

A synoptic key for differentiation of *Monilinia fructicola*, *M. fructigena* and *M. laxa*, based on examination of cultural characters

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A synoptic key for the identification of three morphologically similar species of *Monilinia* principally affecting stone and pome fruit is described. Seven morphological characters were assessed after 10 days incubation under well-defined cultural conditions. No one character was found to separate the species. However, the synoptic key resulted in the correct identification of all isolates tested when compared with a molecular method.

Introduction

Monilinia brown rot is a major fungal disease of stone (*Prunus* spp.) and pome (*Malus* and *Pyrus* spp.) fruit trees causing serious financial losses as a result of blossom and twig blight, formation of cankers and fruit rot (Sinclair *et al.*, 1987). The disease is caused by one or more of three closely related fungi – *Monilinia fructicola*, *Monilinia laxa* and *Monilinia fructigena*.

The distribution of these species differs across the world (Byrde & Willetts, 1977). *M. fructicola* is present primarily in North, Central and South America, Australia and New Zealand (CABI/EPPO, 1999). It is listed as a quarantine pest within the European Union and, until recently, was absent from Europe. In 2001, it was officially reported as present in some peach orchards in the Département du Gard in the south of France (OEPP/EPPO, 2002). *M. fructigena* is present in Europe and parts of Asia, but is absent from South America, Australia and New Zealand (CABI/EPPO, 2000). *M. laxa* is the most common brown rot pathogen and occurs in all major areas of stone and pome fruit production (CABI/EPPO, 1991). Only in central and eastern Asia, where *Prunus*, *Malus* and *Pyrus* spp. originate, do all three species of *Monilinia* occur (CABI/EPPO, 1991, 1999, 2000).

Monilinia fructicola occurs most frequently on peach and nectarine, *M. fructigena* is usually found on apple and pear, and *M. laxa* is most commonly found on apricot and almond (EPPO/CABI, 1997). However, all three species can infect a range of rosaceous fruit trees *Prunus*, *Malus*, *Pyrus*, *Chaenomeles*, *Crataegus*, *Cydonia* and *Eriobotrya*, and there are records of *M. fructicola* on grapes and strawberries (Visarathanonh *et al.*, 1988; EPPO/CABI, 1997; Washington & Pascoe, 2000). Unfortunately, these fungi cannot be distinguished reliably on symptoms alone, and laboratory examination is necessary. The species are morphologically similar in culture, the greatest difficulty perhaps being to separate isolates of *M. fructicola* and *M. laxa*. Previous work has helped to distinguish species by morphological features, e.g. spore size, hyphal diameter and cultural characters such as colony colour, germ tube formation,

colony shape and isolate interactions. However, these features are affected by incubation conditions and media type, which are not sufficiently detailed in existing work (Byrde & Willetts, 1977; Mordue, 1979; EPPO/CABI, 1997) to permit direct comparison of the three species, or only compare two out of three species (Penrose *et al.*, 1976; Sonoda, 1982). Therefore, in practice, accurate diagnosis of *Monilinia* species is at best difficult and at worst unreliable.

Work funded by the EU IVth Framework Programme (Corazza, 1999) endeavoured to solve this problem in part by developing a simple identification protocol and reliable diagnostic key based on examination of cultural characters. Numerous isolates of *M. fructicola*, *M. fructigena* and *M. laxa* were collected from around the world, from culture collections and by direct isolation from naturally infected rosaceous fruits. At the beginning of the project, these were assigned to a species using existing cultural characters (Byrde & Willetts, 1977; Mordue, 1979; OEPP/EPPO, 1988). Parallel work carried out during the project investigated a range of identification techniques including molecular methods (Fulton *et al.*, 1999; Hughes *et al.*, 2000), cultural characters (Corazza *et al.*, 1998; van Leeuwen & van Kesteren, 1998; van Leeuwen, 2000), protein profiles (Belisario *et al.*, 1998) and monoclonal antibodies (Hughes *et al.*, 1996, 1998) to identify the isolates more fully.

