The brown rot fungi of fruit crops (*Monilinia* spp.), with special reference to *Monilinia fructigena* (Aderh. & Ruhl.) Honey

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Dr. ir. L. Speelman,
in het openbaar te verdedigen
op woensdag 4 oktober 2000
des namiddags te 16.00 in de Aula.

Bibliographic data

Van Leeuwen, G.C.M., 2000

The brown rot fungi of fruit crops (*Monilinia* spp.), with special reference to *Monilinia fructigena* (Aderh. & Ruhl.) Honey
PhD Thesis Wageningen University, Wageningen, The Netherlands
With references- With summary in English and Dutch.
ISBN 90-5808-272-5

The research was financed by the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD programme, Fair 1 - 0725, 'Development of diagnostic methods and a rapid field kit for monitoring *Monilinia* rot of stone and pome fruit, especially *M. fructicola*'. It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

Financial support was received from the Dutch Plant Protection Service (PD) and Wageningen University, The Netherlands.

Stellingen

1. De Japanse populatie van *Monilia fructigena* isolaten verschilt zodanig van die van de Europese populatie, morfologisch zowel als genetisch, dat beiden als verschillende soorten beschouwd dienen te worden.
(Dit proefschrift)

2. Ook vruchten die niet (volledig) mummificeren na infectie door *Monilinia fructigena* dragen bij aan het primair inoculum in het volgende seizoen.
(Dit proefschrift)

3. You can't judge a book by looking at the cover (Willie Dixon)
(Dit proefschrift)

4. Moleculair-biologische technieken vormen een belangrijke aanvulling op traditioneel morfologische methoden in schimmeltaxonomie, vormen echter geen vervanging..

5. Wetenschap is ook een soort geloof.

6. Het plannen van werkoverleg tijdens lunchpauzes bevordert de arbeidsvreugde in het algemeen niet.

Zoals mijn oud-leraar klassieke talen placht te zeggen: 'de boog kan niet altijd gespannen staan'.

7. Het is vaak het donkere bos dat ons doet spreken van het open veld.

(Henri Nouwen, 1993; Open uw hart, de weg naar onszelf, de andere en God, Uitgeverij Lannoo nv, Tielt)

8. Voor de Nederlandse consument mag voedsel niet teveel kosten, anders zou die derde of vierde vakantie wel eens in gevaar kunnen komen.

Stellingen behorend bij het proefschrift 'The brown rot fungi of fruit crops (*Monilinia* spp.), with special reference to *Monilinia fructigena* (Aderh. & Ruhl.) Honey'

G.C.M. van Leeuwen
Wageningen, 4 september 2000

Abstract

Van Leeuwen, G.C.M., 2000. The brown rot fungi of fruit crops (*Monilinia* spp.), with special reference to *Monilinia fructigena* (Aderh. & Ruhl.) Honey. PhD thesis, Wageningen University, Wageningen, The Netherlands.

The brown rot fungi of fruit crops (*Monilinia* spp.) cause blossom blight, twig blight, and fruit rot in rosaceous fruit crops in the temperate regions of the world. Three species are distinguished, of which *M. fructicola* and *M. laxa* are predominant in stone fruit culture, whereas *M. fructigena* is in pome fruits. This thesis deals partly with taxonomy and identification of the brown rot fungi, and with the epidemiology of *M. fructigena* in pome fruits.

M. fructicola is considered as a quarantine organism for Europe, and adequate identification tools are essential to prevent the introduction of this species in Europe. The most important pathways that the pathogen can be carried on are imported fruits and nursery stock. An identification protocol was developed based on quantitative colony and germ tube characteristics to distinguish the three brown rot species. In a discriminant analysis, the combination of increase in colony diameter and length of the germ tube resulted in only two misclassifications out of 29 isolates tested. The ITS 1-5.8S-ITS 2 region of ribosomal DNA (rDNA) was sequenced for a wide range of isolates to support identification on the basis of morphology, and four distinct sequences were found. Japanese *M. fructigena* isolates differed from European ones by four transitions within the ITS 1 region and one transition in the ITS 2 region. Morphologically, significant differences were found in stroma formation and conidial dimensions between the Japanese and European group. A new *Monilia* anamorph was defined, *Monilia polystroma* Van Leeuwen, in which the Japanese *M. fructigena* isolates were included.

In a two-years field study in an apple orchard, fruit loss caused by *M. fructigena* was quantified. In cv. James Grieve pre-harvest fruit loss ranged from 4.2 to 4.3 % in both years, in cv. Cox's Orange Pippin this was 4.4 % in 1997 and 2.7 % in 1998. The spatial distribution of diseased fruits among fruit trees, and that of trees with diseased fruits was analysed using Lloyd's index of patchiness (LIP) and spatial autocorrelation analysis respectively. Distinct clustering of diseased fruits among trees was detected in both cultivars in both years, whereas clustering of trees with diseased fruits did hardly occur. The concentration of airborne *M. fructigena* conidia in the orchard was monitored during two seasons, and related to ambient environmental conditions. Relative humidity, temperature, rainfall, wind speed and wind direction were monitored. The highest hourly concentration measured in 1997 was 233 conidia/m^3 and occurred during afternoon hours; in 1998 concentrations were lower than in 1997 throughout the season. Simple and multiple regression analysis was applied to relate weather variables to hourly spore catches. The factors relative humidity and temperature explained the variation in spore catches observed best.

Mummification and sporulation after infection of pome fruits by *M. fructigena* was studied in the field as well as under controlled environment conditions. Fruits of cv. Golden Delicious infected late in the season did not mummify, but sporulated profusely

after overwintering. It was shown that early- as well as late-in-the-season infected fruits contributed to the production of primary inoculum in the next season. Regeneration of conidia was much reduced in previously infected fruits after incubation under conditions of 20 and 25 °C and RH 65-85 % for 8 and 12 weeks.

Voorwoord

Het heeft heel wat zweetdruppeltjes gekost, maar uiteindelijk is het er dan: het proefschrift. Het was voor mij een geweldige uitdaging nu ruim vier jaar geleden om te kunnen gaan werken aan de *Monilinia* schimmels, waarmee ik van huis uit en door mijn korte periode als voorlichter in de fruitteelt goed vertrouwd was. Mijn liefde voor deze schimmels hield me op de been tijdens de perioden van 'zwaar weer' tijdens mijn promotie-onderzoek, vooral in de eerste twee jaren als zo'n beetje alles je wel interessant lijkt om nader te onderzoeken, en je al rondzwemmende bijna verzuipt. Ik kijk met plezier terug op de achterliggende vier jaren, maar ben tegelijk blij dat ik nu aan iets nieuws kan beginnen. De doorstroomstudie in Wageningen gevolgd door promotie-onderzoek heeft me gevormd tot wie ik nu ben.

Als ik terugkijk denk ik aan de mensen van het eerste uur. Herman, mijn eerste dagelijkse begeleider bij de vakgroep Fytopathologie, bedankt voor de interesse die je nog op hebt kunnen brengen voor mijn *Monilinia*-project, terwijl je je spullen al aan het pakken was. Dirk Jan en Marjan, bedankt voor jullie inbreng in het AIO discussiegroepje, onmisbaar om een jonge, onervaren promovendus op weg te helpen in de jungle van het publiceren. Collega's van de Plantenziektenkundige Dienst (PD) van het eerste uur, het kostte me enige moeite mijn draai te vinden in een totaal andere bedrijfscultuur, maar het 'PD-familiegevoel' zal me altijd bijblijven. Wil, bedankt voor de nuttige, praktische tips die je een beginnend mycoloog hebt gegeven. Waarde collega Eelco, ik denk dat ik ook namens Annemarie spreek als ik je nogmaals bedank voor de bed-and-breakfast service tijdens het congres in Edinburgh, augustus 1998. Bedankt voor die positieve kijk op het leven, en nuttige tips tijdens de tijd dat we collega's mochten zijn.

