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Multiple forms of pectin-degrading enzymes produced by intersterile groups P, S and F of *Heterobasidion annosum* (Fr.) Bref

Cecilia Comparini, Lucia Cecere, Paolo Capretti and Aniello Scala

Dipartimento di Biotecnologie Agrarie, Sezione di Patologia vegetale, Università,
P.le delle Cascine 28, 50144 Firenze, Italy

Summary. The time-course of polygalacturonase (PG) and pectin lyase (PNL) activities shown *in vitro* by several isolates belonging to the intersterile groups (IGs) S and F of *Heterobasidion annosum* are reported and discussed in relation to their mycelial growth and the changes in the pH and viscosity of the growth medium they produce. IG-S, characterized by a narrow host range, always produced low levels of enzymatic activity. IG-F grew more abundantly and faster than IG-P and IG-S. In the nutrient medium containing pectin as the sole carbon source. PG activity shown by IG-F per unit of mycelial mass was similar to that of IG-P, but different from that of IG-S; by contrast, the specific PNL activity of IG-F was comparable to that of IG-S, but not to that of IG-P. PG, PNL and pectin methylesterase (PME) activities were separated by non-denaturing isoelectric focusing-polyacrylamide gel electrophoresis; to detect the isozymes, agarose overlays containing the appropriate substrate were stained, in a ruthenium red solution. Each IGs behaved differently from the others, and the variations among them were statistically significant. The isozymatic patterns were also very strongly influenced by the culture period. IG-S, unlike the two other IGs, always showed a single PG band with pI 4.7; an attempt to carry out a partial purification of the PG band with pI 4.7 is shown. The results of the present study open the avenue to obtaining more information about the role of pectinolytic enzymes in the root rot caused by *H. annosum*.

Key words: *Heterobasidion annosum*, polygalacturonase, pectin lyase, pectin methylesterase, isozymes.

Abbreviations used in the text: CF, culture filtrate; CCF, concentrated culture filtrate; GA, galacturonic acid; IEF, isoelectric focusing; IG, intersterile group; PG, polygalacturonase, PME, pectin methylesterase; PNL pectin lyase.

Introduction

Rot of plant tissues is commonly accepted as being the result of the degrading activity of pectinolytic enzymes on the plant cell-wall components. The role of pectinolytic enzymes in the pathogenesis and virulence of phytopathogenic micro-organisms has been widely discussed (Bateman and Miller, 1966; Bateman and Basham, 1976; Cervone *et al.*, 1986; Collmer and Keen, 1986; Alghisi and Favaron, 1995)). It has been suggested that differences in pectinase production in strains of a pathogen may account for differences in the host range or in the virulence and in the extent of the damage and symptoms caused to the host (Bateman and Basham, 1976; Scala *et al.*, 1980; Collmer and Keen, 1986; Rodriguez-Panezuela *et al.*, 1991). Many micro-organisms have multiple forms of pectinolytic enzymes due to multiple genes encoding for them more frequently than post-translational modifications of the protein (Bussink *et al.*, 1992; Caprari *et al.*, 1993; Martel *et al.*, 1996; Annis and Goodwin, 1997). Moreover, molecular forms of an enzyme

Corresponding author: A. Scala
Fax: +39 055 354786
E-mail: aniello.scala@unifi.it

can give a micro-organism greater flexibility in its pathogenicity and/or a competitive advantage over micro-organisms without such forms (Keon *et al.*, 1987; Mikhailava, *et al.*, 1995). The adsorption of pectic polymers to plant host tissues and cell walls, has been evaluated (Cervone *et al*, 1978; Chung *et al.*, 1997). Centis *et al.* (1997) showed that two endopolygalacturonases, CLPG1 and CLPG2, were differentially produced by *Colletotrichum lindemuthianum* during both saprophytic growth and plant infection; this causes each of the two isozymes to play a different pathogenic role. Recent molecular techniques such as gene disruption have been of assistance in defining the functional role during pathogenesis of single isozymes that were separated on the basis of their isoelectric points (pIs) (Barras *et al.*,1994; Annis and Goodwin, 1997; Shieh *et al.*, 1997; Di Pietro and Roncero, 1998; ten Have *et al.* 1998).

Heterobasidion annosum (Fr.) Bref is a basidiomycete causing root and butt rot and extensive mortality to many forest trees, including conifers and angiosperms, in the Northern hemisphere (Hodges, 1969; Hüttermann and Woodward, 1998). Interfertility and pairing tests have shown that *H. annosum* comprises three intersterile groups (IGs), known as P (IG-P), S (IG-S) and F (IG-F). These groups have numerous distinctive features (different, though overlapping, host range, partially different geographical distribution) and morphological characteristics (Koronen and Stenlid, 1998). On the basis of these differences, Niemela and Korhonen (1998) proposed that the three IGs be raised to the status of species. The species epithet "*annosum*" whould remain applicable to IG-P, while IG-S and IG-F would acquire the new names of *H. parviporum* Niemela & Korhonen, species nova, and *H. abietinum* Niemela & Korhonen, species nova, respectively.

H. annosum secretes a wide range of extracellular enzymes that degrade the structural cell wall components of its hosts (Asiegbu *et al.* 1998). Among these, the hemicellulolytic enzymes and laccase activity of IG-P have been compared with those of IG-S and studied to determine their pathological significance for the hosts of these IGs and their differential aggressiveness (Maijala *et al.*, 1995; Johansson *et al.*, 1998; Johansson *et al.*, 1999). Moreover, pectinolytic enzymes are produced differently by IG-P and IG-S (Johansson, 1988). Karlsson and Stenlid (1991) found that various pectinase zymograms were obtained following separation by gel electrophoresis; they suggested that banding patterns might be used as biochemical markers of IG affiliation.

This paper aims to analyse the time-course of polygalacturonase (PG) and pectin lyase (PNL) activities produced by several isolates belonging to IG-P, IG-S and IG-F of *H. annosum*. Differences in enzyme levels among IGs were studied in relation to how well they grow in a liquid medium containing pectin as carbon source. Moreover, for each incubation time the pI values of the forms of PG, PNL and pectin methylesterase (PME) produced by each IG were determined and related to their host range.

Materials and methods

Fungal isolates

Some *H. annosum* isolates were provided by colleagues and some by the Culture Collection of the *Dipartimento di Biotecnologie Agrarie, Sezione di Patologia vegetale*, University of Florence, Italy (Table 1).

