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# Variations in the molecular and physiological characteristics and the virulence of *Monilinia fructicola, M. fructigena* and *M. laxa* isolates

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**Summary.** Twenty-three *Monilinia* isolates of various origin were identified and compared by morphological means and by biomolecular protocols based on random amplified polymorphic DNA (RAPD) analysis, and on PCR detection by species-specific primer pairs. The identification of most *Monilinia* isolates by PCR and RAPD confirmed the identification by morphological criteria, although two isolates initially identified as *M. fructigena* by morphological means were attributed to *M. fructicola* by the biomolecular techniques. *Monilinia* isolates were also compared in terms of their *in vitro* pectolytic activity and isoenzyme patterns. All the isolates produced polygalacturonase and pectin methylesterase, but pectin lyase activity was found only in *M. fructicola* and *M. fructigena,* and not in most *M. laxa* isolates. The *Monilinia* isolates differed significantly in their isoenzyme patterns. Based on RAPD-PCR and PCR amplification and pectolytic isoenzymes, the isolates analysed clustered in three major groups, each corresponding to a *Monilinia* species. Artificial inoculations in peach and pear fruits revealed differences in virulence between the *Monilinia* species and within isolates. No correlation was found between the amount of pectolytic enzymes produced *in vitro* and virulence. Variations between and within the *Monilinia* species may depend at the physiological level on variations in the isoenzyme patterns of the pectolytic enzymes.

**Key words**: brown rot, pectin lyase, PCR-RAPD, pectin methylesterase, polygalacturonase.

## Introduction

*Monilinia fructicola* (Winter) Honey, *M. fructigena* (Aderhold and Ruhland) Honey and *M. laxa* (Aderhold and Ruhland) Honey are causal agents of brown rot of stone and pome fruits with various symptoms including blight of twigs, blossoms and leaves, and fruit rot. Pectolytic enzymes are the main plant cell wall degrading components of these fungi and were reported to play an important role in pathogenicity (Byrde and Willetts, 1977). Pectolytic enzymes include various isoforms of polygalacturonase (PG), pectin methylesterase (PME) and pectin lyase (PNL) ( Snape *et al*., 1997; Willetts *et al*., 1977).

*Monilinia fructigena* and *M. laxa* occur widely in Europe, while *M. fructicola* is found mainly in America, Australia and South Africa. *M. fructicola* is listed as a quarantine disease within the EU since it was thought to be absent from the EU area; but recently it has been detected in Austria and

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France (OEPP/EPPO, 2003). The three *Monilinia* species are commonly distinguished by cultural and morphological characteristics (Byrde and Willetts, 1977; Mordue, 1979). However, *Monilinia* species identification by morphology is unreliable because of many differences between isolates from the same species, and an overlap of colony morphology between species (Ogawa and English, 1954; Penrose *et al*., 1976; Byrde and Willetts, 1977). More recently, techniques based on biochemical and molecular approaches as well as on morphological and quantitative characteristics have increased knowledge about variations between isolates and have improved the identification of the brown rot fungi of pome and stone fruits (Fulton and Brown, 1997; van Leeuwen and van Kesteren, 1998; Belisario *et al*., 1999; De Cal and Melgarejo, 1999; Fulton *et al*., 1999; Snyder and Jones, 1999; Förster and Adaskaveg, 2000; Hughes *et al*., 2000; Ioos and Frey, 2000; Boehm *et al*., 2001; Lane, 2002; Ma *et al*., 2003; Côté *et al*., 2004). Despite the large body of information provided by these studies however, there is still a lack of knowledge about the physiology of brown-rot *Monilinia* species as regards their pectolytic activity and isoenzyme patterns on the one hand, and about how these traits are related to variations between, and the virulence of isolates on the other.

The aim of this study was to compare a set of *Monilinia* isolates between and within each other so as to determine i) variations according to morphological criteria and biomolecular protocols based on random amplified polymorphic DNA (RAPD) analysis and on PCR detection by species-specific primers; ii) the pectolytic activity and the specific isoenzyme patterns produced *in vitro*, as physiological components of pathogenicity and as a means for grouping isolates; iii) the host preference and virulence of isolates.

## Materials and methods

### Fungal isolates

For each of the three *Monilinia* species, a set of isolates of different origin was used (Table 1). Fungal cultures were maintained on potato-dextrose agar (PDA) (Oxoid, Unipath Ltd, Basingstoke, England). A preliminary identification of the *Monilinia* species was carried out using morphological criteria (Corazza *et al*., 1998). Most of the isolates were also included in a previous study in which total mycelium protein profiles were compared to distinguish *Monilinia* species (Belisario *et al*., 1999).

### DNA extraction

Isolates were grown on PDA for 7 days at 25°C in the dark. For each isolate, 7-mm-diameter plugs were transferred to flasks containing potato-dextrose-broth (PDB) (Sigma Chemical Co, St. Louis, MO, USA) and incubated statically at room temperature (20°C) for 5 days.

For each isolate, mycelium was collected by filtration using two layers of cheesecloth, then frozen at -80°C for 2 days, freeze-dried and ground in liquid nitrogen to produce a fine powder (Leclerc-Potvin *et al*., 1999).

The DNA extraction method used was according to the protocol of Lee and Taylor (1990). Briefly, 35–55 mg of freeze-dried mycelium-powder was ground with 600 $\mu$l of lysis buffer (50 mM Tris-HCl, pH 7.2; 50 mM $Na_2EDTA$, pH 8.0; 3% (w:v) SDS; 1% (v:v) 2-mercaptoethanol) and incubated for 60 min at 65°C. Then the DNA was purified by phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v) and chloroform/isoamyl alcohol (24:1 v:v) extraction, precipitated with cold isopropanol and centrifuged (12,000×*g*) for 5 min. The DNA pellet was washed with 70% ethanol, vacuum dried and suspended in 100 $\mu$l TE buffer.

The DNA extracted was stored at -20°C. The DNA was quantified by comparison of DNA with standards by agarose gel electrophoresis. All DNA template amounts were based on gel estimates.

### Amplification conditions for RAPD

RAPD amplification was carried out in a total volume of 25 $\mu$l containing 2.5 $\mu$l of 10× buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl; Pharmacia Biotech, Uppsala, Sweden), 1 unit of red-*Taq*-polymerase (Sigma), 0.2 $\mu$M of each dNTP (Pharmacia Biotech), 0.2 $\mu$M of ten-mer-primers and approximately 10 ng of template DNA.