Rob en Hennie van de PD, jullie namen ook vervroegd afscheid van het *Monilinia*-project. Hennie, initiator van het Europese project waarmee alles begon, bedankt voor zoveel gezelligheid. De contacten met andere Europese onderzoekers binnen het *Monilinia*-project heb ik als zeer nuttig en stimulerend ervaren. Als ik me zo weet te profileren als jij, Hennie, is mijn kostje wel gekocht, fantastisch! Rob, bedankt voor de ruimte en vrijheid die ik heb genoten binnen de sectie Mycologie.

Sommigen gingen, anderen kwamen. Jos, jij hebt me op de rails gezet, niet meer in rondjes zwemmen, maar een bepaalde richting op. Af en toe lekker op zijn Amerikaans. Bedankt Jos, je hebt me geweldig op weg geholpen. Robert, ik herinner me nog goed je enthousiaste aanbod me bij te willen staan gedurende de laatste anderhalf jaar van mijn onderzoek. Bedankt voor alle goede adviezen, door jou heb ik in korte tijd zeer veel geleerd over het bedrijven van wetenschap in deze tijd.

Ja, en sommigen waren er al vóór mij en ga ík nu achterlaten. Corrie, we waren meer collega's op afstand in het laatste jaar, bedankt voor je hulp bij enkele van de experimenten. Angelique, kamergenoot in het begin en aan het eind van mijn AIO-periode, bedankt voor zoveel jaren gezelligheid. Jij weet nu zoveel van mij dat ik wel iets verwacht op het promotiefeest... Verder wil ik al die andere collega's bedanken voor een stukje begeleiding, filosofische gesprekken, en gezelligheid. PD-ers, Brigitta en Karin (jullie zouden zo een relatiebemiddelingsbureau kunnen beginnen), Ilse, Johan, Hans, Mariëlle en Willem, het ga jullie goed. Fytonezen, Jorge, Ellis, Maria, succes in jullie verdere carrières, en een speciaal woord van dank aan onze topsecretaresses Ali en Ria. Ex-fytonezen, nu Biob-ers, Aad, Wim, Dine, Jan-Kees, Trudie, ik heb het erg naar mijn zin gehad in de laatste maanden in jullie groep.

Leo, Herman en Fredy van proefboomgaard De Schuilenburgh, mijn dank voor de gelegenheid die jullie mij boden om de veldexperimenten uit te voeren. Sofia Seixas Basilio, thanks a lot for your excellent work in 1997, muito bem! My dear friend and colleague Imre

Holb, I think you remember well how we started to cooperate in 1998. In time we both improved, and I wish you the best with finishing your PhD thesis. Finian, you have been a great help in analysing the spore catch data, thanks again. Willem, bedankt voor het gepuzzel met de Image Analyser, jij redt het wel! Piet Kostense, bedankt voor je hulp bij het inpassen van foto's en design voor de voorkant van het proefschrift.

Professor Mike Jeger, waarde promotor, jij weet nu hoe paniekerig ik kan reageren als er iets mis dreigt te gaan in veldexperimenten.... Ik heb veel gehad aan je relativerende woorden en positivisme. Jij hebt me geleerd hoe waardevolle informatie te extraheren uit bergen met onderzoeksgegevens en deze resultaten beter te profileren. Bedankt voor de correcties en opmerkingen bij die vele stukken tekst die je voor mij doorgenomen hebt.

Tenslotte, voor mijn familie. Jullie zullen je wel eens afgevraagd hebben, wanneer houdt die jongen eens op met studeren en gaat hij gewoon aan het werk? Welnu, voorlopig zit dat er waarschijnlijk nog niet in. Ik heb jullie nabijheid, soms dichtbij, soms veraf, altijd gevoeld, en ik prijs mezelf gelukkig zo'n geweldig(e) (grote) familie te mogen hebben.

Gerard van Leeuwen

Wageningen, 16 augustus 2000

Contents

Chapter 1

General introduction

The three *Monilinia* spp. which cause blossom and twig blight, and fruit rot in rosaceous fruit crops all over the world, are commonly referred to as "the brown rot fungi of fruits crops". This group includes *Monilinia fructicola* (Wint.) Honey, *Monilinia laxa* (Aderh. & Ruhl.) Honey and *Monilinia fructigena* (Aderh. & Ruhl.) Honey; the anamorph form is referred to as *Monilia*. The former two species are mostly confined to stone fruits, while *M. fructigena* is a major fruit rot pathogen in pome fruits.

History of taxonomy and nomenclature

The first description of a brown rot fungus given, dates back to 1796 when Persoon described a fungus found on decaying fruits of pear, plum and peach (Persoon, 1796). At first he named it *Torula fructigena*, but after careful examination of the material, he changed the generic name into *Monilia (Monilia fructigena)*. During the nineteenth century, several authors have published descriptions of the anamorph of brown rot fungi under different specific names. For example, Kunze & Schmidt (1817) presented a description of a brown rot fungus which produced buff-coloured pustules on fruits, and named it *Oidium fructigenum*. Wallroth (1833) was the first to distinguish two distinct brown rot fungi in Europe. The one producing 'ochraceis' pustules was named *Oospora fructigena*, the one with 'griseis' pustules *Oospora laxa*. Bonorden (1851) changed the latter name into *Monilia cinerea*, a name until recently still used by European workers.

Until the beginning of the twentieth century, many workers still believed that there was only one brown rot fungus in Europe, *Monilia fructigena* Pers.. A brown rot fungus occurring in North America was also denoted as such (Smith, 1889). Woronin (1900) presented convincing evidence that two distinct fungal forms occurred in Europe on the basis of differences in colour of sporogenous tissue on culture media and fruits, spore dimensions and results obtained with cross-inoculation experiments. With Schröter (1893), he was also convinced that both species belonged to the genus *Sclerotinia,* although the perfect state had never been observed. The form producing ash-grey pustules was called *Sclerotinia cinerea* (Bon.) Schröter, the one forming buff-ochreous pustules *Sclerotinia fructigena* (Pers.) Schröter. It was only a few years later that the anamorph-teleomorph connection for these fungi was definitely established by Aderhold & Ruhland (1905). Mummified apple fruits produced apothecia in the second spring after infection, and ascospores sown on culture medium yielded cultures with buff-coloured sporogenous tissue. The species with the buff-coloured pustules was initially called *Sclerotinia fructigena* (Pers.) Schröter, but the name given by Schröter was a nomen nudum, based on the anamorph only. The authors also obtained apothecia on mummified apricot fruits, the ascospores of which gave cultures with ash-grey sporogenous tissue. At that time the brown rot fungus producing ash-grey pustules associated with apricot, was known as *Monilia laxa*. For this, the species obtained from apricot was called *Sclerotinia laxa* (Ehrenb.) Aderh. & Ruhl..