Materials and methods

Fungal cultures

Representative isolates of *M. fructicola* (six isolates), *M. fructigena* (five isolates) and *M. laxa* (six isolates), previously characterized using the polymerase chain reaction (PCR) method of Hughes *et al.* (2000), were used in this study. These isolates were selected from over 200 isolates collected during the EU-funded project to cover the host and geographical diversity of the three species. A further eight isolates obtained from naturally infected fruits during routine diagnostic work were assessed using the synoptic key. After cultural characterization, these were then identified by the PCR method

| Code | CBS reference | Species | Provenance | Host |
|----------------------------------|---------------|----------------------|--------------|-------------------------|
| Representative isolates | | | | |
| 1 | – | <i>M. fructicola</i> | Japan | <i>Malus pumila</i> |
| 2 | CBS 101510 | <i>M. fructicola</i> | New Zealand | <i>Prunus persica</i> |
| 3 | CBS 101503 | <i>M. laxa</i> | Italy | <i>Prunus persica</i> |
| 4 | | <i>M. laxa</i> | Spain | <i>Prunus armeniaca</i> |
| 5 | | <i>M. fructigena</i> | Spain | <i>Prunus domestica</i> |
| 6 | | <i>M. laxa</i> | USA | <i>Prunus armeniaca</i> |
| 7 | – | <i>M. fructicola</i> | USA | <i>Prunus domestica</i> |
| 8 | CBS 101507 | <i>M. laxa</i> | Australia | <i>Prunus armeniaca</i> |
| 9 | – | <i>M. fructicola</i> | Australia | <i>Prunus</i> sp. |
| 10 | | <i>M. laxa</i> | South Africa | <i>Prunus</i> sp. |
| 11 | CBS 101512 | <i>M. fructicola</i> | USA | <i>Prunus domestica</i> |
| 12 | – | <i>M. fructigena</i> | UK | <i>Malus pumila</i> |
| 13 | – | <i>M. fructigena</i> | Portugal | <i>Cydonia</i> sp. |
| 15 | CBS 101505 | <i>M. laxa</i> | Japan | <i>Prunus mume</i> |
| 16 | CBS 101508 | <i>M. fructicola</i> | Japan | <i>Prunus persica</i> |
| 17 | CBS 101500 | <i>M. fructigena</i> | Poland | <i>Prunus domestica</i> |
| 18 | CBS 101501 | <i>M. fructigena</i> | Netherlands | <i>Prunus persica</i> |
| Previously unidentified isolates | | | | |
| I | | <i>M. laxa</i> | France | <i>Prunus domestica</i> |
| II | | <i>M. fructigena</i> | France | <i>Prunus domestica</i> |
| III | | <i>M. laxa</i> | Australia | <i>Prunus domestica</i> |
| IV | | <i>M. fructicola</i> | Australia | <i>Prunus</i> sp. |
| V | | <i>M. fructicola</i> | Australia | <i>Prunus armeniaca</i> |
| VI | | <i>M. fructicola</i> | Australia | <i>Prunus domestica</i> |
| VII | | <i>M. laxa</i> | Australia | <i>Prunus persica</i> |
| VIII | | <i>M. laxa</i> | Australia | <i>Prunus persica</i> |

Species identity was determined by PCR according to Hughes *et al.* (2000).

of Hughes *et al.* (2000). The provenance and identification of all the isolates are given in Table 1.

Inoculation and incubation conditions

A 4-mm-diameter plug from the edge of a 4-day-old colony grown on 4% potato dextrose agar (PDA; Oxoid) at 22 °C in the dark was placed centrally on a 9-cm Petri dish containing 12.5 mL of medium. Three replicates per isolate were incubated at 22 °C with illumination of 12 h near-UV (wavelength 365.5 nm)/12 h dark. After 10 days, the plates were assessed for seven critical characters as described below.

