The Taxonomy of the Brown Rot Fungi (*Monilinia* spp.) Related to their Extracellular Cell Wall-degrading Enzymes

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Patterns of extracellular enzymes secreted by the brown rot fungi *Monilinia fructigena*, *M. fructicola*, *M. laxa* and *M. laxa* f. sp. *mali* were studied by column isoelectric focusing. Of the four enzymes assayed, α -L-arabinofuranosidase (AF) showed the greatest differences between species. Thus *M. fructicola* had high AF activity at isoelectric point (pI) 5.1, *M. fructigena* had AF peaks at pI 3.0 and 7.0, *M. laxa* showed activity over the pI range 6.0 to about 7.3 with distinct peaks at 6.7 and 7.2, and *M. laxa* f. sp. *mali* had very high AF activity at pI 7.2. The pectinlyase (PL) patterns were also useful for identification: the characteristic molecular form of PL in cultures of *M. laxa* and *M. laxa* f. sp. *mali* was at pI 9.1, while the two other species showed single peaks of activity at about pI 8.5. However, some *M. laxa* isolates also produced a peak at pI 8.5, although this was less active than the characteristic one at pI 9.1. The polygalacturonase and pectinesterase enzyme patterns were not useful for distinguishing between the *Monilinia* fungi. The AF and PL enzyme patterns from known isolates were used to identify several 'unknown' brown rot cultures, including *M. laxa* isolates from stone fruits in Australia and *M. laxa* from *Stranvaesia undulata*, a new host in England.

INTRODUCTION

The usual criteria for identifying the brown rot fungi [*Monilinia* (= *Sclerotinia*) spp.] of pears, apples and stone-fruits include spore colour, type of conidial pustules produced on infected fruits, mode of branching of germ tubes arising from conidia, cultural characteristics, and host and tissue specificities (Wormald, 1954). When using these criteria to identify an isolate, it is often necessary to compare unknown with known cultures. This may be inconvenient and also dangerous, as exotic cultures must be collected and new pathogens may be introduced. Furthermore, the characters vary even within species and are, therefore, not always satisfactory for accurate identification.

In the pathogenicity of these fungi, extracellular enzymes that degrade host cell walls play an important role (Byrde & Willetts, 1977). This investigation was aimed to determine whether patterns of these enzymes, obtained by the use of column isoelectric focusing, could distinguish between brown rot fungi.

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Table 1. *Details of isolates of Monilinia spp.*

Reference code	Host	Country of origin	Date of isolation	Species name given, based on general criteria
<i>Isolates used as standards</i>				
L1	Peach fruit	France	1974	<i>M. laxa</i>
L2	Peach fruit	England	1974	
L3	Peach fruit	Italy	1974	
L4	Apricot fruit	Turkey	1962	
L5	Plum twig	England	1975	
L6	Nectarine fruit	England	—	
Lm1 (CBS290.31)	Apple twig	Netherlands	—	<i>M. laxa</i> f. sp. <i>mali</i>
FC1	Peach fruit	Australia	1965	<i>M. fructicola</i>
FC2	Single ascospore isolate – from apothecium on peach mummy	Australia	1974	
FC3	Apricot fruit	Australia	1972	
FI (ATCC26106)	Apple fruit	England	1969	
F2 (Herb. IM1162408)	<i>Malus pumila</i>	England	1971	<i>M. fructigena</i>
<i>Unidentified cultures</i>				
X ₁ (75/63)	Flowering quince	N.S.W., Australia	1975	
X ₂ (74/175)	Flowering quince	Victoria, Australia	1974	
X ₃ (75/23)	Flowering cherry	N.S.W., Australia	1975	
X ₄ (74/159 ^A)	Cherry	Victoria, Australia	1974	
UI	<i>Stranvaesia undulata</i>	England	1975	

METHODS

Details of the isolates used are given in Table 1. Isolates of the three common brown rot species, *M. fructigena* (Aderh. & Ruhl.) Honey, *M. laxa* (Aderh. & Ruhl.) Honey and *M. fructicola* (Wint.) Honey were obtained from Europe and Australia. Only one culture of *M. laxa* f. sp. *mali* (Wormald) Harrison was available.