The IG of each isolate was determined by mating tests, using two homokaryotic testers each from IG-S, IG-P and IG-F (Capretti *et al.*, 1990).

Production of pectinolytic enzymes

H. annosum was grown in the liquid medium described by Johansson (1988), supplemented with citrus pectin (Sigma, St. Louis, MO, USA) as the sole carbon source, to a final concentration of 5 g l^{-1}. The medium was adjusted to pH 5.5 with 1N NaOH. Autoclaving at 120°C for 20 minutes changed the pH of the medium to about 5.1. The inoculum consisted of single plugs (5-mm diameter) of mycelium picked up from the edge of 5 day-old cultures on malt extract agar (MEA). The cultures were grown for 3, 6, 9, 13, 16 and 20 days at 23°C in 100 cc Erlenmeyer flasks containing 25 ml medium. One flask was prepared per isolate per incubation time. For each incubation time, the cultures were centrifuged at 13,000 *g* at 4°C for 20 min. The supernatants obtained were filtered through 0.45 µm cellulose membranes and immediately used for pH and viscosity determinations.

The pH of culture filtrate (CF) was estimated with a Crison pH meter (Alella, Spain).

Table 1. Isolates of *Heterobasidion annosum* used in this study, separated into the intersterile groups (IGs) P, S and F.

Intersterile groups	Strain number	Code in the present paper	Place of isolation	Date of isolation	Host plant	Source[a]
IG-F						
	931111 (3.11)	1f	Molise, Italy	1993	*Abies alba*	PC
	921218 (4.4)	2f	Foggia, Italy	1993	*Abies alba*	PC
	930519 (4.1)	3f	Trento, Italy	1993	*Picea abies*	PC
	931030(4.15)	4f	Aspromonte, Italy	1993	*Abies alba*	PC
	H27 14.1.2.1	5f	France	1993	*Abies alba*	CD
	H6 2.2.2.1	6f	Piemonte, Italy	1993	*Abies alba*	GN
	H11 2.3.2.2.1	7f	Trentino, Italy	1993	*Picea abies*	GN
	931014 (2.8)	8f	Cadore, Italy	1993	*Picea abies*	PC
IG-S						
	931007 (2.4)	9s	Cadore, Italy	1993	*Picea abies*	PC
	92057/2	10s	Munchen, Germany	1992	*Picea abies*	KK
	92186/2	11s	Denmark	1992	*Picea abies*	KK
	930830 (1.1)	12s	Trento, Italy	1995	*Picea abies*	PC
	B540	13s	Estonia	1991	*Populus tremula*	MH
	B234	14s	Estonia	n.a.[b]	*Picea abies*	MH
	LAV.5.2	15s	Lavazè, Italy	1997	*Picea abies*	NLP
	LAV.5.1	16s	Lavazè, Italy	1997	*Picea abies*	NLP
	LAV.4.2	17s	Lavazè, Italy	1997	*Picea abies*	NLP
IG-P						
	930930 (2.5)	18p	Maresca, Italy	1993	*Pinus laricio*	PC
	911116 (1.1)	19p	Tirrenia, Italy	1991	*Pinus pinea*	PC
	8F	20p	Calabria, Italy	1997	*Fagus sylvatica*	PC
	970907.4.9	21p	Pirenei, France	1997	*Abies grandis*	PC
	9705-4	22p	The Netherland	1997	*Picea abies*	WS
	930519 (1/9)	23p	Cadore, Italy	1993	*Picea abies*	PC
	970702(2.3)	24p	Consuma, Italy	1997	*Pinus laricio*	PC
	970702(3.2)	25p	Consuma, Italy	1997	*Pinus laricio*	PC
	B336	26p	Estonia	1990	*Pinus silvestris*	MH

[a] PC, Paolo Capretti; CD, Claude Delatour; GN, Giovanni Nicolotti; KK, Kari Korhonen; MH, Märt Hanso; NLP, Nicola La Porta; WS, Wouther Schuring.
[b] Not available.

The percentage diminution of CF viscosity (V) was determined according to the equation:

$$V = \frac{Vo - Vt}{Vo - Vs} \times 100$$

where Vo is the flux time in seconds of 6 ml uninoculated medium, Vt the flux time in seconds of 6 ml CF, and Vs the flux time (in seconds) of 6 ml distilled water. The flux time was calculated using an Ostwald viscosimeter (Brand GMBH & Co, Wertheim, Germany) mod. NO II 85 sec K 0,011 immersed in a water thermostat at 30°C.

Pellets obtained after centrifugation were dried at 80°C overnight. The mass of dried mycelium was expressed in mg and represented the growth of each isolate at each incubation time.

Enzyme assay

Ten ml of the CFs above-described were dialysed (cut off 1,000 Da) for 18 h at 4°C against distilled water, concentrated in the same dialysis tubes against polyethylenglycol (PEG) 6,000, and adjusted to a final volume of 200 µl to obtain a 50-fold

concentrated CF (CCF). CCFs were stored at -30°C. Prior to assay, the CCFs were centrifuged at 10,000 *g* for 2 minutes.

The determination of PG activity was based on the hydrolytic release of reducing groups from polygalacturonic acid according to the method of Nelson (1994). The reaction mixture contained 108 µl of 0.41% (w/v) polygalacturonic acid in 100 µM Na-acetate buffer (pH 5.0) and 2 µl of CCF. The reaction mixture was incubated at 30°C for 1 h. Galacturonic acid (GA) was used as a standard. A linear relation was obtained between the optical density at 575 nm (OD_{575}) and GA quantities for values ranging between 0.1 and 4 µmoles. One µmol of GA caused an OD_{575} of 0.428. One reducing unit of PG activity catalysed the formation of 1 µmol GA equivalent min^{-1} under the assay conditions described above.

PNL activity was measured by determining the release of unsaturated uronide from pectin according to the method of Zucker and Hankin (1970). The reaction mixture contained 1.475 µl of 0.25% (w/v) cytrus pectin (Sigma) in 100 mM Tris-HCl buffer (pH 8.0), 5 µl of 0.5 M $CaCl_2$ and 20 µl of CCF. In the blanks, CCF was replaced with distilled water. The samples and the relative blanks were incubated at 30°C directly in quartz cuvettes. Since an increase of 1.730 in optical density at 235 nm (OD_{235}) indicated the release of 1 µmole of unsaturated uronide (Zucker and Hankin 1970), one unit of PNL activity catalysed the release of 1 µmole of unsaturated uronides min^{-1} under the assay conditions described above.