Thirteen 10-mer oligonucleotides were randomly chosen from among the series OPA, OPB, OPL and OPAN (Operon Technologies, Alameda, CA, USA) and tested as primer sequences. Eight primers (OPA 2, OPB 15, OPL 1, OPL 4, OPL 12, OPAN 3, OPAN 7, OPAN 11) were selected as generating the 145 most informative markers, and as being

Table 1. *Monilinia* isolates analysed. Species identification was by morphological criteria.

| ISPaVe Isolate | Origin | Host | *Monilinia* species |
|---|---|---|---|
| 997 | New Zealand | *Prunus persica* | *M. fructicola* |
| 1000 | New Zealand | *P. domestica* | *M. fructicola* |
| 1001 | Australia | *P. persica* | *M. fructicola* |
| 1002 | Australia | *P. avium* | *M. fructicola* |
| 1006 | Japan | *P. persica* | *M. fructicola* |
| 1258 | Japan | *Malus pumila* | *M. fructicola* |
| 1268 | USA | *P. domestica* | *M. fructicola* |
| 1273 | Japan | *P. persica* | *M. fructicola* |
| 989 | Spain | *P. domestica* | *M. fructigena* |
| 990 | Spain | *P. persica* | *M. fructigena* |
| 991 | Spain | *P. domestica* | *M. fructigena* |
| 995 | Spain | *P. persica* | *M. fructigena* |
| 1270 | Portugal | *Cydonia* sp. | *M. fructigena* |
| 1271 | Japan | *M. pumila* | *M. fructigena* |
| 1274 | Poland | *P. domestica* | *M. fructigena* |
| 516 | Italy | *P. avium* | *M. laxa* |
| 893 | Italy | *P. persica* | *M. laxa* |
| 953 | Italy | *P. persica* | *M. laxa* |
| 983 | Spain | *P. armeniaca* | *M. laxa* |
| 987 | Spain | *P. persica* | *M. laxa* |
| 988 | Spain | *P. persica* | *M. laxa* |
| 993 | Portugal | *P. domestica* | *M. laxa* |
| 1261 | Spain | *P. armeniaca* | *M. laxa* |

the most suitable to distinguish between *Monilinia* isolates.

RAPD amplification was conducted in a Gene AMP PCR System 9600 (Applera, Norwalk, CT, USA) programmed for initial denaturation at 94°C for 1 min followed by 40 cycles at 94°C for 1 min, 37°C for 1 min and 72°C for 2 min, and with a final elongation step at 72°C for 10 min.

Ten $\mu$l of each amplification product was resolved by electrophoresis on agarose (1.5%) gel, stained with ethidium bromide, and gel images were acquired with a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA , USA). In all electrophoresis tests, a 1 Kb DNA ladder (GIBCO-BRL, Inchinnen, Scotland) was used as a molecular size standard. All experiments were repeated at least two times.

**Molecular diagnosis of *Monilinia* species based on species-specific primer pairs**

The sequences of the rDNA internal transcribed spacer (ITS) as species-specific PCR primer pairs used for each of the three *Monilinia* species and the cycling profiles were those described by Ioos and Frey (2000), except for the annealing temperature, which, as recommended by the authors, was raised to 65°C in order to minimise the cross-reactions observed at lower annealing temperatures.

**Fungal cultures for enzyme extraction**

For enzyme extraction, isolates were surface cultured in Czapek's liquid medium (pH 5.0) containing $NaNO_3$ (2 g $l^{-1}$), $KH_2PO_4$ (1 g $l^{-1}$), $MgSO_4 \times 7H_2O$ (0.5 g $l^{-1}$), KCl (0.5 g $l^{-1}$), $FeSO_4 \times 7H_2O$ (0.01 g $l^{-1}$), $ZnSO_4 \times 7H_2O$ (0.01 g $l^{-1}$) and 5 g $l^{-1}$ of citrus pectin (Sigma, P-9135) as the sole carbon source. The inoculum was one agar disc (6 mm diameter) cut from the edge of 5-day-old cultures on PDA. The cultures were grown statically at 22°C in 250 ml Erlenmeyer flasks containing 50 ml medium.

Mycelium from 14-day-old cultures of each isolate, from three independent experiments, was removed by filtration using a Büchner funnel and utilised for mycelial d wt determination. The culture filtrates were centrifuged at 15.000 *g* for 15 min at 4°C and the supernatants were dialysed

against several changes of distilled water at 4°C.

**Pectolytic enzyme assays**

Polygalacturonase activity was determined as the increase in reducing end-groups over time. Reducing end-groups were measured by the method of Nelson (1944), using D-galacturonic acid (Sigma) as a standard. Activity was expressed as reducing units (RU). One RU was defined as the amount of enzyme that at 30°C produced 1 $\mu$mol of reducing groups $min^{-1}$ d wt $g^{-1}$ from 0.25% (w:v) polygalacturonic acid (PGA) (Sigma) or pectin (Sigma) from citrus in Na-acetate buffer (0.1 M, pH 5.0). PNL activity was determined spectrophotometrically by measuring the increase of absorbance at 235 nm. An increase in absorbance of 1.73 indicated the formation of 1 $\mu$mol of unsaturated uronide (Zucker and Hankin, 1970). One unit of PNL enzyme activity (U) catalysed the formation of 1 $\mu$mol of unsaturated uronide $min^{-1}$ d wt $g^{-1}$ from 0.25% (w:v) citrus pectin (Sigma) in Tris-HCl buffer (0.1 M, pH 8.0) at 30°C. PME activity was monitored spectrophotometrically by the method of Hagerman and Austin (1986) at 615 nm using bromocresol green as pH indicator. One PME Unit (U) was the amount of enzyme that released 1 $\mu$mol $H^+$ ions $min^{-1}$ d wt $g^{-1}$ at 30°C from 0.5% (w:v) citrus pectin (Sigma) solution at pH 5.5.