During the investigation *M. laxa* was reported to be infecting ornamental quince, cherry plum and commercial peaches and plums at the flowering stage in the Orange district of New South Wales, Australia, where the fungus was previously unknown. To test the extracellular cell wall-degrading enzyme patterns as taxonomic criteria, we assayed isolates of the new brown rot pathogen from New South Wales and cultures from Victoria, where *M. laxa* had previously been reported (Jenkins, 1965). A brown rot fungus isolated from twigs of *Stranvaesia undulata* growing in England was also tested.

Cultures were grown for 11 days in ammonium tartrate/sodium polypectate liquid medium (Byrde & Fielding, 1968) before the mycelia were filtered off and the filtrates were dialysed against distilled water for 24 to 48 h at 4 °C and then freeze-dried. As a quarantine precaution, filtrates of the Australian cultures were passed through a Millipore filter to remove mycelial and other fungal fragments before lyophilization and sealing in small glass ampoules. This treatment did not change the pectolytic enzyme patterns. The culture filtrates were electrofocused on an LKB 110 ml column (Laborda, Fielding & Byrde, 1973) for 3 days. Elution was controlled by a peristaltic pump and the eluted fractions were assayed for the extracellular enzymes, pectinesterase [PE; pectin pectyl-hydrolase, EC 3.1.1.11], pectinlyase [PL; poly(methoxygalacturonide) lyase, EC 4.2.2.10; formerly pectin methyl-*trans*-eliminase], polygalacturonase [PG; poly(1,4- α -D-galacturonide) glycanohydrolase, EC 3.2.1.15] and α -L-arabinofuranosidase [AF; α -L-arabinofuranoside arabinohydrolase, EC 3.2.1.55].

Estimation of enzyme activities. PL, PG and AF were assayed by the methods described by Byrde & Fielding (1968). PE was estimated by back-titration with 0.01 M-NaOH to pH 5.0 at 25 °C, using a substrate mixture of 0.5 % (w/v) pectin (Brown Ribbon brand, Union Crystalex Gelatine Ltd, London) in 0.1 M-NaCl containing 0.1 % (w/v) phenol, adjusted to pH 5.0 with 1 M-NaOH (NaCl activates PE, and phenol prevents microbial contamination). The reaction mixture consisted of 10 ml substrate with 0.1 ml sample.

Table 2. Summary of average pI values (in the range 3 to 10) of extracellular enzymes of isolates of the brown rot fungi

Some indication of the relative activity of each enzymic form is shown: $\equiv$, very high; $\equiv$, high; $-$, moderate; no underline, low activity. Numbers in parentheses indicate that the form of the enzyme at that pI was detected only occasionally and, when observed, the activity was low.

Species	α -L-Arabinofuranosidase			Pectinlyase		Polygalacturonase			Pectin- esterase	
<i>M. fruticola</i>	3.0	<u>5.1^a</u>	7.1	<u>8.3^a</u>				<u>9.7^b</u>	5.0 ^a	
<i>M. fructigena</i>	3.0		7.0	8.4		(2.8)	4.4	(5.5)	<u>9.7</u>	4.8
<i>M. laxa</i>		6.0	<u>6.7</u>	<u>7.2^a</u>	8.5	9.1 ^a	(3.0)	[(4.6)]	<u>10.1^a</u>	4.8 ^a
<i>M. laxa</i> f. sp. <i>mali</i>			<u>7.2</u>		9.1			<u>5.1</u>	9.7	4.7

Standard errors of mean: a, ± 0.1 ; b, ± 0.2 .

RESULTS

Standard isolates

The isoelectric points (pI) of the AF, PL, PG and PE enzymes of isolates identified morphologically as *M. fruticola*, *M. fructigena*, *M. laxa* and *M. laxa* f. sp. *mali* are shown in Table 2. The enzymic forms of the isolates of any one species had very consistent pI values but they differed in absolute activity. Isolates of different species often differed greatly. Patterns of the AF, PL and PG enzymes for typical isolates of the four brown rot fungi are illustrated in Fig. 1. The heights of the peaks varied between isolates of the same species, indicating different enzyme activities, but the overall patterns were similar.