The size of asci and ascospores found for *Sclerotinia fructigena* by Aderhold & Ruhland (1905) differed from the description of the perfect state of a brown rot fungus collected in the USA (Norton, 1902). According to Conel (1914), the fungus described by Norton could not be *Sclerotinia fructigena* Aderh. & Ruhl., as the American form did not produce buff-coloured pustules, but instead ash-grey ones. Then, *Sclerotinia cinerea* became the name commonly accepted for the American form. However, Wormald (1920) distinguished the

American form with ash-grey pustules from the European form on the basis of sporulation intensity and its mode of growth when cultivated on prune juice agar. He proposed the name *Sclerotinia cinerea* forma *americana* for the American form. Later, Norton & Ezekiel (1924) proposed the name *Sclerotinia americana* for the American form. The rediscovery of an early description of the perfect state of a brown rot fungus found in Pennsylvania (USA) by Winter (1883) given the name *Ciboria fructicola*, made Roberts & Dunegan (1924) decide that the only correct name for the American form was *Sclerotinia fructicola* (Wint.) Rehm.

In an excellent study of the related genera *Sclerotinia* Fuckel, *Ciboria* Fuckel and *Stromatinia* Boud., Honey (1928) erected a new genus, *Monilinia*, comprising the monilioid species formerly placed in the genus *Sclerotinia*. He distinguished this new group from the other *Sclerotinia* species on the basis of nature of the stroma/sclerotial tissue and the type of conidial fructification. The conidial stage within *Monilinia* is monilioid, macroconidia are arranged as beads on a string. The author declared *Monilinia fructicola* (Wint.) Honey (= *Sclerotinia fructicola* (Wint.) Rehm) as the type species. Later, Honey (1936) subdivided the group of *Monilinia* spp. in two sections: Junctoriae and Disjunctoriae. In the Disjunctoriae macroconidia in the conidial chain are separated by disjunctors, in the Junctoriae these structures are absent. The Disjunctoriae comprise species with a very narrow host range (monotrophs, oligotrophs), the group of brown rot fungi belong to the Junctoriae (polytrophs).

Geographical distribution

As the history of nomenclature within the group of brown rot fungi indicates, initially there was a distinct geographical distribution of the three species worldwide. At the beginning of the twentieth century, *M. fructicola* was known to occur in the New World (North America, Australia), while the other two species were endemic in Europe and the Far East (Manchuria, Japan). During the last century however, brown rot species have become established in regions where they were not previously known. Jackson (1915) recorded the presence of *M. laxa* in Oregon at the west coast of the USA, and since that time its distribution has extended northwards and southwards in the Pacific Coast region (Hewitt & Leach, 1939; Ogawa *et al.*, 1954). Spread to eastern parts of the USA (Wisconsin) was reported by Keitt *et al.* (1943), and later by Kable & Parker (1963) in New York State. Until 1962 only *M. fructicola* had been recorded as the brown rot species causing losses in Australia. Samples of blighted blossoms and twigs of cherry collected in Victoria district in 1962, yielded cultures of *M. laxa* (Jenkins, 1965a). In a limited survey, *M. laxa* appeared to be present only in southern Victoria, not in northern Victoria. Later, it was also reported from New South Wales, Australia (Penrose *et al.*, 1976), and from New Zealand (Boesewinkel & Corbin, 1970). In 1965, Terui & Harada (1966) detected *M. fructicola* for the first time in Japan, where it has since become established. At the present time, Japan is the only country where the three brown rot species coexist, as far as we know. In the 1970s *M. fructigena* was found in Maryland, USA, on pear fruits (Batra, 1979). The complete orchard was destroyed to eradicate the disease and no further records of its occurrence in the USA have been made.

During the last century the brown rot fungi have invaded new geographical regions, probably favoured by an intensified exchange of commodities and goods between countries and continents. Fortunately, *M. fructicola* has not yet been detected in Europe (Corazza *et al.*, 1999), though on numerous occasions the pathogen has been intercepted at ports of entry (Wormald, 1954; Byrde & Willetts, 1977).

Identification, detection

Identification of the three brown rot fungi has always relied heavily on general colony characteristics when grown on natural agar media (Wormald, 1920; Byrde & Willetts, 1977). On potato dextrose agar (PDA), sporogenous tissue in *M. fructigena* is distinct buff-coloured

(Rayner, 1970), while it has a brownish to ash-grey colour in *M. fructicola* and *M. laxa*. *M. fructicola* is delineated from *M. laxa* on the basis of differences in mode of growth, growth rate, and sporulation intensity on PDA. Conidial dimensions have been determined extensively (Wormald, 1920; Ezekiel, 1924; Hewitt & Leach, 1939), and it was shown that mean conidium size of *M. fructigena* was greater than that for *M. fructicola* and *M. laxa*. Features of germ tubes sprouting from conidia have also been used for delineation. *M. fructicola* and *M. fructigena* produce long, straight germ tubes before branching of the germ tube occurs, while in *M. laxa* branching starts close to the conidium (Ezekiel, 1924; Jenkins, 1965a; Schlagbauer & Holz, 1987). Furthermore, hyphal anastomosis of germinating conidia has been used to separate *M. fructicola* from *M. laxa*; it commonly occurred in *M. fructicola*, whereas in *M. laxa* anastomosis was lacking or rare (Ogawa & English, 1954). Because of the occurrence of intermediate colony types, Sonoda *et al.* (1982) experienced difficulties to separate *M. fructicola* from *M. laxa* on the basis of general colony characteristics. They proposed the use of interaction of cultures to delineate the two species. When isolates of both species were grown in one Petri dish filled with oatmeal agar, dark lines appeared at the junctions between *M. laxa* and *M. fructicola* isolates, but not between *M. laxa* isolates.

As outlined above, classification of the brown rot fungi is entirely based on differences in the anamorph *Monilia*. Only the teleomorph of *M. fructicola* is regularly found in the field (Huber & Baur, 1941; Terui & Harada, 1966; Zehr, 1982), apothecia of the other two species are very rare (Wormald, 1921; Solkina, 1931; Batra & Harada, 1986). Aderhold & Ruhland (1905) compared apothecial material from *M. fructigena* and *M. laxa*, and concluded that size and colour of the apothecia and asci were identical, and that only a slight difference in shape of the ascospores existed. Ascospores in *M. fructigena* were gently tapered at both ends, while those of *M. laxa* had rounded ends. However, Harrison (1935) stated that ascospores of *M. fructigena* were not always pointed, and Batra (1991) concluded that ascospore shape is variable in the brown rot fungi, and unsuitable as diagnostic feature. A thorough comparison of apothecia of *M. laxa* and *M. fructicola* by Harrison (1935), revealed no morphological differences.

As well as cultural and morphological methods to delineate the brown rot species, differences in soluble proteins and enzymes have been studied. Penrose *et al.* (1976) compared the electrophoretic patterns of soluble proteins and several enzymes between isolates of *M. fructicola* and *M. laxa*, and found that both species could be readily distinguished with the enzymes arylesterase and acid phosphatase. Extracellular cell wall-degrading enzymes, like pectin esterase and polygalacturonase, also proved to be useful in separating the brown rot species (Willetts *et al.*, 1977; Gupta & Byrde, 1988).

More recently, genetic differences between the brown rot species have been exploited for delineation of the species. Sequence analysis of the ITS-region of ribosomal DNA (rDNA) revealed three distinct sequences corresponding with the three species (Holst-Jensen *et al.*, 1997a), though some intra-specific variation occurred in *M. fructicola* and *M. laxa*. The occurrence of an intron in the small subunit (SSU) rDNA gene in *M. fructicola* has been used to construct species-specific primers to detect this species *in vitro* as well as *in planta* (Fulton & Brown, 1997).