Isoenzyme separation by isoelectric focusing (IEF) was performed horizontally on a Multiphor II apparatus (Pharmacia Biotech) using 0.4-cm-thick polyacrylamide gels containing 5% (v:v) ampholytes (Pharmacia Biotech) covering the pH range 3.5–10.0 or 4.0–6.0. The same amount of total protein was loaded for each extract. The gels were run at a constant power of 5 W for 1.5 h. After IEF, gels were overlaid with ultra-thin (0.4 mm) agarose gels prepared as described by Ried and Collmer (1985). For PG isoenzymes detection, a 1% (w:v) agarose gel containing 0.1% (w:v) PGA and 10 mM EDTA was buffered at pH 5.0 with 50 mM Na-acetate. For PNL detection, 1% (w:v) agarose gel containing 0.1% (w:v) pectin (Sigma) in 50mM Tris-HCl buffer, pH 8.0 was used. For PME isoenzyme detection, 1% (w:v) agarose gel containing pectin (Sigma) 0.1% (w:v) with high degree of esterification (93%) was buffered at pH 5.0 with 50 mM Na-acetate. IEF polyacrylamide gels overlaid with ultra-thin agarose gels were incubated at 100% humidity for 30 to 60 min at 30°C. Activity bands were visualised by staining the agarose overlay in 0.5 g $l^{-1}$ ruthenium red (Sigma) solution for 10 min, followed by rinsing in distilled water. PNLs or PGs appeared as white bands, whereas PMEs appeared as dark-red bands. The pI values of pectolytic isoenzymes were estimated from a regression equation of standard proteins (Pharmacia Biotech) versus the distance of migration.

**Host inoculation and disease assessment**

Host preference and virulence were determined by inoculating each isolate on peach (cv. Springbell) and pear (cv. Williams) fruits at commercial maturity. Before inoculation, fruits were surface-disinfected with 1% sodium hypochlorite. Three peach and pear fruits were inoculated on opposite sides with 6-mm-diameter and 2-mm-thick PDA plugs containing mycelium from the edge of a five-day-old culture of each isolate. The inoculum was inserted into holes (around 2 mm deep) made on the fruits with a 6 mm cork borer. Control fruits were inoculated with sterile PDA discs. Inoculated fruits were incubated in the dark at 22°C. The disease was assessed 72 h after inoculation as mean diameter length (mm) of the lesions on the inoculated fruit.

**Statistical analysis**

Isoenzyme patterns of polygalacturonase, PME and PNL were used to evaluate the relationships between the *Monilinia* isolates. A binary matrix was generated and analysed by the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc version 1.70/1992, Applied Biostatistics Inc., Setauket, NY, USA). Similarity relationships were calculated between all pairs of isolates using the following formula: S=2a/(2a+b+c), where "S" is the Dice coefficient (Dice, 1945), "a" is the common bands between two isolates, and "b" and "c" are the unique bands in each of the two isolates respectively. Similarity values were subjected to phenetic analysis using the unweighted pair-group arithmetic average method (UPGMA) (Sneath and Sokal, 1973).

Of the profiles of amplicons obtained by the eight most informative RAPD primers, those obtained by the same primers were scored as identical. The data were used to calculate average taxonomic distance coefficients by the formula of Dice

(1945). A dendrogram was constructed by the UPGMA using the NTSYS-pc.

Data on PG, PME and PNL activity and those on the disease assays were subjected to analysis of variance. Average enzyme activity data were distinguished by the Scheffè test at $P$=0.05.

## Results

**RAPD characterisation**

The profiles generated by the genomic DNAs of isolates using the eight selected primers were different for each *Monilinia* species. Each primer amplified reproducible fragments, with faint or ambiguous bands being excluded from the analysis (Fig. 1). *M. fructicola, M. laxa* and *M. fructigena* possessed a substantial homogeneity with each other, clustering with a similarity coefficient of 0.81, 0.77 and 0.57 respectively (Fig. 2). By cluster analysis, isolates 990 and 991, that had been attributed to *M. fructigena* by morphological characterisation, were clearly included in the *M. fructicola* group, as shown by the profile generated by each deca-primer used in the analysis (OPAN3) (Fig. 1 and 2).

**Comparison of *Monilinia* isolates by PCR using ITS primer pairs**

Three specific primer pairs (Ioos and Frey, 2000) were tested with DNA extracted from the *Monilinia* isolates. The specificity of the primers was confirmed by the analysis of both *M. fructigena* and *M. laxa*. Each isolate yielded one amplification product with only the specific primer pairs (Fig. 3b, c), apart from one faint band generated by *M. fructicola* isolates 1268 and 1273. A fairly large number of cross-reactions with both *M. fructigena* and *M. laxa* were found when isolates were analysed with *M. fructicola* primer pairs (Fig. 3a). By PCR primer pair analysis, isolates 990 and 991 were clearly not related to *M. fructigena*, but showed a clear amplification product when the specific primers for *M. fructicola* were used.

**Quantitative pectolytic enzymes production**

A liquid mineral medium at pH 5.0 supported consistent fungal growth and pectolytic enzyme production (Table 2). In the cultural conditions described, all the *Monilinia* isolates produced both PG and PME. PNL activity was found in most of *M. fructicola* and *M. fructigena* isolates but not in