All the spectrophotometric assays were performed in a Shimadzu UV-VIS (Kyoto, Japan) recording spectrophotometer mod. UV-160.

Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) and isozyme pattern detection

Patterns of PG, PNL and PME isozymes were separated in non-denaturing isoelectric focusing gels and were detected in ultrathin layer substrate agarose overlays following the methods of Ried and Collmer (1985) with the modifications of Chilosi and Magro (1998).

Ultrathin polyacrylamide gels (total acrylamide concentration, 5.6%; degree of cross-linking, 2.2%; 0.4 mm thickness) were cast for isoelectric focusing (IEF) using a Model 111 IEF-CELL (Biorad, CA, USA) in accordance with manufacturer's instructions.

Polyacrylamide gels containing 5% (v/v) ampholytes (pH 3 to 10) were loaded with 5 ml of pI markers (Biorad, CA, USA) or 2 µl of CCFs. The run was carried out under the following conditions: 15 min at 100 V and 6 mA; 15 min at 200 V and 6 mA; 60 min at 450 V and 4 mA.

After IEF, the polyacrylamide gels were divided into two sections for processing. The lane containing pI markers was immediately stained for proteins using a Biorad Silver stain. The lanes loaded with CCFs were overlaid with ultrathin (0.4 mm) agarose overlay gels containing 1 g l^{-1} polygalacturonic acid and 10 mM EDTA buffered at pH 5.0 with 50 mM Na-acetate for PG activity determination or containing 1 g l^{-1} citrus pectin (Sigma) buffered at pH 8.5 with 50 mM Tris-HCl for both PNL and PME activities determination.

The sandwiched gels were incubated for 60 min at 35°C and 100% RH, then the agarose overlay gels were stained for 10 minutes in a 0.5 g l^{-1} ruthenium red solution, rinsed abundantly with distilled water and dried at 30°C for a few hours.

Bands corresponding to PG and PNL activities were white, as the ruthenium red did not stain where the PG and PNL had degraded the substrate. PME bands appeared as dark red bands, as a consequence of the enhanced binding of ruthenium red with the free acid groups of pectin.

The pI values of the PG, PNL and PME bands were determined from a regression equation of the pI values of markers versus the relative mobility of the same markers forward to the phycocyanin (pI, 4.65) as reference (relative mobility, 0).

There were two replicates for each experiment.

Statistical analysis

Isolates from each of the IG-P, S or F were assumed as individuals belonging to the same population.

Data on fungal growth, pH of CFs, percentage viscosity of medium and PG and PNL activity were analysed by ANOVA using the Graph PAD INSTAT 2 software. Means were compared using Tukey's test (P=0.05).

The pIs of PG, PNL and PME isozymes produced by all the isolates were used to construct a binary matrix of absence/presence (0/1) of each isozyme for all the isolates tested at 3, 6, 9, 13, 16 and 20

days after inoculation. Dendrograms were constructed with unweighted pair-group method averaging (UPGMA) using the SIMQUAL programme of the NTSYS-pc v. 1.60 package and subjected to AMOVA (Excoffier *et al.*, 1992) in order to know the statistical robustness of the variation found.

Results

Fungal growth, pH and viscosity of CFs

The time-course of fungal growth, expressed as mg of dry weight mycelium of isolates from each IG of *H. annosum* is shown in Figure 1. The pH and the % diminution of viscosity of CFs are also reported. Nine days after inoculation, IG-F mycelial growth was significantly more abundant than that of IG-S or IG-P. IG-F produced 34.9±2.6 mg of mycelial mass by day 16, while for IG-P and IG-S maximum mycelial mass was only 22.4+2.2 and 22.5+2.5 respectively at day 20. However, all the IGs reached a plateau between 16 and 20 days after inoculation. In a much the same manner, IG-F caused an enhanced variation of the pH of the CF from day 13. Starting with a pH of 5.1 for the non-inoculated medium, all three IGs first caused a decrease in the pH of the CF after 3, 6 and 9 days, up to values ranging from 3.6 to 3.7. After that, however, the pH curves of IG-P and IG-S continued to remain roughly parallel, reaching, respectively, pH 3.6 and 3.8 on day 13, 4.2 and 4.3 on day 16 and 4.8 and 5.1 on day 20. In contrast, the pH curve caused by IG-F was much higher, reaching pH values of 5.2 on day 13, 5.8 on day 16 and 5.9 on day 20. Differences between the three IGs in ability to degrade pectin by reducing the viscosity of the nutrient medium were less evident. IG-F was always similar to IG-S here, with a high viscosity of the CF at 98.2 and 95.7% by day 3, decreasing drastically to 31.8 and 39.6% by day 6, and more slowly to 11.1 and 13.7% by day 20. IG-S reduced CF viscosity more slowly than the other two IGs, particularly in the logarithmic growth phase; this difference was sta-