α -L-Arabinofuranosidase. A form of the AF enzyme with a pI about 7.1 was common to all isolates, being particularly active in filtrates of *M. laxa* f. sp. *mali* where it was the only form detected (Fig. 1d). In *M. laxa* cultures, AF activity was detected between pI 6.0 and 7.2 (Fig. 1c) with little activity at the bottom of the range but with two distinguishable peaks at 6.7 and 7.2. Cultures of *M. fruticola* and *M. fructigena* had a pattern different from the above, but the heights of the three peaks differed between the two species (Fig. 1a, b); in the former there was high activity at pI 5.1 with low peaks at 3.0 and 7.1, while in cultures of *M. fructigena* there was only occasional low activity at pI 5.0 compared with the distinct peaks at pI 3.0 and 7.1.

Pectinlyase. There was a common, active form of the PL enzyme at pI 8.2 to 8.5 in *M. fruticola*, *M. fructigena* and *M. laxa* (Fig. 1e, f, g) but *M. laxa* had an additional more active enzymic form at pI 9.1. *Monilinia laxa* f. sp. *mali* (Fig. 1h) had only one detectable enzymic form at pI 9.1.

Polygalacturonase. The characteristic feature of the PG patterns of all isolates was the common peak at pI 9.7 to 10.1. This was substantially the only peak detected in PG patterns from *M. fruticola* cultures (Fig. 1i) and was very high. In *M. fructigena* cultures (Fig. 1j), apart from the moderately active, common peak at pI 9.7, three forms of the PG enzyme with low activity were sometimes detected at pI values of about 2.8, 4.4 and 5.5, with a shoulder at pI 8. The PG pattern for *M. laxa* isolates (Fig. 1k) was similar to that of *M. fructigena* with low activities at pI 3.0 and 4.6 and a moderately active peak at about 10.1. The PG enzyme pattern for *M. laxa* f. sp. *mali* (Fig. 1l) had two peaks, a large one at pI 5.1, and a moderate one at pI 9.7.

Pectinesterase. Only one form of the PE enzyme with pI about 4.8 was detected. It was common to all the brown rot cultures studied.

Fig. 1. Cell wall-degrading enzyme patterns obtained by isoelectric focusing of filtrates from cultures of selected brown rot fungi. (a-d) α -L-Arabinofuranosidase; (e-h) pectinlyase; (i-l) polygalacturonase; inset in (i) shows PG activity over pI range 3 to 8 with the activity scale expanded 10-fold. (a, e, i) *Monilinia fructicola* (FC1); (b, f, j) *M. fructigena* (F2); (c, g, k) *M. laxa* (L1); (d, h, l) *M. laxa* f. sp. *mali* (LM1).

Unidentified isolates

Patterns for AF and PL enzymes for four of the five unidentified isolates are shown in Fig. 2 (a-h). The excluded isolate (x_2) gave results very similar to those of isolate x_1 (Fig. 2 b, f). Patterns for PG showed no characteristics that could be used to distinguish between these cultures.

The AF and PL enzyme patterns of isolate x_4 (Fig. 2 a, e) were very similar to those of *M. fructicola* (Fig. 1 a, e). Isolates x_1 (Fig. 2 b, f), x_2 and x_3 (Fig. 2 c, g) had AF patterns characteristic of the standard *M. laxa* cultures; also all the PL patterns (Fig. 2 f, g) had peaks at pI 9.1, comparable with those detected in the standard *M. laxa* and *M. laxa* f. sp. *mali*

Fig. 2. Cell wall-degrading enzyme patterns obtained by isoelectric focusing of filtrates from cultures of selected brown rot fungi. (a-d) α -L-Arabinofuranosidase; (e-h) pectinlyase. (a, e) isolate x_4 ; (b, f) isolate x_1 ; (c, g) isolate x_3 ; (d, h) isolate u_1 .

cultures, but had no activity at pI 8.5, unlike the patterns obtained from the standard *M. laxa* cultures.

Isolate u_1 had an AF pattern (Fig. 2d) similar to that of *M. laxa*, with moderate activity over a pI range of 6.0 (5.6) to 7.1, but with two additional small peaks with pI values of about 3.0 and 4.4. The PL pattern for u_1 (Fig. 2h) had the characteristic *M. laxa* peak at pI 9.1 with a shoulder at about 8.3.