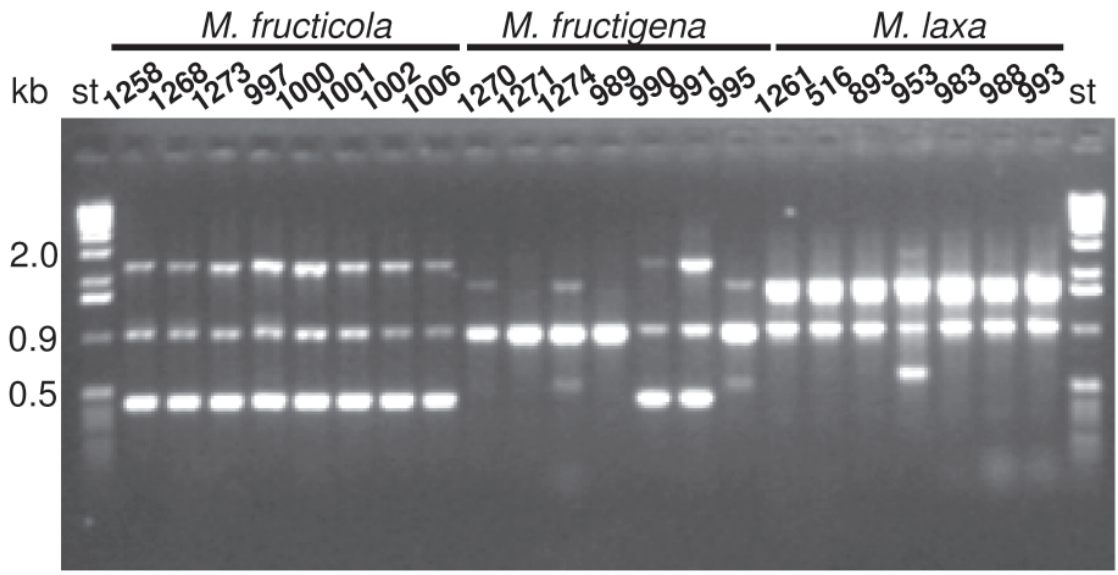


Fig. 1. RAPD analysis of *Monilinia fructicola, M. fructigena* and *M. laxa* isolates previously identified on the basis of morphological criteria using the random 10-mer oligonucleotide OPAN 3. Isolate 987 of *M. laxa* was not included in the present gel. The 1Kb DNA ladder was used as a molecular size standard.

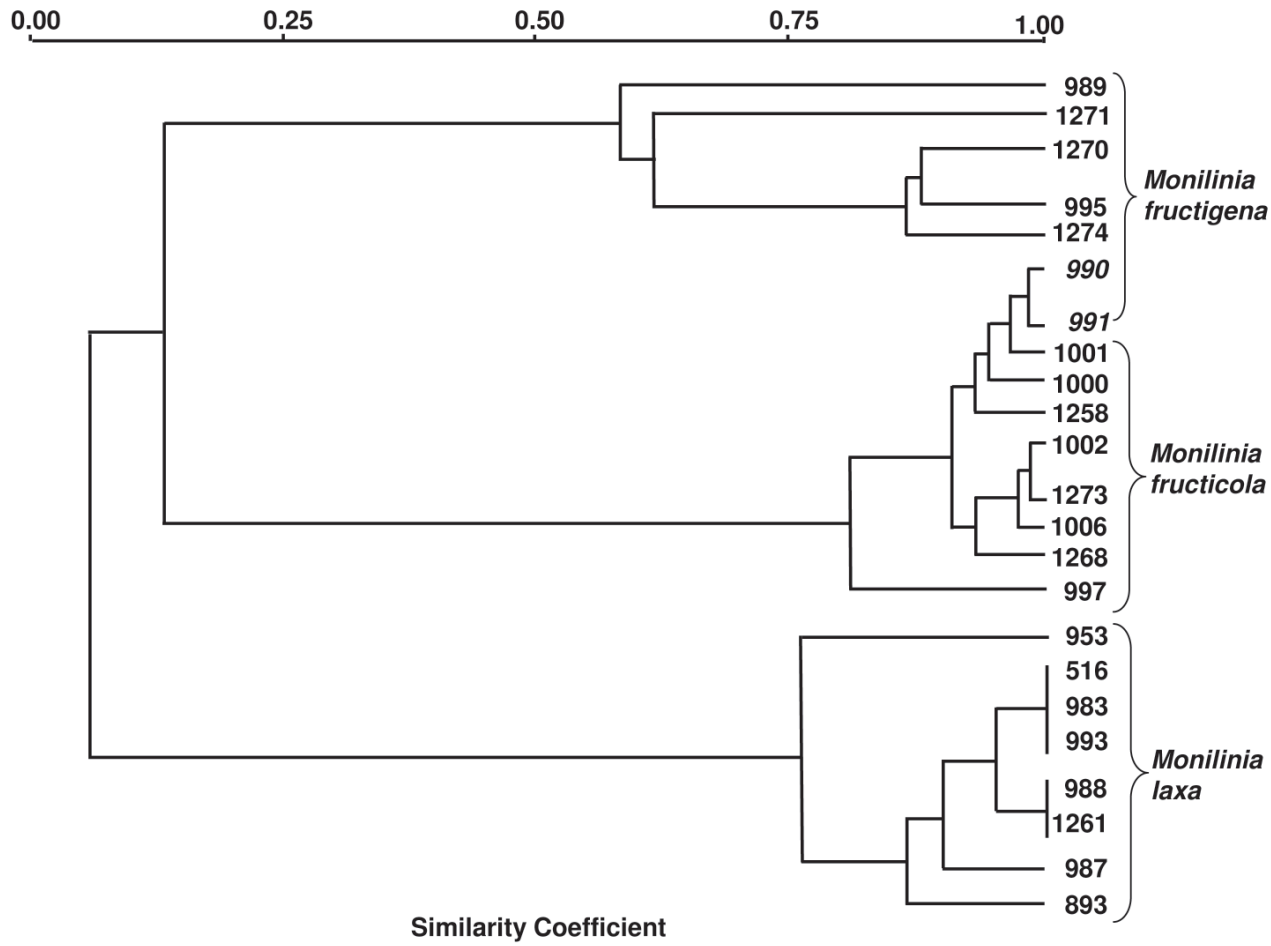


Fig. 2. Dendrogram representing differences between *M. fructigena, Monilinia fructicola* and *M. laxa* isolates, generated on the basis of RAPD markers. Isolates 990 and 991 (in italic type) previously attributed to *M. fructigena* by morphological characterisation, were included in the *M. fructicola* group.

the *M. laxa* isolates, apart from isolate 987. The *Monilinia* isolates in those cultural conditions produced significant differences in pectolytic activity.

**Qualitative pectolytic enzymes production**

Culture filtrates of *Monilinia* isolates were subjected to thin layer polyacrylamide gel IEF and evaluated for the isoenzymes PG, PME and PNL (Fig. 4). The *M. fructicola* pectolytic isoenzyme patterns were similar, with five PGs (pIs <4.0, 4.1, 4.4, 4.6 and >6.0), five PMEs (pIs 4.7, 5.0, 5.6, 5.9, 6.2) and two PNLs. One PNL isoform with pI 4.1 was present in all isolates, but the other, with pI 4.8, was detected only in isolates 1000, 1002, 1006 and 1273.