Critical characters

- 1 Colony colour: upper surface of plate grey (A), yellow (B) or cream/white (C).
- 2 Growth rate: mean colony diameter > 80 mm – fast (D), 70–80 mm – medium (E), or < 70 mm – slow (F).
- 3 Sporulation: upper surface of colony, viewed with a dissecting microscope, showing sporulation abundant (G) or sparse (H).
- 4 Concentric rings of sporulation: upper surface of colony, viewed with a dissecting microscope, showing concentric rings present (I) (see Fig. 1a) or absent (J).
- 5 Colony margin: colony, when examined from the underside of the plate, showing margin lobed (K) (see Fig. 2b) or non-lobed (L) (see Fig. 3b).

Table 1 Provenance of representative isolates of *Monilinia fructicola* (six isolates), *Monilinia fructigena* (five isolates), *Monilinia laxa* (six isolates) and eight previously unidentified isolates (I–VIII)

- 6 Rosetting: upper surface of colony ‘rosetted’, i.e. showing mycelium in distinct layers (petals) on top of each other, with the appearance of an open rose flower (M) (see Fig. 2a) or not (N).
- 7 Black arcs: lower surface of colony showing black arcs or rings associated with the ‘petals’ of a rosetted isolate (see Fig. 2b) (O), black dotted areas or brown arcs or rings (Fig. 1b) (P) or no black arcs or rings absent (Q).

Synoptic key

The following synoptic key was constructed to identify the species (letters in brackets indicate a character that is not usually produced but can occur in some isolates):

M. fructicola A, D, (E), G, I, (J), L, (M), N, (P), Q

M. laxa A, (C), (E), F, H, J, K, M, (N), O

M. fructigena B, (C), (D), E, (F), (G), H, (I), J, L, N, Q

Results

Assessment of colony characters is presented in Table 2. Colony colour consistently helped to separate isolates of *M. fructicola* and *M. laxa* (‘grey’) from *M. fructigena* (principally ‘yellow’ with two ‘cream/white’ in colour (isolates 5 and 12). Growth rate was variable between and within species and could not be used to separate species. However, in general, colony diameter for *M. fructicola* was greater than for *M. fructigena*,