Epidemiology of the brown rot fungi

General

Although the three brown rot species are considered polytrophs and are usually parasitic on all rosaceous fruit crops, a certain specialisation exists. *M. fructigena* is mainly a fruit rot

pathogen, although it is able to infect and colonise blossoms as well. However, ingression of mycelium into twigs from out of colonised flowers does not take place in *M. fructigena* (Woronin, 1900; Byrde & Willetts, 1977). *M. fructigena* occurs predominantly in pome fruit orchards (*Malus* spp., *Pyrus* spp.), but also in *Prunus* spp.. On the contrary, *M. laxa* is especially a blossom and twig pathogen, and causes damage in stone fruits. Under warm, humid conditions in the blooming period, severe infection of blossoms can take place, especially in sour cherry, apricot and almond (Calavan & Keitt, 1948; Ogawa & English, 1960; Gupta & Byrde, 1988). Subsequently, twigs are invaded by the pathogen, and the upper parts of these twigs die as soon as twigs are girdled. *M. laxa* also infects fruits in stone fruit crops, but there it is often encountered together with *M. fructigena* (Europe) or *M. fructicola* (USA, Australia). Finally, *M. fructicola* is mostly confined to stone fruits and is especially a fruit rot pathogen. In peach especially, *M. fructicola* has caused tremendous losses in the past, often exceeding 50 % (Smith, 1889; Hutton & Leigh, 1956). Nowadays, average yield losses are lower, though occasionally still high under adverse weather conditions during harvest time (Zehr, 1982; Hong *et al.*, 1997). Much effort has been done to assess the relative pathogenicity of *M. fructicola* and *M. laxa* on different stone fruit crops (Huber & Baur, 1941; Ogawa & English, 1960; Michailides *et al.*, 1987). *M. fructicola* is the only species in which life cycle apothecia play a role in nature, though the occurrence of apothecia varies per region (Kable, 1965b; Landgraf & Zehr, 1982; Sanoamuang *et al.*, 1995).

Monilinia fructigena (Aderh. & Ruhl.) Honey

M. fructigena is a major fruit pathogen in pome fruit culture, in pre-harvest as well as post-harvest stage. In the field, the first infections of unripe, green fruitlets usually occur 6-8 weeks after the blooming period (Fig. 1).

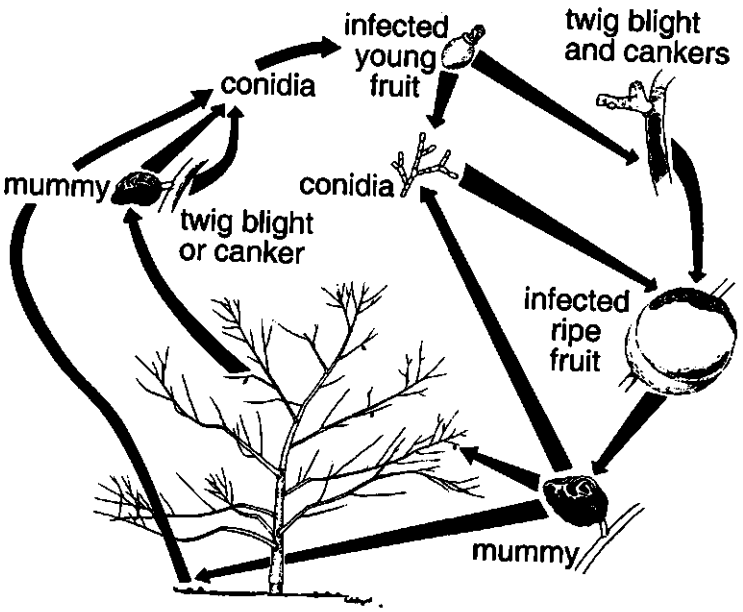

Fig. 1. Life cycle of *M. fructigena* (adapted from Byrde & Willetts, 1977).

M. fructigena is mainly a wound pathogen, although apples have been successfully infected via lenticels (Horne, 1933). Fruit skin injuries can be caused by abiotic (spring frosts, hail) as well as biotic factors (insects, birds, man). After infection, a brown lesion develops on the fruit, which is later ruptured by numerous sporodochia (conidial pustules) bursting through the cuticle of the fruit. In the first crop of conidia, millions of spores are produced per fruit, which are subsequently dispersed by air, water or vectors such as insects and birds (Pauvert *et al.*, 1969; Lack, 1989). A part of these conidia initiate new infections, though healthy fruits are also infected by fruit-to-fruit contact, as described for *M. fructicola* (Michailides & Morgan, 1997). No exact data are available for pre-harvest losses caused by *M. fructigena* nowadays, but in older literature moderate losses (10-15 %) to extremely high losses (50-60 %) are reported (Wormald, 1954). Diseased fruits either stay in the tree or fall to the ground. In the tree canopy, diseased fruits gradually dry out and shrivel, and turn into firm structures ('mummies', mummified fruits). These mummified fruits are the main survival structures of the pathogen during wintertime (Byrde & Willetts, 1977), though it also survives as mycelium in infected twigs and branches (Fig. 1). In springtime, periods of rain stimulate the uptake of moisture by the dried-out, mummified fruits. Subsequently, these mummies produce a new crop of conidia when temperature during daytime rises to 18-20 °C (Byrde, 1954a); this forms the primary inoculum to start a new epidemic (Fig. 1).

About this thesis

Background to the research

M. fructicola is listed on the EPPO A1 list of quarantine organisms for Europe (CABI/EPPO, 1997). Correct and quick identification and detection is necessary at entry points throughout Europe (airports, dock sites, harbours) to prevent the introduction of this pathogen in Europe. A project, involving the Dutch Plant Protection Service in Wageningen, which focussed on improvement of identification and detection methods was funded by the European Union and started in 1996 in cooperation with several other European institutes and universities. A study of epidemiological aspects of the brown rot diseases, was embedded in the overall PhD-research project in cooperation with the Department of Phytopathology (later with the group of Biological Farming Systems) of Wageningen University. This part of the project focussed on the epidemiology of *M. fructigena* in apple and pear orchards. In the Netherlands, 95 % of the area of pome and stone fruit culture consists of apple and pear and in 1998 about 500 million kg of apples and 140 million kg of pears were produced (Anonymous, 1999a). Though many aspects of the etiology and epidemiology of *M. fructigena* have been studied in the past (Wormald, 1954; Byrde & Willetts, 1977), some aspects have only been given minor attention.

Objectives, approach, and outline of the thesis

As mentioned before, identification of the three brown rot species relies heavily on qualitative colony characteristics (colour, shape of colony margin, etc.). This makes correct identification difficult for people without former experience with cultures of the brown rot fungi. It was our aim to develop an identification protocol based on unambiguously defined quantitative characteristics of colony and germ tube growth (Chapter 2). In cooperation with workers from The Queen's University of Belfast experienced in molecular biology, genetic variation among and within the brown rot species was investigated. Especially among isolates from Japan, considerable genetic variation was found (Chapter 3). Based on these results, subsequently we focused our attention on a comparison of cultural, morphological and biological

characteristics between Japanese and European *M. fructigena* strains. This resulted in the description of a new *Monilia* anamorph (Chapter 4).

In the second part of this thesis certain aspects of the epidemiology of *M. fructigena* are presented. Increase of disease incidence of *M. fructigena* in apple was studied in time and in space during two growing seasons. It was our aim to quantify nowadays yield losses caused by *M. fructigena*, and to study the spatial distribution of diseased fruits in time (Chapter 5). Spatial dynamics give insight into the underlying mechanism of dispersal of a disease. The role which aerial dispersal of conidia plays in *M. fructigena* is not clear, but it might facilitate dispersal over long distances. We determined the concentration of *M. fructigena* conidia in the air during the season in an apple orchard, and related this to ambient environmental conditions (Chapter 6).