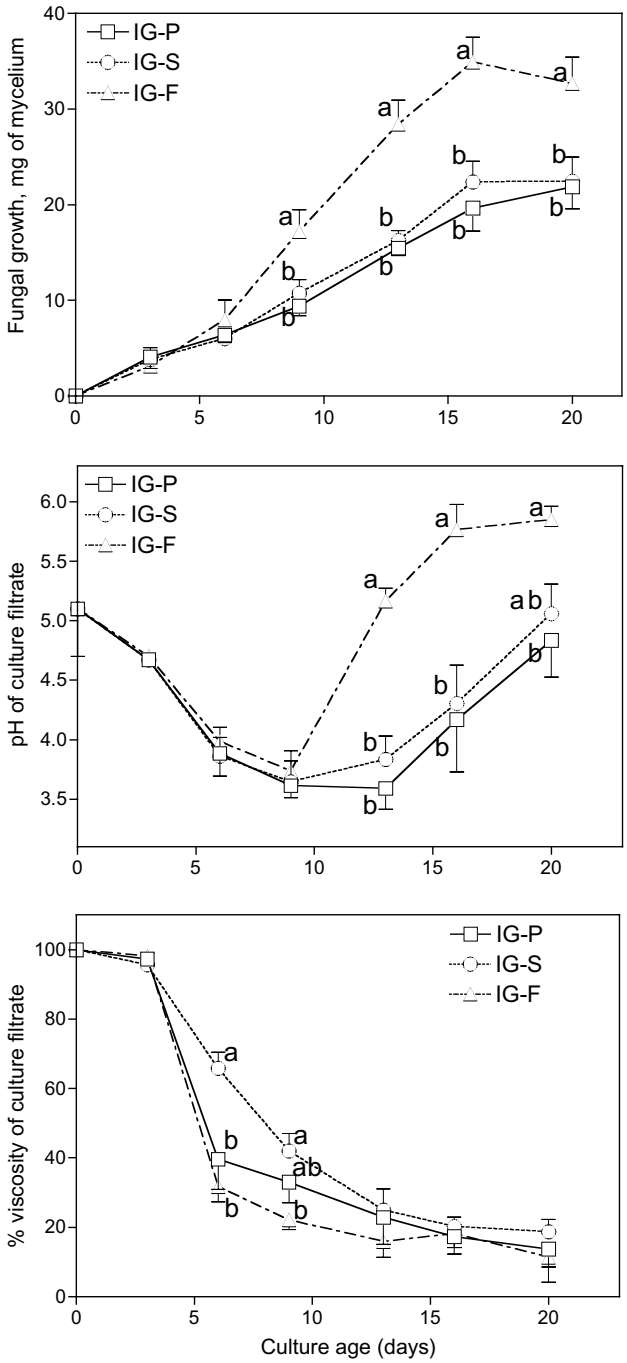

Fig 1. Time-course determination of fungal growth, pH and viscosity of the culture filtrate of the intersterile groups (IGs) P (-□-), S (-○-) and F (-△-) of *Heterobasidion annosum* grown in liquid nutrient medium supplemented with 1% pectin as the sole carbon source. Values are means of 9 isolates of IG-P and IG-S and 8 isolates of IG-F; bars represent SE. Means followed by the same letter at each incubation time do not significantly differ at P=0.05, according to Tukey's test; absence of letters indicates no significant differences among the IGs. Fungal growth is expressed as mg of dry weight biomass. Viscosity of culture filtrate is expressed as the percentage of sample viscosity as compared with the uninoculated control medium.

tistically significant against both IG-P and IG-F on day 6, and only against IG-F on day 9.

PG and PNL assays

Figure 2 shows the time-course of PG and PNL activity produced by IG-P, IG-S and IG-F. PG activity was scarcely produced by all the isolates during the first three days of growth. Starting from day 6, IG-P, IG-S and IG-F began to produce from small to larger amounts of PG activity, ranging from 0.002 units ml^{-1} for IG-S to 0.015 units ml^{-1} for IG-F. For the duration of the experiment, IG-S produced only small amounts of PG, never exceeding a maximum of 0.007 units ml^{-1} (day 13). In contrast, IG-F considerably increased its PG activity from day 6 (0.015 ml^{-1} units), arriving at a plateau of 0.067 and 0.068 units ml^{-1} after 16 and 20 days. IG-P had produced 0.009 units ml^{-1} by day 6, and 0.015 units ml^{-1} by day 9; these levels were at each date mid-way between those produced by IG-F (0.015 and 0.028 units ml^{-1}) and by IG-S (0.002 and 0.006). Later, there was a marked increase in IG-P activity, to 0.020, 0.044 and 0.062 units ml^{-1} after 13, 16 and 20 days of culture respectively. PNL activity began to be appreciably produced by IG-P and IG-F from day 9 (0.002 and 0.002 U ml^{-1} respectively), while IG-S produced measurable PNL activity (0.001 U ml^{-1})only from day 13. PNL activity produced by IG-S was barely noticeable even at 16 and 20 days from inoculation (about 0.001 U ml^{-1}). The greatest PNL activity was produced by IG-P, with levels of 0.043 and 0.050 U ml^{-1} after 16 and 20 days of growth. IG-F tended to display PNL activity values mid-way between those of IG-P and IG-S at 13 (0.005 U ml^{-1}) and 16 days (0.021 U ml^{-1}) from inoculation, even though on day 20 PNL activity (0.013 U ml^{-1}) was not statistically different from that registered by IG-S.

Figure 3 shows the regression lines of PG and PNL activities produced by IG-P, IG-S and IG-F against the mycelial mass dry weight measured at the various observation dates. For every mg of mycelial mass dry weight, IG-P and IG-F produced about 11 and 8 times more PG activity than IG-S. IG-P and IG-F produced respectively 63 and 5 times more PNL activity than IG-S. When the specific PG activities were examined, substantial differences only existed between IG-P and IG-F, whereas in the case of specific PNL activityes IG-F behaved as IG-S.

IEF-PAGE and isozyme patterns

Typical isozyme patterns of several isolates belonging to the IGs F, P and S are shown in Figure 4 (PG activity: white bands) and in Figure 5 (PNL activity: white bands, PME activity: dark red bands). Tables 2, 3 and 4 sum up - for PG, PNL and PME activities - the isoelectrophoretic forms displayed at 3, 6, 9, 13, 16 and 20 days from inoculation of isolates on the medium.

