, to determine whether some clones have a selective advantage that can be measured. A selective advantage is not the only explanation of differences in clone frequency and distribution. Common clones may be older, spanning more generations over a longer period of time, with more opportunities to disperse over a wide geographic area than rare clones or genotypes. Nevertheless, common clones would have to be fit to persist.

This study is based on in vitro observations of isozymes of PG and PME produced by isolates of different clones of *S. sclerotiorum*. Comparative studies under controlled conditions offer some advantages, especially in screening large numbers of isolates. However, because enzyme production is sensitive to environmental factors within the infected plant, the isozyme profiles produced on pure substrate culture may differ, most probably in the amount of activity (9) and perhaps in the number of isozymes (5,12,24) and the sequence of isozyme production (12,24), from those produced in living plants. Further studies in living plants are needed, especially concerning the role of two isozymes that have shown variability, pI 7.2 and 8.3.

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