The role of mummified fruits as the main survival structures of the pathogen, offers possibilities to control the disease by removing or destroying mummified fruits before these start to resporulate in springtime. Detailed studies of factors governing resporulation of mummified fruits in springtime, are lacking. Initially, it was our aim to study the effect of temperature and moisture conditions in springtime on resporulation of mummified fruits. However, it soon appeared that there was an enormous variation in potential sporulation intensity between mummified fruits collected in the field. Therefore, we designed experiments to obtain more insight into the process of mummification and subsequent resporulation in springtime (Chapter 7).

Chapter 8 of this thesis deals with risk analysis. One of the outcomes of the EU-project was that it would be very useful to quantify the risk of introduction of *M. fructicola* into EU membership countries. Guidelines on Pest Risk Analysis (PRA) developed by the European and Mediterranean Plant Protection Organisation (EPPO) were followed to assemble relevant biological and commercial information (trade pathways).

Chapter 2

Delineation of the three brown rot fungi of fruit crops (*Monilinia* spp.) on the basis of quantitative characteristics

Abstract

The three *Monilinia* spp., known as the brown rot fungi of fruit crops, are usually distinguished from each other on the basis of (qualitative) colony characteristics. We linked these qualitative features to unambiguously defined, quantitative colony and germ tube characteristics. A wide collection of isolates of *Monilinia fructicola*, *M. laxa* and *M. fructigena* was used to determine growth rate and sporulation intensity on potato dextrose agar (PDA) at 22 °C under two light regimes (darkness, 12 h light/ 12 h darkness). The following germ tube characteristics were determined on water agar after incubation for 18 h at 22 °C in darkness: length of the (leading) germ tube, distance to the first branch, and the number of germ tubes per conidium. Increase in colony diameter from day 3 to day 5 and sporulation intensity measured after 14 days, was the highest in *M. fructicola*, whilst *M. laxa* and *M. fructigena* showed considerable overlap in these features. The length of the germ tube after 18 h incubation was shortest in *M. laxa*, ranging from 161 µm to 466 µm. In *M. fructicola* and *M. fructigena* this range was 465 to 851 µm and 307 to 806 µm, respectively. The occurrence of more than one germ tube per conidium was most prominent in *M. fructigena*. Discriminant analysis on the basis of different combinations of the quantitative characteristics measured, showed that the combination of growth rate on PDA and length of the germ tube was sufficient to delineate the three brown rot fungi. One out of 11 *M. fructicola* isolates was misclassified, the same held for *M. fructigena* (one misclassification out of nine isolates). No misclassifications occurred in *M. laxa*.

Introduction

Considerable losses in fruit crops are caused in the temperate regions of the world by three *Monilinia* species, *Monilinia fructicola* (Winter) Honey, *Monilinia laxa* (Aderhold & Ruhland) Honey and *Monilinia fructigena* (Aderhold & Ruhland) Honey, commonly referred to as the brown rot fungi of fruit (Byrde & Willetts, 1977; Batra, 1991). The brown rot fungi are polytrophs; they attack a wide range of members of the Rosaceae. Other *Monilinia* species are oligotrophs and monotrophs and their host plant plays an important role in identification of these species (Batra, 1991). Several diagnostic features have been used to separate the three brown rot species. The part of the host plant infected, the colour of pustules and the time of appearance of the first pustules, give preliminary information for determination of the species. *M. laxa* is considered to be more a pathogen of blossoms and twigs than of fruit, *M. fructigena* is mainly a fruit pathogen (Calavan & Keitt, 1948; Wormald, 1954). *M. fructicola* is a pathogen of both blossom/twigs and fruits, and mainly affects stone fruits (Ogawa & English, 1960). The colour of the pustules on infected plant tissue is buff in *M. fructigena*, greyish-brown in *M. fructicola* and *M. laxa* (Byrde & Willetts, 1977). However, observations in the field

only are not sufficient to delineate the three brown rot species accurately; laboratory-based techniques are indispensable for an accurate identification.

Several laboratory-based techniques have been described in the literature to separate the three species. Growth characteristics on natural media (e.g. potato dextrose agar (PDA)), germ tube morphology and hyphal anastomosis between germ tubes, as well as interaction of cultures on oatmeal agar have been used as features for identification. From the very beginning, the growth characteristics on agar media have been used for identification (Wormald, 1919; Ezekiel, 1924; Roberts & Dunegan, 1932). Wormald (1920) was the first to report that there were differences in germ tube morphology among the species. He found that *M. fructicola* and *M. fructigena* produced a long, straight germ tube before branching, while *M. laxa* branched closely to the conidium. Calavan & Keitt (1948) stressed the fact that the mode of germination depends strongly on environmental conditions. An additional feature to distinguish *M. fructicola* from *M. laxa* is anastomosis between germ tubes. *M. fructicola* shows abundant anastomosis between germ tubes, in *M. laxa* it rarely occurs.

M. laxa and *M. fructicola* coexist in several regions of the world (Japan, USA, Australasia), and although host preference is reported (Ogawa *et al.*, 1954), the two species can be encountered together on nearly every stone fruit crop. Problems have arisen concerning the identification of so called 'atypical' isolates, isolates which do not have the typical characteristics as described for the type cultures (Aderhold & Ruhland, 1905; Honey, 1928). Different opinions have appeared in the literature concerning the characteristics which are most typical for the two species (Ogawa & English, 1954; Sonoda *et al.*, 1982). A proper identification and separation between these two species is of the utmost importance, as *M. fructicola* is a quarantine organism for Europe.

It is striking to see that the characteristics for identification of the brown rot fungi are almost exclusively qualitative traits, depending heavily on colony characteristics and interaction between colonies. For this, diagnostic personnel less familiar with cultures of the three species, encounter difficulties while interpreting described qualitative characteristics for cultures under study. The main goal of this study was to provide several quantitative, unambiguously defined characteristics for delineation of the three brown rot species solely on the basis of quantitative characteristics. Moreover, our study includes a world-wide set of isolates for all three brown rot species. To our knowledge this is the first comparative, quantitative study of all three species done at the same time.

Material and methods

Set of isolates

Isolates were received from several workers in different regions of the world. Additionally, during summer 1996 samples were collected in Dutch orchards. After subculturing on PDA to identify the isolates, all isolates were lyophilized. Isolates were identified by the first author on the basis of general qualitative colony characteristics (colony margin, lobing, colour of sporogenous tissue) on a commercial PDA formulation (Oxoid, Basingstoke, England). The set of isolates used in the experiments is shown in Table 1.

Colony characteristics

A commercial PDA formulation was used to prepare PDA medium (39 g/L distilled water), on which growth characteristics of the three species were studied. Nine cm diameter, plastic Petri dishes were used, which were filled with 12.5 ml PDA.

Table 1. List of isolates of *Monilinia* spp. used to determine quantitative characteristics.