The pectolytic isoenzyme patterns of *M. fructigena* and *M. laxa* were more complex, and were distinctive for particular groups of isolates. *M. fructigena* isolates 990 and 991 shared common PGs (pIs <4.0, 4.3, 4.4, and > 6.0) and PMEs (pIs 4.7, 5.0, 5.6, and 6.2). Isolates 989, 995, 1270 and 1274 had common PMEs (pIs 4.4, 4.7, 5.0, 5.6, 5.7 and 8.6); isolates 989 and 995 also shared a common PG pattern (pIs <4.0 and >6.0). Isolate 1274 expressed a distinct PG pattern characterised by a single faint band (pI >6.0). A peculiar isolate was *M. fructigena* 1271, which displayed PG bands at pI <4.0, 4.3, 4.4, 4.6 and >6.0, and PME bands at pI 3.9, 4.4, 4.7, 5.0, 5.6. Like *M. fructicola* isolates, *M. fructigena* isolates produced PNL isoenzymes

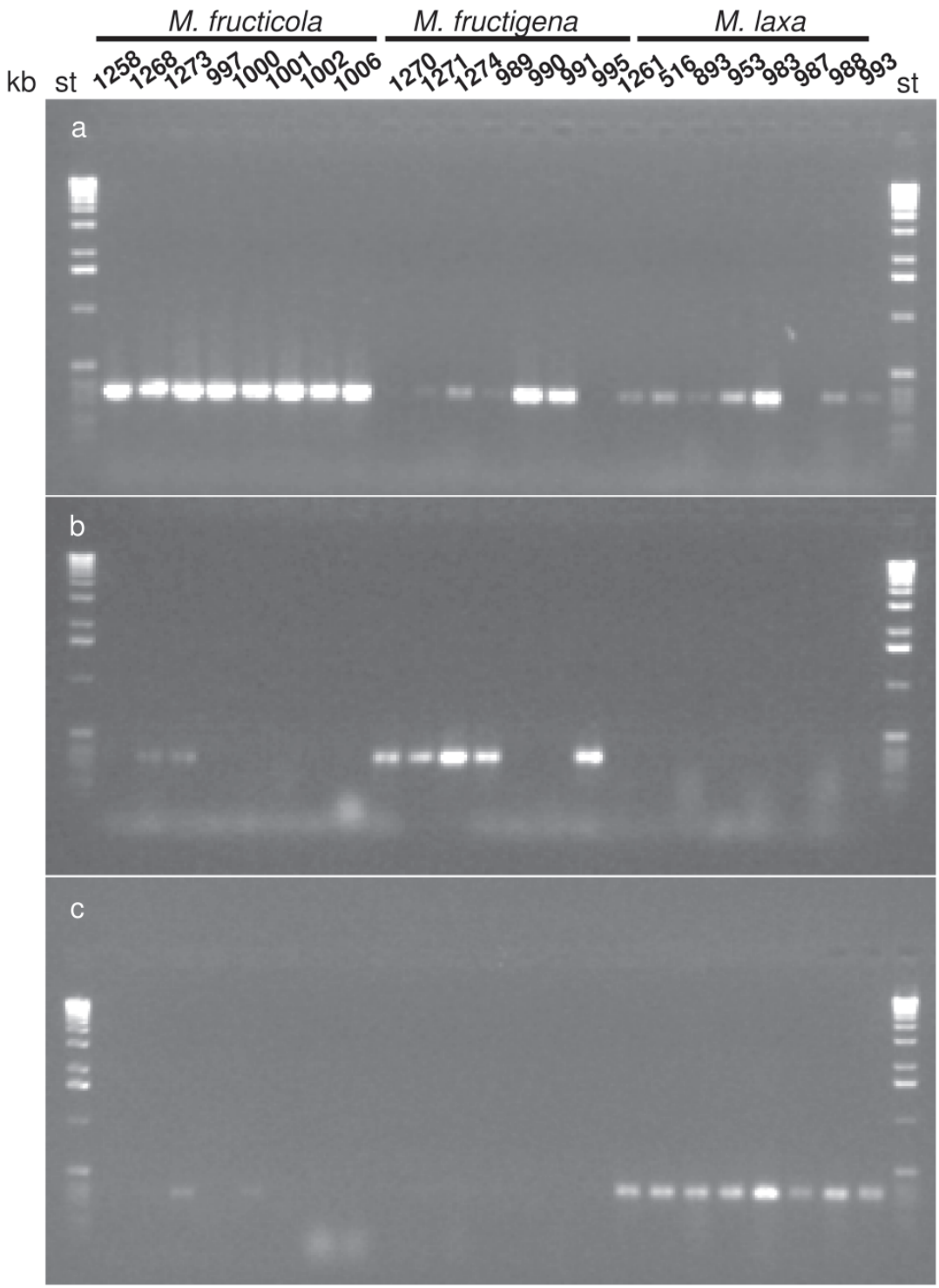


Fig. 3. Amplification products generated with ITS species-specific pairs of primers from *Monilinia fructicola* (a), *M. fructigena* (b) and *M. laxa* (c) isolates previously identified on the basis of morphological criteria. The 1 Kb DNA ladder was used as a molecular size standard.

Table 2. Pectin methylesterase (PME), polygalacturonase (PG) and pectin lyase (PNL) activity produced in liquid medium by *Monilinia fructicola* (eight isolates), *M. fructigena* (seven isolates) and *M. laxa* (eight isolates). For each species, values within columns followed by the same letter are not significantly different ($P \geq 0.05$).