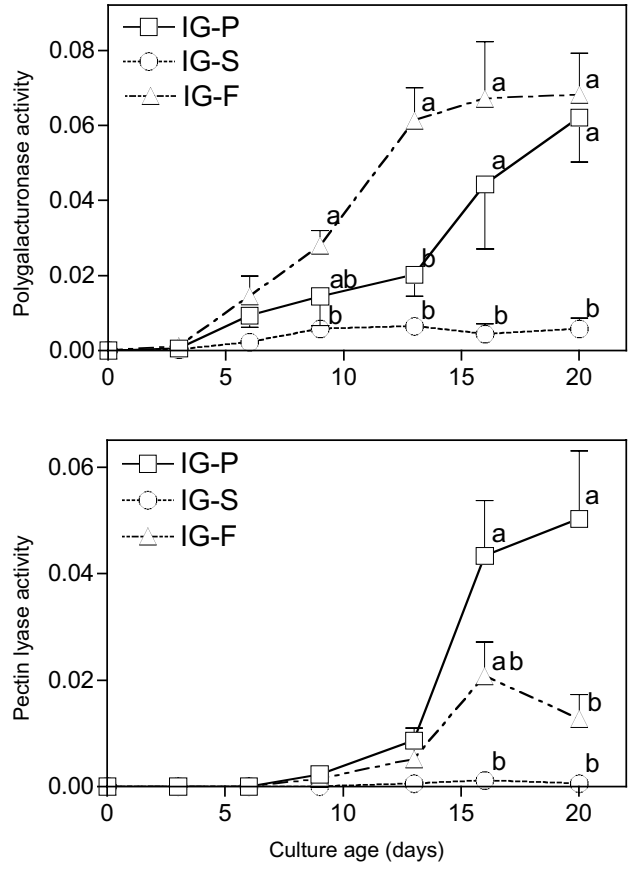

Fig. 2. Time-course determination of polygalacturonase and pectin lyase activities produced by the intersterile groups (IGs) P (-□-), S (-○-) and F (-△-) of *Heterobasidion annosum* grown in liquid nutrient medium with 1% pectin as the sole carbon source. Polygalacturonase activity is expressed as µmoles of galacturonic equivalents min^{-1}; pectin lyase activity as µmoles of unsaturated uronides min^{-1}. Values are means of 9 isolates of IG-P and IG-S and 8 isolates of IG-F; bars represent SE. Means followed by the same letter at each incubation time do not significantly differ at P=0.05, according to Tukey's test; the absence of letters indicates no significant difference among the IGs.

With regard to the PG (Table 2), it was noted that, while IG-S displayed a single band with pI 4.7 from day 6, IG-P and IG-F presented 5 and 6 isozymes (from pI 4.0 to pI 8.7), of which 4 were shared. IG-P presented three neutral bands from the earliest culture periods (one band with pI 7.3 from day 3, one band with pI 7.0 from day 6 and one band with pI 6.5 from day 9), whereas two acid isozymes with pIs of 4.0 and 4.7 were consistently detected as intense bands on IEF gels from day 9. At all these culture periods, IG-F displayed an acid band with pI 4.7, which was very intense from day 9; in addition, a band with pI 4.0 was present on day 6. Neutral and/or basic bands with pIs of 8.7, 7.7 and 7.3 were present

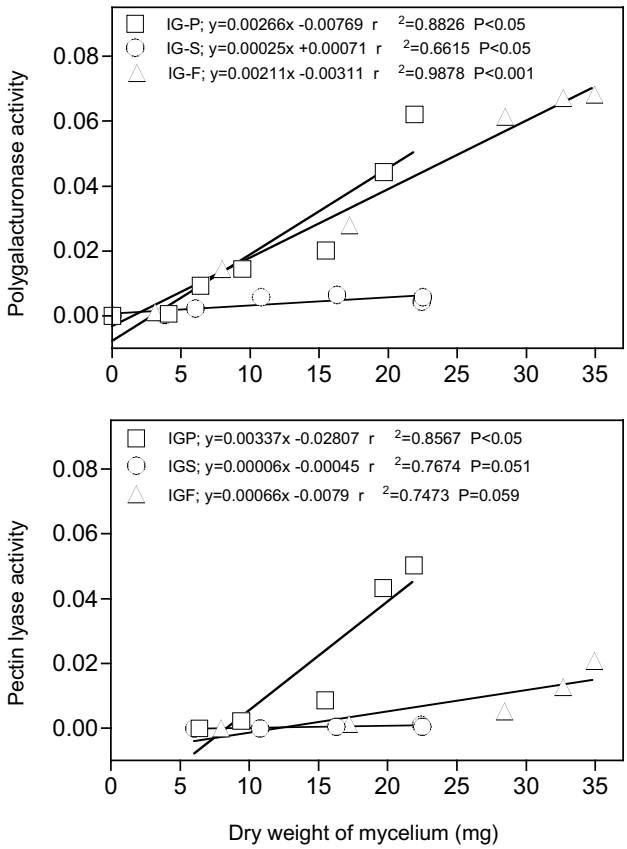

Fig. 3. Relation between growth, expressed as mg of dry weight mycelial mass, and polygalacturonase and pectin lyase activity, expressed as reducing units and units per ml culture filtrate respectively, by the intersterile groups (IGs) P (-□-), S (-○-) and F (-△-) of *Heterobasidion annosum* grown in liquid nutrient medium with 1% pectin as the sole carbon source.

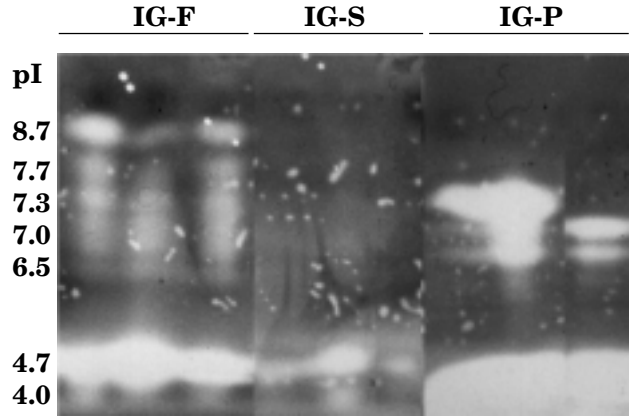

Fig. 4. Isoelectric focusing (IEF) zymograms of polygalacturonase (PG) produced by different isolates of the intersterile groups (IGs) F, S and P of *Heterobasidion annosum* on day 16 from inoculation in a liquid medium containing pectin as the sole carbon source. Samples (2 ml of 50-fold concentrated culture filtrate) were separated by broad-range analytical IEF. PG activity, detected by the activity stain overlay technique at pH 5, appear as white bands. The pI values of bands are indicated on the left.

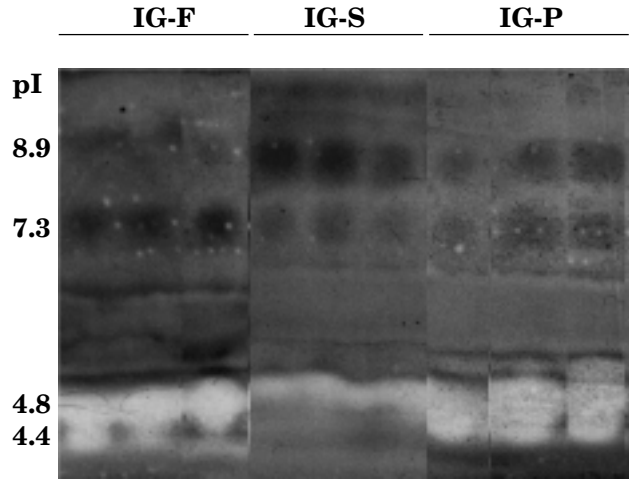

Fig. 5. Isoelectric focusing (IEF) zymograms of pectin lyase and pectin methylesterase produced by different isolates of the intersterile groups (IGs) F, S and P of *Heterobasidion annosum* on day 16 from inoculation in a liquid medium with pectin as the sole carbon source. Samples (2 ml of 50-fold concentrated culture filtrate) were separated by broad-range analytical IEF. PNL and PME activities, detected by the activity stain overlay technique at pH 8, appear as white and dark red bands respectively. The pI values of bands are indicated on the left.