Isolate	Host	Species	Origin	Year of isolation
dar 27029	Prunus persica	fructicola	Australia	1976
dar 27031	Prunus persica	fructicola	Australia	1975
dar 27033	Prunus avium	fructicola	Australia	1976
dar 27036	Prunus avium	fructicola	Australia	1976
cc 778 [a)]	Prunus sp.	fructicola	Australia	1971
nz 12.89	Prunus persica	fructicola	N-Zealand	1989
nz 17.90	Prunus domestica	fructicola	N-Zealand	1990
nz 22.94 [b)]	Prunus armeniaca	fructicola	N-Zealand	1994
nz 23.94	Prunus armeniaca	fructicola	N-Zealand	1994
jap 1438	Malus pumila	fructicola	Japan	1989
jap 1829	Prunus persica	fructicola	Japan	1992
jap 2636 [b)]	Prunus cerasus/ avium	fructicola	Japan	1995
cc 865 [a)]	Prunus domestica	fructicola	USA	1994
cc 866	Prunus domestica	fructicola	USA	1994
cc 867 [b)]	Prunus domestica	fructicola	USA	1994
cc 953 [a)]	Prunus domestica	fructicola	USA	1996
cbs 203.25	Malus sylvestris	fructicola	USA	1925
dar 41474	Prunus armeniaca	laxa	Australia	1978
dar 41543	Prunus armeniaca	laxa	Australia	1978
nz 1.90 [b)]	Prunus persica	laxa	N-Zealand	1990
jap 1390	Prunus mume	laxa	Japan	1989
usa 55 [a)]	Prunus amygdalus	laxa	USA	- [c)]
cc 954 [a)]	Prunus persica	laxa	South Africa	1996
es-7	Prunus persica	laxa	Spain	1987
es-12 [a)]	Prunus armeniaca	laxa	Spain	1996
es-18	Prunus armeniaca	laxa	Spain	1996
cc 682 [a)]	Prunus persica	laxa	Italy	1992
utad F1 [a)]	Prunus domestica	laxa	Portugal	1996
pd 17.96	Prunus sp.	laxa	The Netherlands	1996
pd 17.96m1	Prunus sp.	laxa	The Netherlands	1996
pd 20.96	Prunus cerasus	laxa	The Netherlands	1996
pd 20.96m4	Prunus cerasus	laxa	The Netherlands	1996
cbs 165.24	Cydonia vulgaris	laxa	- [c)]	1924
jap 2317	Malus pumila	fructigena	Japan	1995
pd 4.96	Malus pumila	fructigena	The Netherlands	1996
pd 4.96m1	Malus pumila	fructigena	The Netherlands	1996
pd 8.96	Prunus persica	fructigena	The Netherlands	1996
pd 8.96m1 [b)]	Prunus persica	fructigena	The Netherlands	1996
pd 15.96	Malus pumila	fructigena	The Netherlands	1996
pd 15.96m2 [b)]	Malus pumila	fructigena	The Netherlands	1996
pd 27.96 [b)]	Prunus domestica	fructigena	The Netherlands	1996
pd 27.96m1	Prunus domestica	fructigena	The Netherlands	1996
es-48 [a)]	Prunus domestica	fructigena	Spain	1996
utad LL1	Malus pumila	fructigena	Portugal	1995
utad B9 [b)]	Malus pumila	fructigena	Portugal	1995
utad B11 [a)]	Malus domestica	fructigena	Portugal	1995
Pt M2 [a)]	Cydonia sp.	fructigena	Portugal	1996
cc 747 [b)]	Malus pumila	fructigena	UK	1969
cc 782	Malus sp.	fructigena	UK	1994
cc 752	Prunus domestica	fructigena	Poland	1993

Note: Unless otherwise indicated, both germ tube and colony characteristics were determined for the isolates.

[a)] only germ tube characteristics were determined.

[b)] only colony characteristics determined

[c)] not known

After 3 days of growth on PDA at 22 °C in darkness, plugs of *M. fructicola*, 4 mm diameter, were taken from the edge of the colony and placed at the centre of new PDA dishes. Subsequently, the dishes were incubated at 22 °C either in darkness or in 12 h light/12 h dark regime. Similarly, plugs of 4 mm diameter were taken from the edge of colonies of *M. fructigena* and *M. laxa* after 6 days of growth on PDA. Light during incubation was provided by two nUV (near ultraviolet) tubes (Philips TLD, 18W/08), hanging approximately 15 cm above the Petri dishes; three replicates were used per treatment.

After 12-13 days incubation, the shape of the colony margin, site and colour of sporulating areas in the colony, and the presence of zonation were examined. Only dishes from the light/dark regime were observed, as *M. fructigena* and *M. laxa* sporulated sparsely in darkness.

Quantitative characteristics were assessed as follows: mean colony diameter after 3 and 5 days, number of discrete sclerotial plates (stromata) present after 12-13 days, and the number of macroconidia (spores) per dish after 14 days incubation. Mean colony diameter included the 4 mm agar plug placed at the centre of the dish. Spores were removed from the dishes by gently scraping the surface with a sterile needle, after adding 8-10 ml of distilled water to the dishes. Three droplets of Tween-80 (wetting agent) were added per litre distilled water. The resultant suspensions were whirled on a rotary shaker at 1500 rpm for 30 s, the suspension was passed through two layers of cheesecloth to remove large mycelial fragments, and the number of conidia per ml was determined using a haemocytometer.

Germ tube characteristics

Conidia of all three species were grown on PDA under nUV-light (12 h light/12 h dark) at 22 °C. Depending on the species, conidia were washed from the dishes either 4 days (*M. fructicola*) or up to 14 days (*M. fructigena, M. laxa*) after incubation. Conidia were harvested in 3-5 ml distilled water, after scraping the cultures with a needle. The conidial suspension was whirled on a rotary shaker for 30 sec at 2200 rpm, and then poured through a double layer of cheese cloth. The suspension was adjusted to 1×10^5 spores/ml, and 0.1 ml was used to inoculate a dish of water agar (1.5 % WA, Oxoid, Basingstoke, England). Two dishes per isolate were incubated for 18 h at 22 °C in darkness. Germ tube morphology of 25 conidia per dish was examined. To avoid biased selection of conidia, the four conidia surrounding the first conidium chosen were assessed, and the process was repeated on four other parts of the dish. Thus a total of 50 conidia per isolate were assessed. The following characteristics were determined: the distance from conidium to the tip of the longest germ tube (longest straight length), the distance from conidium to the first branch in the germ tube, and the presence of more than one germ tube per conidium (Fig. 1). A branch was defined as a ramification of the leading germ tube with a length (of the side branch) of at least 20 µm. Measurements were done at 100x magnification with a calibrated micrometer. The percentage of conidia showing branching prior to 100 µm, as well as the percentage of conidia showing more than one germ tube was calculated ($n = 50$).

Statistics

Discriminant analysis (Fisher, 1936) was used to construct classification rules based on different linear combinations of the observed quantitative characteristics. The procedure DISCRIM, part of the statistical program SAS (SAS Institute Inc., Cary, NC, USA) was used to perform the calculations. Data from each set of isolates were used to construct discriminant functions for colony and germ tube characteristics separately.

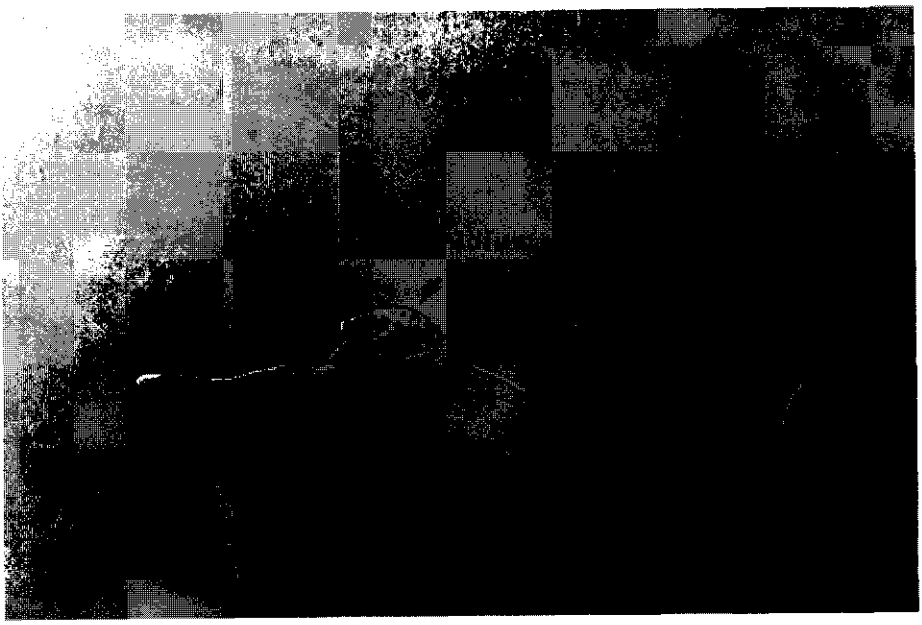

Fig. 1. Conidium of *M. fructigena* germinating with two germ tubes. Branching of the (leading) germ tube takes place at approximately 75 µm from the conidium. Scale bar = 10 µm.