| *Monilinia* isolate | PME | | PG | | PNL | |
|---|---|---|---|---|---|---|
| | U $g^{-1}$ d wt | St.Dev. | RU $g^{-1}$ d wt | St.Dev. | U $g^{-1}$ d wt | St.Dev. |
| *M. fructicola* | | | | | | |
| 997 | 39.6 a | 2.1 | 83.2 a | 18.4 | 16.3 abc | 5.8 |
| 1000 | 16.4 ef | 0.7 | 20.9 cd | 5.8 | 6.5 bc | 0.8 |
| 1001 | 15.1 f | 1.2 | 40.2 bc | 2.1 | 0.0 c | 0.0 |
| 1002 | 41.0 a | 2.4 | 11.9 cd | 2.8 | 33.0 a | 2.2 |
| 1006 | 21.3 bcd | 1.9 | 36.2 c | 5.3 | 9.5 bc | 3.4 |
| 1258 | 19.8 de | 0.7 | 42.3 bc | 23.5 | 6.4 bc | 1.0 |
| 1268 | 23.4 bcd | 2.2 | 3.8 d | 2.0 | 27.8 ab | 11.1 |
| 1273 | 25.3 ab | 0.5 | 69.6 ab | 8.8 | 3.7 c | 1.0 |
| *M. fructigena* | | | | | | |
| 989 | 19.3 c | 0.4 | 64.6 a | 8.3 | 19.4 ab | 3.6 |
| 990 | 12.4 d | 1.4 | 4.7 b | 0.2 | 7.2 b | 0.4 |
| 991 | 15.8 cd | 1.6 | 9.5 b | 2.6 | 37.1 ab | 33.3 |
| 995 | 29.3 b | 1.8 | 16.1 b | 1.2 | 32.0 ab | 12.0 |
| 1270 | 31.0 b | 2.0 | 24.3 b | 4.6 | 77.3 a | 14.2 |
| 1271 | 52.2 a | 3.7 | 63.3 a | 18.2 | 28.3 ab | 2.6 |
| 1274 | 33.4 b | 2.0 | 4.7 b | 2.3 | 52.3 ab | 8.2 |
| *M. laxa* | | | | | | |
| 516 | 44.9 b | 2.8 | 28.5 a | 6.0 | 0.0 b | 0.0 |
| 893 | 26.2 c | 1.9 | 19.2 bc | 1.4 | 0.0 b | 0.0 |
| 953 | 27.1 c | 0.5 | 18.9 bc | 4.1 | 0.0 b | 0.0 |
| 983 | 17.5 d | 0.4 | 15.9 c | 2.8 | 0.0 b | 0.0 |
| 987 | 25.8 c | 0.9 | 18.1 c | 2.6 | 1.9 a | 0.5 |
| 988 | 3.0 e | 1.3 | 3.6 d | 0.5 | 0.0 b | 0.0 |
| 993 | 4.6 e | 4.1 | 3.1 d | 0.8 | 0.0 b | 0.0 |
| 1267 | 54.4 a | 2.5 | 27.8 ab | 3.8 | 0.0 b | 0.0 |

at pI 4.1 and 4.8, except for isolate 1274 which produced only the pI 4.1 isoenzyme.

*Monilinia laxa* isolates 893, 953 and 983 shared a common PME isoelectric pattern (pIs 4.4, 4.7, 5.0, 5.6, 5.9, 6.5, 7.3). The other isolates showed slight differences between each other. The *M. laxa* isolates with common PG isoenzyme patterns were 516 and 1261 (sharing pIs 4.3, 4.4, 4.6, 5.0), 893 and 983 (pIs <4.0, 4.1, 4.3, 4.4), and 988 and 993 (pIs 4.3, 4.4). The PG profiles of the other *M. laxa* isolates had some common isoforms with the four isolates mentioned. PNL isoenzymes were detected only with isolate 987 (pIs 4.1, 4.8).

According to the similarity index (SI) calculated on the basis of the presence or absence of the PG, PME and PNL bands, a dendrogram was produced (Fig. 5). *M. fructicola* isolates were clearly similar (SI=0.96). *M. laxa* isolates clustered with a similarity of 0.71, whereas *M. fructigena* isolates were not included in one single cluster, but in different groups. Two *M. fructigena* isolates (990, 991) appeared to be close to *M. fructicola* isolates. Isolates 989, 995, 1270, 1274 were grouped in a separate cluster, which was quite distant from the *M. fructigena* isolates of the other cluster, with a similarity to it of only 0.60.

**Host preference and virulence analysis**

Seventy-two hours after inoculation, both pear and peach fruits showed the typical brown rot lesions. The virulence of each isolate was assessed by measuring the lesion diameters. Results were