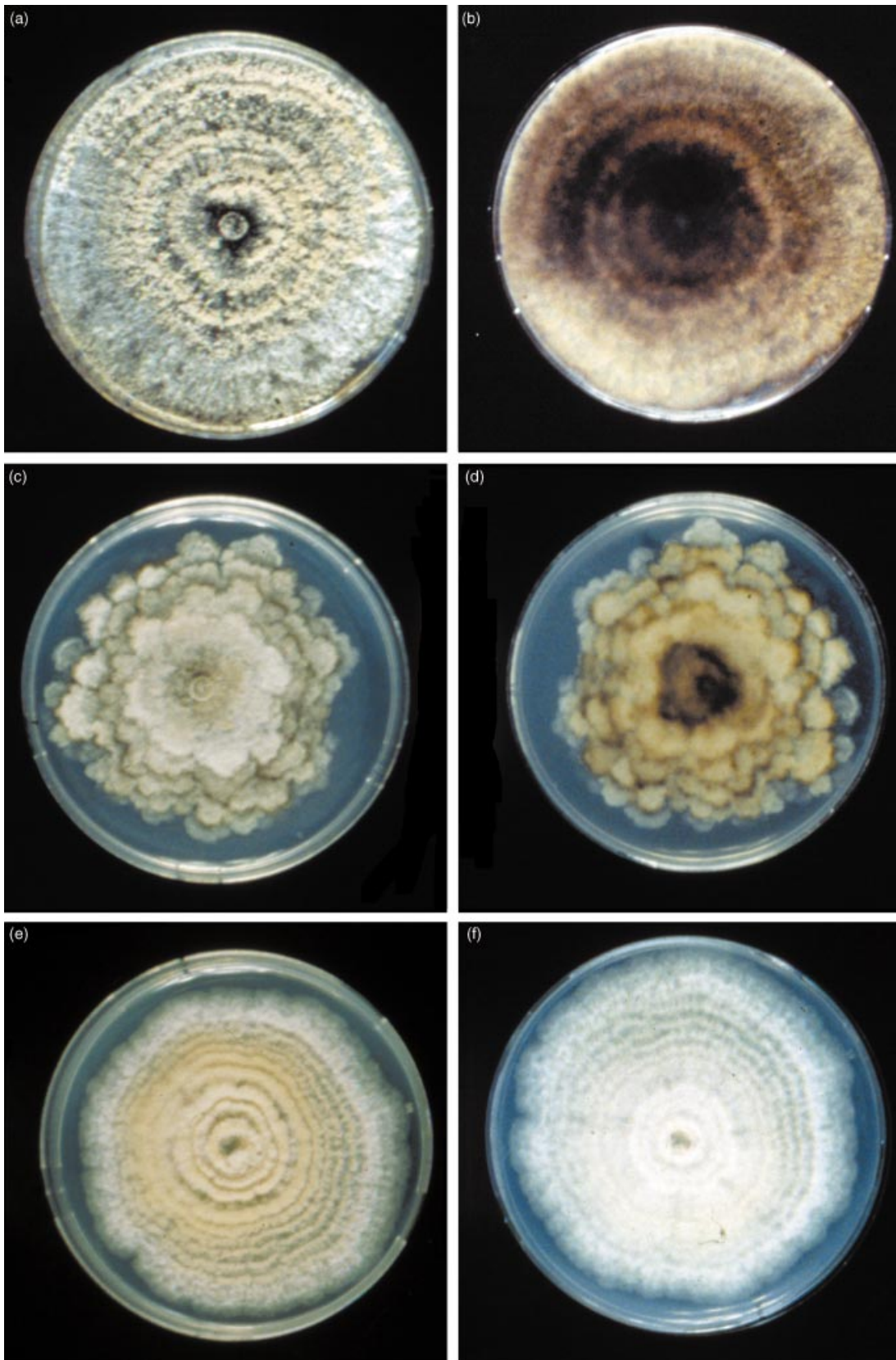


Fig. 1 Colonies of *Monilinia* spp. grown on 4% PDA (10 days, 12 h light/12 h dark at 22 °C). *M. fruticola* isolate 1: (a) upper surface; (b) lower surface; *M. laxa* isolate 8: (c) upper surface; (d) lower surface; *M. fructigena* isolate 18: (e) upper surface; (f) lower surface.

Table 2 Assignment of synoptic key letters (A–Q) in order to identify isolates of *Monilinia* (Mfc, *M. fruticola*; Mfg, *M. fructigena*; Mlx, *M. laxa*)

| Culture reference | Colony colour | Growth rate | Sporulation | Concentric rings of spores | Lobed colony margin | Rosettes | Black arcs associated with rosettes | Identity based on synoptic key |
|-------------------|---------------|-------------|-------------|----------------------------|---------------------|-----------|-------------------------------------|--------------------------------|
| | A grey | D fast | abundant | I present | K lobed | M present | O black | |
| | B yellow | E medium | G abundant | J absent | L smooth | N absent | P brown | |
| | C cream/white | F slow | H sparse | | | | Q absent | |
| 1 | A | D | G | I | L | N | Q | Mfc |
| 2 | A | E | G | I/J | L | N | Q | Mfc |
| 7 | A | D | G | I | L | N | Q | Mfc |
| 9 | A | D | G | I | L | N | Q | Mfc |
| 11 | A | E | G | I | L | N | Q | Mfc |
| 16 | A | D | G | I | L | N | P/Q | Mfc |
| 5 | C | F | H | J | L | N | Q | Mfg |
| 12 | C | E | H | J | L | N | Q | Mfg |
| 13 | B | F | H | J | L | N | Q | Mfg |
| 17 | B | F | G | J | L | N | Q | Mfg |
| 18 | B | E | G | J | L | N | Q | Mfg |
| 3 | A | E | H | J | K | M | O | Mlx |
| 4 | C | F | H | J | K | M | O | Mlx |
| 6 | C | E/F | H | J | K | M | O | Mlx |
| 8 | A | F | H | J | K | M | O | Mlx |
| 10 | A/C | F | H | J | L | N | Q | Mlx |
| 15 | A | F | H | J | K | M | O | Mlx |
| I | C | F | H | J | K | M | O | Mlx |
| II | B | E | H | J | L | N | Q | Mfg |
| III | A | F | H | J | K | M | O | Mlx |
| IV | A | D | G | I | L | N | Q | Mfc |
| V | A | D | G | I | L | N | Q | Mfc |
| VI | A | E | G | I/J | L | N | Q | Mfc |
| VII | A | E | H | J | K | M | O | Mlx |
| VIII | A | E | H | J | K | M | O | Mlx |