In addition discriminant functions were constructed on the basis of the group of isolates for which both colony as well as germ tube characteristics were determined (Table 1). Validation of the obtained functions was done using the so called 'jackknife technique' (cross-validation), resulting in error-rate estimates for each species. With this technique, the isolate to be classified is not included in the construction of the discriminant functions. This isolate is classified on the basis of classification rules fixed by the remaining group of isolates from the set of isolates under study. An isolate was considered to be misclassified when it was designated to a group different from its initial identification on the basis of general colony characteristics. Each isolate was designated to the group with the highest resulting value of the discriminant function (*d* value). The increase in colony diameter from day 3 to day 5 and the log10-transformed number of conidia per dish after 14 days were used as quantitative colony characteristics. Among the germ tube characteristics, the distance from conidium to the tip of the longest germ tube (longest straight length) in µm was used as a quantitative characteristic. Arcsine transformation was applied to the percentages of conidia branching prior to 100 µm and to the percentages of those showing more than one germ tube. Values of 0 % were substituted by (1/4*n*) %, where *n* was 50 (Gomez & Gomez, 1984).

Results

Qualitative colony characteristics
Qualitative colony characteristics of the three brown rot fungi differed considerably. *M. laxa* showed a distinctive growth rhythm; radial growth was checked several times, after which growth resumed. This resulted in a typical rosette pattern of the colony, with the formation of dark, intramatrical rings at the sites where growth had been checked (Figs. 2e and 2f). The other two species did not show this growth rhythm; radial growth was continuous and the colony margin was more or less even. In darkness, however, some *M. fructigena* isolates also formed a kind of rosette colony pattern. Sporulation in *M. fructicola* took place over the entire surface of the colony. Distinct concentric rings of aerial mycelium were formed by *M. fructigena* on which sporulation occurred (Figs. 2c and 2d). Colonies of *M. laxa* initially produced spores closely appressed to the agar; however, after 10-14 days little tufts of aerial mycelium developed on which sporulation also occurred. The colour of sporogenous tissue was distinctively buff/luteous in *M. fructigena*, and hazel/isabelline in the other two species (Rayner, 1970).

Quantitative colony characteristics
Mean colony diameters measured after 3 and 5 days are shown in Table 2.

Table 2. Colony diameter (mm) of three *Monilinia* spp. on PDA after 3 and 5 days incubation at 22 °C under two different light regimes.

	Darkness		12 h light/ 12 h darkness	
	3 days	5 days	3 days	5 days
M. fructicola ($n = 14$) [a)]	40.5 (33-51) [b)]	62.7 (45-75)	43.6 (31-53)	70.1 (48-83)
M. laxa ($n = 11$)	22.7 (17-30)	34.2 (28-46)	21.5 (17-30)	30.8 (23-42)
M. fructigena ($n = 14$)	16.0 (10-26)	23.4 (14-40)	21.1 (15-29)	33.6 (24-46)

[a)] number of isolates.
[b)] range of colony diameter given in parentheses.

Colony diameter of *M. fructicola* was considerably higher than that of the other two species. The range in colony diameter for the set of *M. laxa* and *M. fructigena* isolates showed a considerable overlap. There was a significant difference in colony diameter for *M. fructigena* between both light regimes, after 3 as well as after 5 days (*t*-test, $\alpha = 0.05$).

The number of discrete sclerotial plates showed both inter- and intraspecific variation. In *M. laxa* no discrete sclerotial plates were observed in either light regime. In *M. fructicola* 3 out of 14 isolates showed some sclerotial plates after 12 days in darkness, and 7 out of 14 in light/dark regime (Figs. 2a and 2b). The opposite occurred with *M. fructigena*, where sclerotial plates were favoured by darkness.

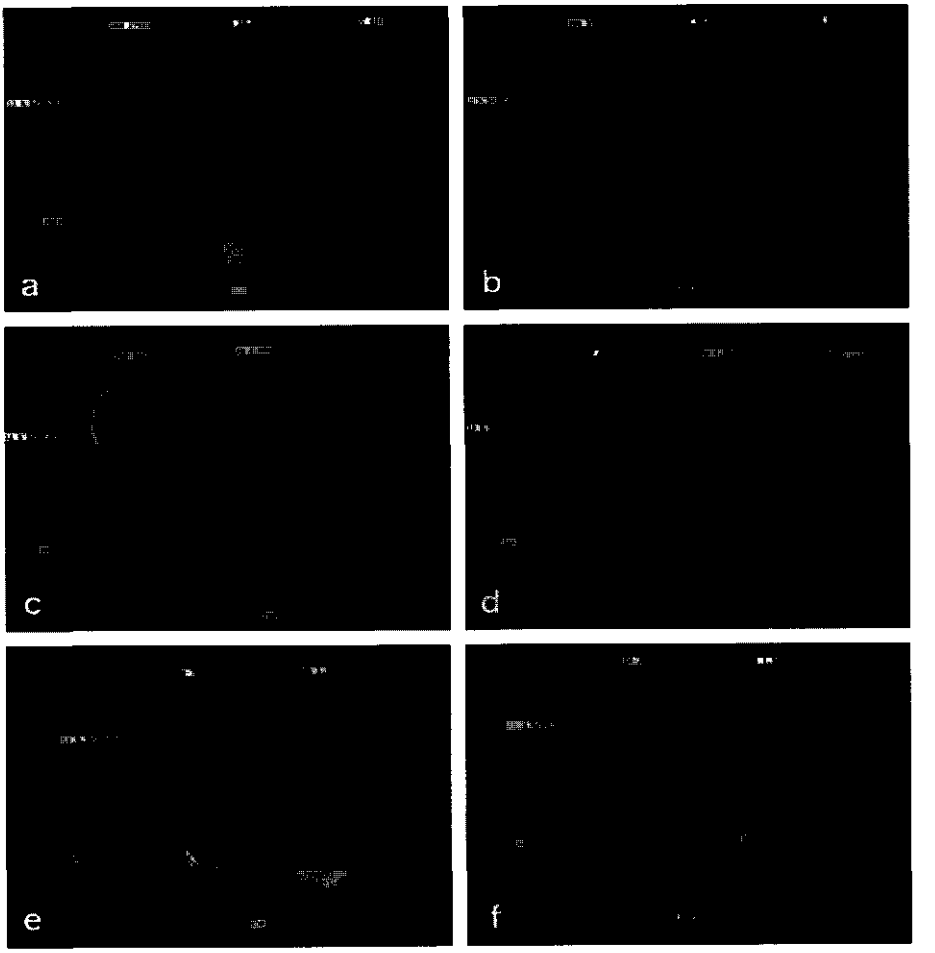

Fig. 2. Top (a, c, e) and bottom sides (b, d, f) of Petri dish cultures (PDA) after 12 days of incubation at 22 °C. *M. fructicola* (a, b), *M. fructigena* (c, d) and *M. laxa* (e, f).