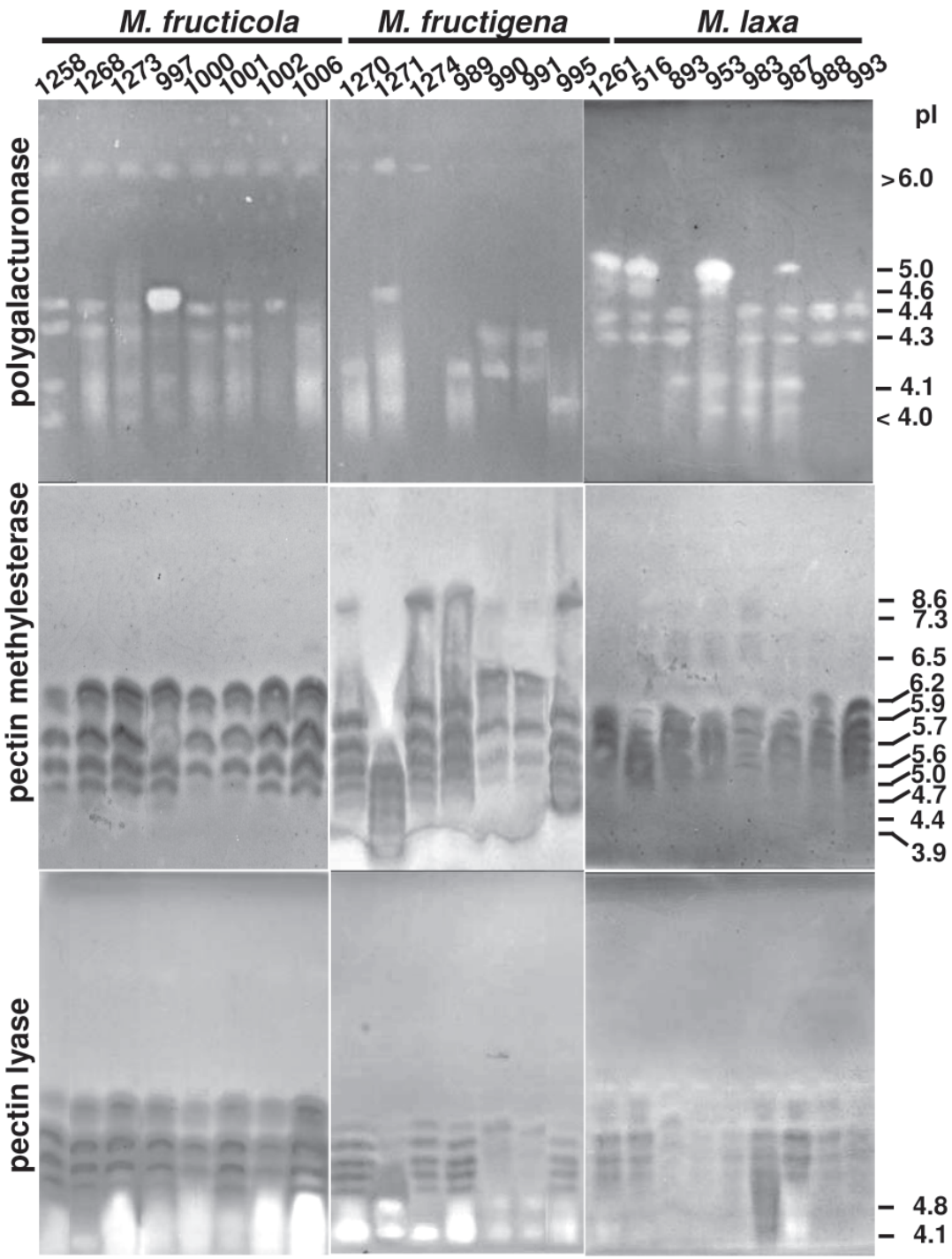


Fig. 4. Polygalacturonase (PG), pectin-methylesterase (PME) and pectin-lyase (PNL) isoenzyme patterns from liquid medium by *Monilinia fructicola*, *M. fructigena* and *M. laxa* isolates. Samples were separated on an isoelectric focusing (IEF) gel (pH 4.0-6.0 or 3.5-10.0), followed by agarose overlay activity staining. Position of pI values and pectolytic isoenzyme bands (white for PG and PNL, and dark for PME) are indicated on the right.

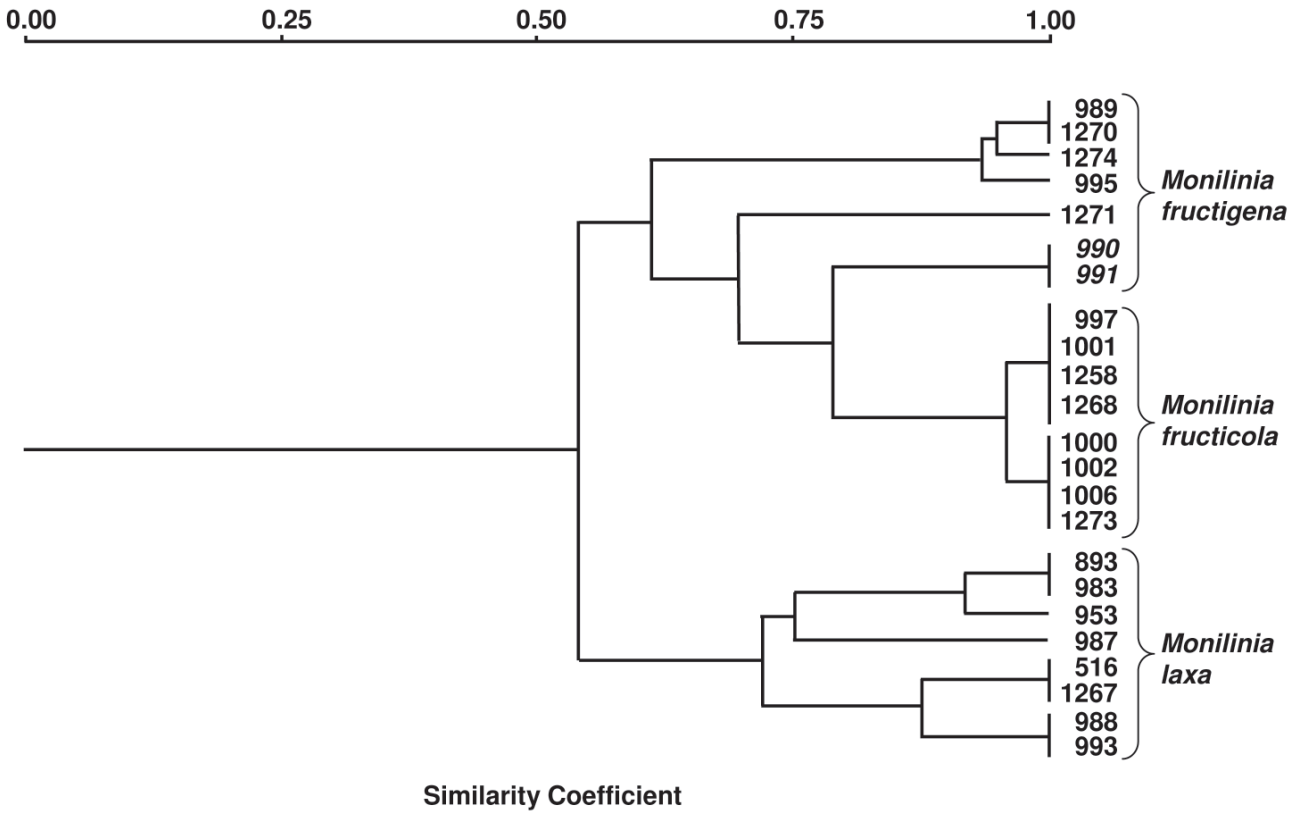


Fig. 5. Dendrogram representing differences among *M. fructigena, Monilinia fructicola* and *M. laxa* isolates, generated on the basis of polygalacturonase, pectin-methylesterase and pectin-lyase isoenzyme patterns. Isolates 990 and 991 (in italic type) previously attributed to *M. fructigena* by morphological characterisation, were included in the *M. fructicola* group.

subjected to analysis of variance. Virulence values differed significantly between isolates in most cases, except for *M. fructigena* isolates on peach fruits, where only isolate 1271 differed significantly from the others (Table 3). The average lesion diameters within each host species did not differ significantly. On the other hand, there were significant differences between average lesion diameters on peach and pear.