which in turn was greater than for *M. laxa*. Abundant sporulation was observed with all isolates of *M. fruticola*, two out of five isolates of *M. fructigena*, but never for *M. laxa*. Concentric rings of spores were never observed for *M. laxa* or *M. fructigena*, but were seen clearly in four out of five isolates of *M. fruticola*. Some isolates of *M. fructigena* produced rings in culture, but these were primarily thick rings of mycelium producing few spores. This could potentially cause confusion with *M. fruticola*. The presence of a lobed colony margin helped to separate *M. laxa* from *M. fruticola* and *M. fructigena*.

Unfortunately, although no rosettes with black arcs were seen in any *M. fruticola* or *M. fructigena* isolates, one isolate of *M. laxa* (isolate 10) failed to develop black arcs, thus preventing clear separation. The identity of eight previously unidentified isolates obtained using the synoptic key agreed with molecular characterization.

Discussion

The synoptic identification key accurately identified all 25 isolates of *Monilinia* tested in this study. The same success has been achieved in all recent diagnostic work at the Central Science Laboratory as part of routine plant health monitoring on behalf of DEFRA Plant Health Inspectors. The method is

simple to set up, and the characters are easy to identify and quick to record. The use of a synoptic key allows compensation for atypical isolates as well as for inaccurate assessment of characters, so is preferable to a dichotomous key. Although ideal for accurate identification of *Monilinia* species isolated from stone and pome fruits, it is not suitable for rapid diagnosis directly from infected plant material. Currently, this can be best achieved using the PCR test developed by Hughes *et al.* (2000). However, the cultural protocol and synoptic identification key require only basic microbiological facilities and skills.

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Clé synoptique pour différencier *Monilinia fructicola*, *M. fructigena* et *M. laxa*, d'après l'examen des caractères en culture

Une clé synoptique d'identification de trois espèces de *Monilinia* morphologiquement similaires affectant principalement des arbres fruitiers à noyau et à pépins est décrite. Sept caractères morphologiques ont été évalués après 10 jours d'incubation dans des conditions de culture bien définies. Aucun caractère pris séparément ne permettait de séparer les espèces. Cependant, la clé synoptique a permis l'identification correcte de tous les isolats testés par comparaison avec une méthode moléculaire.

Синоптический ключ для дифференцирования *Monilinia fructicola*, *M. fructigena* и *M. laxa*, основанный на изучении характеристик культуры

В статье описывается синоптический ключ (комбинированная определительная таблица) для идентификации трех морфологически подобных видов *Monilinia*, главным образом поражающих косточковые и семечковые плодовые. Семь морфологических признаков оценивались после 10 дней инкубации при четко определенных условиях культуры. Не было найдено ни одной характеристики, позволяющей самостоятельно разделить виды патогенов. Однако синоптический ключ привел к правильной идентификации всех проверенных изолятов при сравнении с молекулярным методом.

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