DISCUSSION

The investigation suggests that there are consistent differences in AF and PL patterns that could help in distinguishing between species of the brown rot fungi. Confirmation is needed

that the enzymes at the same isoelectric points are indeed the same. This might be achieved by immunoelectrophoresis and determination of molecular weights.

The AF patterns were the best for identifying the brown rot fungi. Thus, *M. fructicola* was distinguished by high activity at pI 5.1, *M. fructigena* had maximum activity at 3.0 and 7.0 (cf. Laborda *et al.*, 1973), *M. laxa* showed activity over a wide range of pI values but with most from pI 6.0 to about 7.3, and *M. laxa* f. sp. *mali* had very high activity at 7.2.

The PL patterns were useful for distinguishing *M. laxa* and its forma specialis from *M. fructicola* and *M. fructigena*. The two last fungi had one form of the PL enzyme of pI 8.2 to 8.5. Although the standard *M. laxa* isolates had one enzymic form at pI 8.5, a second, more active form occurred at pI 9.1 and this was the only one detected in *M. laxa* f. sp. *mali* filtrates. The later studies on initially unidentified material indicate that the main PL enzymic form in *M. laxa* cultures has a pI of 9.1 and the form at 8.5 is not always active.

The PG enzyme patterns were less useful for identification; the very high peak at 5.1 for *M. laxa* f. sp. *mali* may help to distinguish this fungus from other members of the group, but more isolates need to be studied.

The PE enzyme patterns had no taxonomic value.

Comparing the AF and PL patterns of the four 'unknown' brown rot cultures from Australia with standard isolates of *M. laxa* and *M. fructigena* from Europe, and *M. fructicola* from Australia, indicated that three were *M. laxa* and one was *M. fructicola*. These results support the conclusions of Penrose, Tarran & Wong (1976) on morphological and electrophoretic evidence, that *M. laxa* is now established on growing crops in New South Wales, Australia. Our findings also support Jenkins' (1965) report that *M. laxa* is a pathogen of stone fruits in Victoria, Australia. The fungus isolated in England from *Stranvaesia undulata*, a host on which *Monilinia* spp. had not been recorded before, was characteristic of *M. laxa* although the AF pattern showed activity over a wider range of pI than observed in other *M. laxa* isolates.

Although the enzyme patterns differ characteristically between species, a basic pattern for the genus is discernible in the PE, AF and PG enzymes. Perhaps the ancestor(s) from which the brown rot fungi evolved had simple enzyme patterns, and subsequent changes in them would be related to adaptation of variant forms of the fungus to particular hosts and/or habitats. From some of the records of the establishment of brown rot species in new areas (Barss, 1923; Jenkins, 1965; Boesewinkel & Corbin, 1970; Penrose *et al.*, 1976) it seems that *M. laxa* adapts more readily to different environmental conditions than either *M. fructigena* or *M. fructicola*. The *M. laxa* isolates showed activity over wider pI ranges for both AF and PL enzymes, and possibly this applies to other enzymes not included in the investigation. This could confer on the species a greater enzymic plasticity than is found in the other two species. However, activity was not detected at low pI values. The least number of enzymic forms were observed in cultures of *M. laxa* f. sp. *mali*, and this could be related to the narrow host and tissue specificities and restricted regional distribution of the fungus. Probably the relationships between enzyme forms with varying host and tissue specificities could be determined from detailed studies on a range of fungal material collected from selected areas.

The association between the brown rot species and their hosts is thought to have originated in the Far East with secondary centres in the Transcaucasian region of the Middle East and in North America (Byrde & Willetts, 1977). Studies on enzyme patterns of isolates of the brown rot fungi from these regions could help explain the evolutionary affinities of *Monilinia* spp. Of particular interest would be the patterns obtained from cultures of *Monilinia mali* (Tak.) Whetzel and several of the other interesting brown rot fungi reported from Japan (Wormald, 1954).

It is concluded that AF and PL enzyme patterns are useful for confirming morphological identifications of the brown rot fungi. Further studies with isoelectric focusing and

complementary techniques, such as immunoelectrophoresis, could help explain the intra- and inter-specific relationships within this and other groups of fungi.

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