## Discussion

Identification of most *Monilinia* isolates by the molecular PCR technique using ITS primer pairs (Ioos and Frey, 2000) confirmed the identification made by morphological criteria. However, isolates 990 and 991, that had previously been attributed to *M. fructigena*, were now identified as *M. fructicola*, a species in the EU quarantine list. This grouping was consistent with that found by the total mycelium protein SDS-PAGE (Belisario *et al*., 1999) where isolates 990 and 991 were also classed with *M. fructicola* isolates. The attribution of these isolates 990 and 991 by morphological means to *M. fructigena* rather than to *M. fructicola* may have been due to the overlap of morphological and cultural traits as a consequence of the high variation in qualitative and quantitative morphological characters between the *Monilinia* species (Byrde and Willetts, 1977), confirming the value of identification based on molecular methods. With PCR using the ITS primer pairs, there were a number of cross reactions in the sequences specific for *M. fructicola*; however, the specificity of the other primer pairs and the PCR-RAPD analysis left no doubt about the species determination.

Table 3. Mean lesion diameters produced on peach and pear fruits by eight isolates of *Monilinia fructicola,* seven isolates of *M. fructigena,* and eight isolates of *M. laxa,* and the average lesion diameter for each species. For each species, values within columns followed by the same letter are not significantly different ($P \leq 0.05$).

| Isolate | Mean lesion diameter (mm) | |
|---|---|---|
| | Peach | Pear |
| *M. fructicola* | | |
| 997 | 38.3 bc | 26.0 b |
| 1000 | 42.7 ab | 26.1 b |
| 1001 | 44.3 a | 33.0 a |
| 1002 | 37.4 bc | 26.5 b |
| 1006 | 37.3 c | 29.9 ab |
| 1258 | 27.3 d | 20.5 c |
| 1268 | 39.4 abc | 26.8 b |
| 1273 | 40.4 abc | 31.5 a |
| *M. fructigena* | | |
| 989 | 37.5 a | 18.8 d |
| 990 | 36.9 a | 26.2 c |
| 991 | 40.5 a | 29.1 bc |
| 995 | 40.0 a | 30.3 abc |
| 1270 | 39.1 a | 34.3 ab |
| 1271 | 21.3 b | 34.1 ab |
| 1274 | 40.2 a | 35.3 a |
| *M. laxa* | | |
| 516 | 18.0 d | 17.8 c |
| 893 | 44.7 ab | 29.0 ab |
| 953 | 47.9 a | 27.1 ab |
| 983 | 32.1 c | 29.7 ab |
| 987 | 42.1 ab | 31.8 a |
| 988 | 39.3 b | 22.8 bc |
| 993 | 42.5 ab | 26.6 ab |
| 1261 | 44.6 ab | 17.4 c |
| Species: | | |
| *M. fructicola* | 38.3 ab | 27.5 b |
| *M. fructigena* | 36.5 b | 29.7 a |
| *M. laxa* | 38.9 a | 25.3 c |

The dendrogram based on the RAPD patterns exhibited three major groups of isolates, which corresponded to the three *Monilinia* species. This dendrogram did not show clustering according to the geographic origins, but like the results from PCR using the ITS primers pairs, it grouped isolates 990 and 991 with *M. fructicola*.

Of the numerous cell wall degrading enzymes produced by plant pathogenic fungi, most studies have focused on the pectinases, since these enzymes are typically produced first, in the largest amount, and are the only cell wall degrading enzymes that can macerate plant tissue and kill plant cells on their own (Collmer and Keen, 1986; Alghisi and Favaron, 1995). Moreover, pectinases produced by pathogenic fungi have also been reported to act as pathogenicity or virulence factors (Shieh *et al*. 1997; Ten Have *et al*. 1998). Previous studies have shown that *Monilinia* species produce pectolytic enzymes as multiple isoenzymes (Willetts *et al*., 1977; Snape *et al*., 1997). The present study provides a novel picture of all the pectolytic

enzymes produced by a considerable number of *Monilinia* isolates of different geographic origin. *M. fructigena, M. fructicola* and *M. laxa* all produced *in vitro* PG, PME and, except for most *M. laxa* isolates, PNL. Substantial differences between the amount of pectolytic enzymes produced by the isolates tested were found, but these differences did not appear useful to differentiate the three species.

The PG and PME isoenzyme profiles of the *Monilinia* isolates revealed differences within an overall common pattern. The PG isoenzymes produced by all the isolates were always slightly neutral or acidic, so that variations in the pI values were best resolved when the IEF was performed in the 4.0–6.0 pH range. This finding was not consistent with that of other authors, who reported that the PG produced by *Monilinia* species was basic (Willetts *et al.*, 1977; Snape *et al.*, 1997). The absence of a basic PG from the isolates tested in the present study may have been due to the experimental conditions chosen to grow isolates. The dendrogram constructed on the basis of the pectolytic enzyme patterns revealed that the *M. fructicola* isolates were similar (SI=0.96), while the *M. laxa* isolates were less uniform, clustering in two main groups. Pectolytic isoenzyme pattern analysis did not reveal a geographically linked grouping between *M. fructicola* and *M. laxa* isolates, suggesting that isolates with a common isoenzymatic pattern occur worldwide. The *M. fructigena* isolates were divided into different groups; specifically, one Japanese isolate (1271, from *Malus pumila*) did not cluster with the other *M. fructigena* isolates. Molecular analysis, also grouped isolates 990 and 991 with *M. fructicola*. These facts taken together revealed that variation between and within the isolates considered in this study may depend at the physiological level on differences in the pectolytic isoenzyme patterns.

Artificial inoculations on two hosts revealed differences in virulence between both the *Monilinia* species and the isolates. This difference may be associated with the regulation of pectolytic isoenzymes, which are reported to be affected by a number of factors, such as the composition of free sugars, the presence of enzyme inhibitors, and the pH level of the host (Holtz and Knox-Davies 1986a, 1986b; De Lorenzo *et al*. 2001; Prusky and Yakobi, 2003).

Pectolytic isoenzyme profiles have proved to be a ready means to characterise and group fungal and bacterial isolates (Cruickshank 1983, 1990; Ried and Collmer, 1986; Bonde *et al*., 1993; Nicoletti *et al*., 1999). *M. fructicola* isolates are distinguished from *M. laxa* isolates by having the pI>6.0 PG band. *M. fructigena* isolates differed from *M. fructicola* isolates by having the pI 8.6 PME band. An additional feature to distinguish *M. fructicola* and *M. fructigena* from *M. laxa* isolates was that *M. laxa* lacked PNL isoforms. PG, PME and PNL isoenzyme patterns, even though more difficult to interpret than the rDNA-based method, can be an additional method to characterise *Monilinia* brown-rot species, in particular where molecular strategies are not feasible.

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