Table 2. Extracellular polygalacturonase isozymes produced by the intersterile groups (IGs) P, S and F of *Heterobasidion annosum* at various days from inoculation in a liquid growth medium containing pectin as the sole carbon source.

pI	IG-P						IG-S						IG-F					
	3	6	9	13	16	20	3	6	9	13	16	20	3	6	9	13	16	20
4.0	-	-	++	++	++	++	-	-	-	-	-	-	-	+	-	-	-	-
4.7	-	-	++	++	++	++	-	+	+	+	+	+	+	+	++	++	++	++
6.5	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
7.0	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-
7.3	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+
7.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
8.7	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+

+, presence of bands.
++, presence of intense bands.
-, absence of bands.

Table 3. Extracellular pectin lyase isozymes produced by the intersterile groups (IGs) P, S and F of *Heterobasidion annosum* at various days from inoculation in a liquid growth medium with pectin as the sole carbon source.

pI	IG-P						IG-S						IG-F					
	3	6	9	13	16	20	3	6	9	13	16	20	3	6	9	13	16	20
4.4	-	+	+	++	++	++	-	-	-	-	-	+	-	-	-	+	+	+
4.8	-	+	++	++	++	++	-	-	+	+	+	+	-	+	+	++	++	+
7.0	-	-	+	+	++	++	-	-	-	-	-	+	-	-	-	-	-	-

+, ++, - see Table 2.

from day 6 to day 20. The band with pI 7.0 only appeared on day 16.

Table 3 shows the patterns of the PNL isozymes produced by IG-P, IG-S and IG-F. These patterns of isozymes produced by the three groups are not particularly complex, even though each is different from the others. IG-P had from day 6 a band with pI 4.4, which became more intense from day 13 and, a band with pI 4.8, which became more intense from day 9. A band with pI 7.0 was present from day 9, and became intense from day 16. IG-F had only two acid bands, one with pI 4.8 from day 6 and very intense on days 13 and 16 and one with pI 4.4, present from day 13 but with a consistently low intensity. The isozyme for IG-S was banded at pI 4.8 from day 9; two other bands, with pI 4.4 and 7.0, appeared only on day 20.

Table 4 shows the pattern of PME isozymes of IG-P, IG-S and IG-F. Four acid isozymes, with pI 3.7, 4.6, 4.9 and 5.1, appeared in all three groups but only during the earlier growth phases: on days 3 and 6 for IG-S and IG-F, and on days 3, 6 and 9 for IG-P. The isozyme with pI 3.7 often appeared as an intense band. The neutral and basic isozymes with pI 7.3 and 8.9 appeared with IG-P from day 13, and with IG-S from days 6 and 3 respectively. IG-F displayed the isozyme with pI 8.9 only on day 3, as an intense band, and the isozymes with pI 7.3 from day 13.

Since not all the isolates from each IG produced all the isozymes shown in Tables 2, 3 and 4, variation within IGs was studied by means of a binary matrix and a dendrogram constructed with the UPGMA method (Fig. 6). The isozyme patterns of

Table 4. Extracellular pectin methylesterase isozymes produced by the intersterile groups (IGs) P, S and F of *Heterobasidion annosum* at various days from inoculation in a liquid growth medium with pectin as the sole carbon source.

pI	IG-P						IG-S						IG-F					
	3	6	9	13	16	20	3	6	9	13	16	20	3	6	9	13	16	20
3.7	++	++	+	-	-	-	++	++	-	-	-	-	++	+	-	-	-	-
4.6	+	+	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-
4.9	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
5.1	-	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-
7.3	-	-	-	+	+	+	-	+	+	+	+	-	-	-	-	+	+	+
8.9	-	-	-	+	+	+	++	+	+	+	+	+	++	-	-	-	-	-

+, ++, - see Table 2.

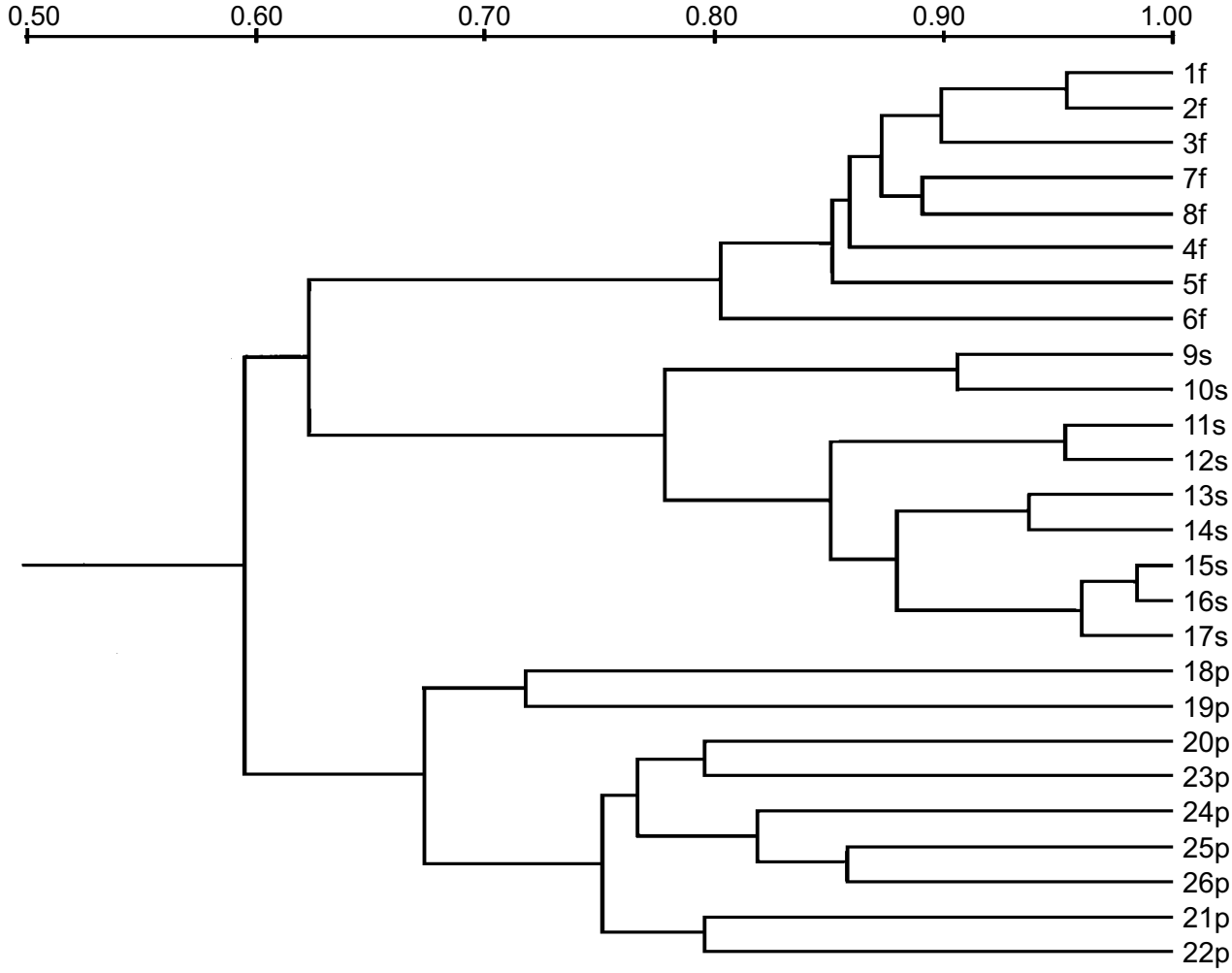

Fig. 6. UPGMA dendrogram based on all isozymes of polygalacturonase, pectin lyase and pectin methylesterase produced by the isolates belonging to the intersterile groups P, S and F of *Heterobasidion annosum* at 3, 6, 9, 13, 16 and 20 days of culture in a growth medium with pectin as the sole carbon source.

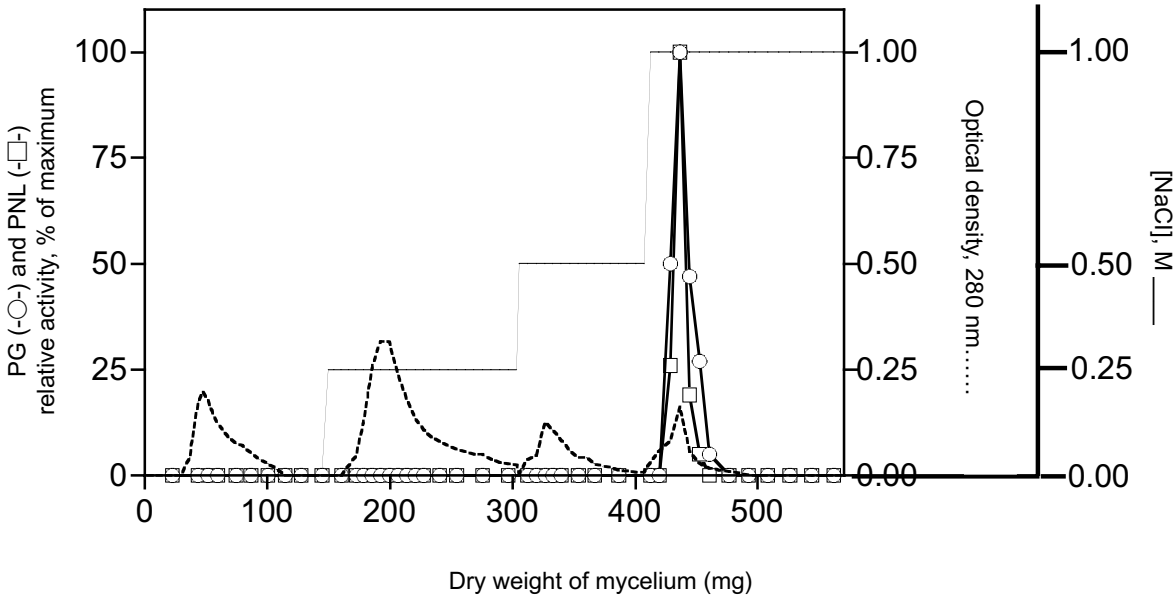

Fig. 7. Elution profile of the desalted concentrated culture filtrate of *Heterobasidion annosum* intersterile group S, strain 931007 (2.4) grown in liquid growth medium with 1% pectin as the sole carbon source from an Ion exchange chromatography column eluted with a discontinous gradient of NaCl; the polygalacturonase (PG) and pectin lyase (PNL) relative activities are expressed as percentage of maximum.

PG, PNL and PME activities at various days of culture yielded a total of 64 bands: 32 for PG, 14 for PNL and 18 for PME. Fifty bands were detected in IG-P (23 for PG, 14 for PNL and 13 for PME), 35 in IG-F (19 for PG, 8 for PNL and 8 for PME), and 28 in IG-S (6 for PG, 6 for PNL and 16 for PME). Three statistically different ($P<0.05$) groups of isolates were found, corresponding to the three IGs of *H. annosum*.

Figure 7 shows the elution profile of a desalted concentrated solution starting from the 13-day-old CF of *H. annosum* IG-S strain 931007 (2.4) from an Ion Exchange DEAE Sephadex A-50 column. PG activity was eluted together with the PNL activity in the NaCl 1M fractions. The IEF zymogram of the NaCl 1M fractions confirmed the presence of the PG pI 4.7 and the PNL pI 4.8 bands (data not shown).

Discussion

This work reports on the activity of PG and PNL produced by numerous isolates from the IGs P, S and F of *H. annosum* after varying days of growth on a medium with pectin as the only source of carbon. The behavior of each IG was easily distinguished from the others. The PG and PNL activities of IG-S were always low. PG activity of IG-F was higher than that of IG-P, but PNL activity was not. Differences between IGs also emerged in mycelial growth, pH and percentage viscosity of the CF. IG-F grew faster than the other IGs and caused earlier alkalization of the CF, from day 13. Regarding the percentage viscosity of the culture filtrate, IG-S displayed a lesser ability to decrease the viscosity of medium on days 6 and 9 of culture. Specific PG and PNL activities, obtained by comparing the enzymatic activity with the mass of dry mycelium produced on various days of growth, indicated that IG-F displayed a non-uniform behavior, with specific PG activity similar to that of IG-P, but specific PNL activity similar to that of IG-S. Our data agreed with Johansson (1988) as regard mycelial growth values, pH and viscosity of the CF of IG-P and IG-S. In addition, our data included an examination of IG-F and extended all observations until day 20 of culture, a time period required for the three IGs

to reach their plateau of growth. Johansson (1988) found that IG-P caused a significantly greater loss of viscosity in the culture liquid per mg of mycelial mass produced than IG-S did. Our results extended the analysis to IG-F and detected PG and PNL enzymatic activity as a direct parameter measuring the ability of the isolates to degrade pectic substances.

In our study, we separated out the pectinolytic activityes by means of non-denaturing IEF-polyacrylamide gel electrophoresis, and detected the PG and PNL enzymes in ultrathin layer substrate agarose overlays containing, respectively, polygalacturonic acid buffered at pH 5.0 and pectin 8.5. This is a technique that has been widely used (Di Pietro and Roncero, 1996a; Di Pietro and Roncero, 1996b; Chilosi and Magro, 1997; Snape *et al.*, 1997; Zhang *et al.*, 1997; Kollar, 1998). Most of the isolates used in the present study produced multiple forms of PG and PNL. The method employed to stain the agarose overlay gels, after processing the IEF polyacrylamide gels, allowed us also to highlight the PME isozymes, which appeared as dark red bands.

The time-course study of PG, PNL and PME isozymes produced by all the isolates from all the IGs of *H. annosum* suggested that the isozymatic patterns sufficiently characterized each IG. However, this patterns were very strongly influenced by duration of the culture period. In the case of PG isozymes, IG-P produced neutral isozymes, with pI 7.3 from day 3 and pI 7.0, from day 6 of culture respectively, while its acid isozymes were only detected after 9 days of culture. In contrast, IG-F produced an acid isozyme, pI 4.7, from the very first days of growth and other PG isozymes (pI 8.7 and pI 7.3 and 7.7) from days 6 and 9. In the case of PME isozymes, the acid bands pI 3.7, 4.6, 4.9 and 5.1, were detected only in the first 6 days, with the exception of the isozyme pI 3.7, which was also detected in IG-P after 9 days of culture. The isozymes pI 7.3 and/or pI 8.9 were the only ones detected after 9 and 13 days of culture, even though they were already present in IG-S and IG-F after just three days of culture. The differences in pectinolytic enzymes detected after various days of culture confirmed the adaptability of *H. annosum* to a wide range of hosts. Our results were in agreement with Errampalli and Kohn (1995), who detected two PG isozymes, pI 4.85 and 5.1, after 1 day in a culture of *Sclerotinia sclerotiorum,* and an additional PG isozyme after 2 days. Also, Guevara *et al.* (1997) found that the number of PG, pectate lyase and PNL isozymes increased with the culture age of *Fusarium oxysporum* f. sp. *radicis lycopersici*. Frequently, not all the isozymes produced by plant pathogens grown in culture are detected in the extracts from spores and/or plant tissues colonized by them (Gao *et al.*, 1996; Centis *et al.*, 1997; Zhang *et al.*, 1997; Chilosi and Magro, 1998).

The PG isozymatic pattern of IG-S was much simpler than that of IG-P or IG-F. With regard to the isozymatic profiles of PNL, only on day 20 of culture did IG-S present more than one isoform. Bands of PG and PNL displayed by IG-S were always weaker and sometimes barely visible. Only a few isozymes were specific to one IG (PG isozyme pI 6.5 to IG-P; PG isozymes pI 7.7 and 8.7 to IG-F); some occurred in 2 IGs (for example, PG pI 4.0, 7.0 and 7.3 in IG-P and IG-F; PNL pI 7.0 in IG-P and IG-S, PME pI 4.8 in IG-P and IG-S). Among the PG isozymes, only one, with pI 4.7, occurred in all the IGs; in IG-S it was the only band. With the PNL and PME isozymatic patterns, isozymes found in all three IGs were more frequent. AMOVA analysis showed that variation among IGs was statistically significant, while variation within them was not.

Karlsson and Stenlid (1991) had separate PG and PME enzymes by electrophoresis at a constant pH of 8.7, incubating the pectin-acrylamide gels in 0.1 M malic acid; they thus differentiated the isozymes on the basis of their relative mobility, but they did not detect the PNL isozymes. Johansson (1988) and Karlsson and Stenlid (1991) suggested that the moderate pectinolytic activity of IG-S, compared with the strong PG and PNL activity produced by IG-P, might result in a less efficient degradation of host pectin, and consequently reduced pathogenicity. Another hypothesis was that the difference of these IGs was due to the fact that IG-P strains grow in the pectin-rich cambial zone of pine trees, where IG-S strains have not been observed.

In the present paper we showed that IG-F, which has a host range between IG-S and IG-P, differed from the other two IGs in most of the parameters considered. In the nutrient medium with pectin as the sole carbon source IG-F grew, more abundantly and faster than IG-P and IG-S. PG activity shown by IG-F per unit of mycelial mass was similar to

that shown by IG-P, but different from that of IG-S; by contrast, specific PNL activity of IG-F was comparable to that of IG-S, but not to that of IG-P. In the isozymatic patterns obtained at various days of culture, IG-F was also clearly distinct from IG-P and IG-S. All these considerations strengthen the proposal of Niemela and Korhonen (1998) to raise the three IGs to the status of species. Moreover, a role for pectinolytic enzymes in causing *H. annosum* virulence cannot be excluded; the data of the present study suggest that IG-S is a good candidate for studies on mutagenesis involving the destruction of the gene that codifies for the isozymes PG pI 4.7 or PNL pI 4.8, which were the only PG and PNL isozymes produced in culture by this particular IG in the first 16 days from inoculation. The first step in the purification of the PG pI 4.7 and PNL pI 4.8 bands, reported in this paper, opens the way to a greater understanding of the role of these enzymes in the root rot disease caused by *H. annosum*.

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