The Japanese isolate (JAP 2317), especially, produced sclerotial plates abundantly in concentric circles around the inoculum plug (Figs. 2c and 2d).

Sporulation in all three species was more abundant in the light/dark regime (Fig. 3). In darkness sporulation was highest in *M. fructicola*, but in the light/dark regime sporulation intensity of some of the isolates of *M. fructicola* overlapped with some of the profusely sporulating isolates of *M. laxa*. Isolates of *M. laxa* from Australia and Japan tended to produce more spores than those from Europe in both light regimes. Under light/dark conditions the sporulation intensity of *M. laxa* lies just in between that of *M. fructigena* and *M. fructicola*.

Germ tube characteristics

Frequency distributions were calculated for total germ tube length and the percentage of conidia showing branching of the germ tube prior to 100 μm (Figs. 4 and 5). The mean length of the germ tube ranged from 465 to 851 μm for *M. fructicola*, and from 161 to 466 μm for *M. laxa*. *M. fructigena* had an intermediate range; the minimum was 307 μm while the maximum was 806 μm. There was much intraspecific variation in germ tube branching (Fig. 5). In general, the highest percentages of conidia branching prior to 100 μm occurred in the *M. laxa* isolates; 6 out of the 15 isolates showed percentages higher than 50 %. The presence of more than one germ tube per conidium was most prominent in *M. fructigena*. Every *M. fructigena* isolate tested had some conidia with more than one germ tube. In *M. fructicola*, this occurred with 5 out of 14 isolates, and with *M. laxa* 4 out of 15 isolates.

Discriminant analysis

Discriminant analysis was applied separately to both sets of quantitative characteristics (colony and germ tube features, two and three quantitative characteristics, respectively), and also to the common set of isolates, including a combination of colony and germ tube characteristics. From the common set of isolates, isolate CBS 165.24 was excluded from the analysis, because it was an old isolate (1924) of unknown origin.

The two sets of data (dark and light/dark regime) for the colony characteristics were described by the following linear classification functions in darkness for *M. fructicola*:

$$d_1 = -106.4 + 0.86x_1 + 27.2x_2$$

M. laxa:

$$d_2 = -47.8 + 0.33x_1 + 18.9x_2$$

and *M. fructigena*:

$$d_3 = -43.9 + 0.08x_1 + 18.6x_2$$

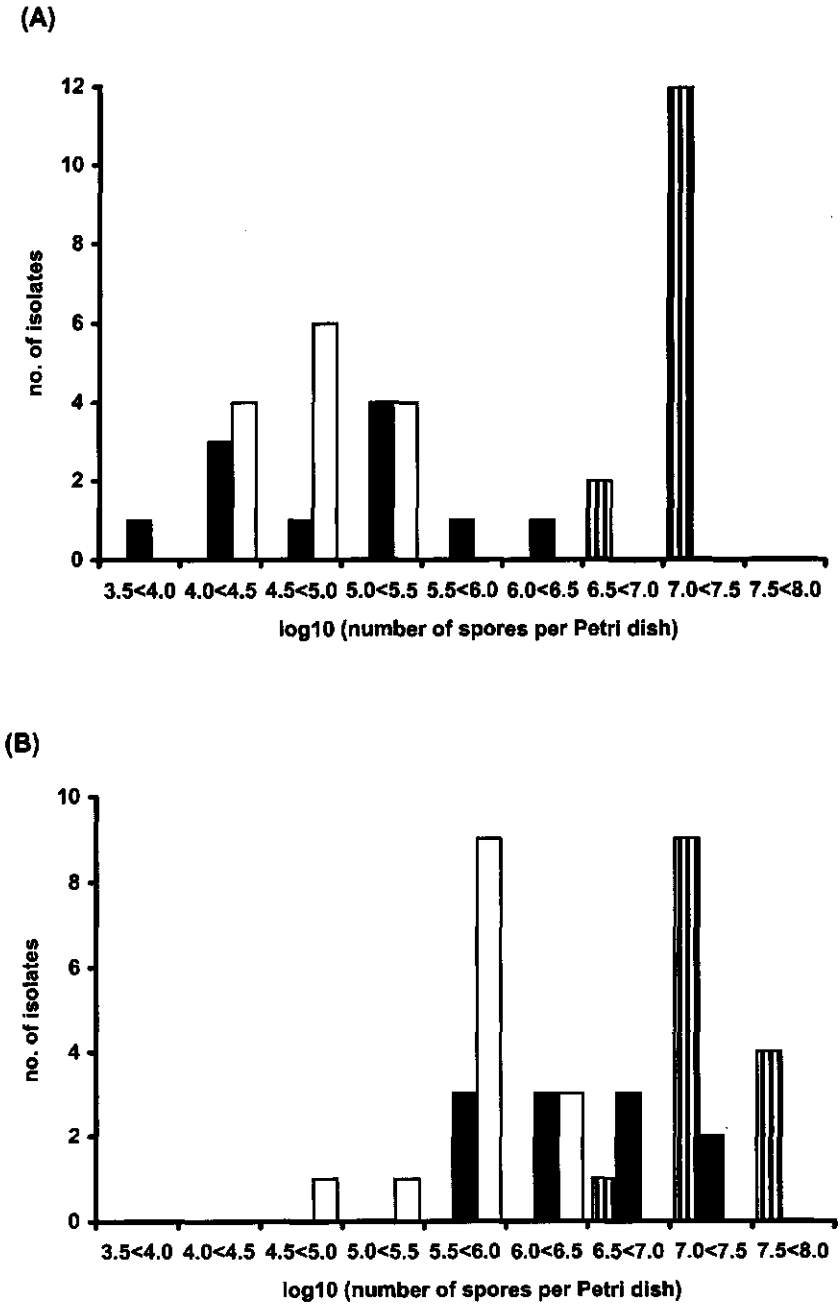

Fig. 3. Frequency distribution of log10-transformed sporulation intensity of *Monilinia fructicola* (▥), *M. fructigena* (□), and *M .laxa* (■) after 14 days incubation at 22 °C
(A) continuous darkness
(B) 12 h light/12 h darkness

and in the light/dark regime for *M. fructicola*:

$$d_1 = -211.5 + 2.13x_1 + 49.9x_2$$

M. laxa:

$$d_2 = -144.3 + 0.28x_1 + 45.0x_2$$

and *M. fructigena*:

$$d_3 = -117.2 + 0.73x_1 + 39.6x_2$$

where x_1 is the increase in colony diameter (mm) from day 3 to day 5, and x_2 is the log10-transformed number of spores per dish after 14 days. Cross-validation of the functions obtained showed that the error-rate estimates for the individual species were lowest in the light/dark regime. In darkness, error-rate estimates were 0, 0.36 and 0.29 for respectively *M. fructicola*, *M. laxa* and *M. fructigena*. From the 11 isolates tested in the *M. laxa* group, 4 were assigned to the *M. fructigena* group; in *M. fructigena* 4 out of 14 isolates were designated as *M. laxa*. No misclassifications occurred in the *M. fructicola* group. The classification functions in light/dark regime turned out to be more robust and reliable. Out of the total of 39 isolates, only two isolates were misclassified. Error-rate estimates were 0.07 (1 out of 14), 0.09 (1 out of 11) and 0 for respectively *M. fructicola*, *M. laxa* and *M. fructigena*. In the *M. fructicola* group, isolate DAR 27031 was designated to *M. laxa*, most likely because of its comparatively low growth rate (colony diameter 31 mm after 3 days in light/dark regime). *M. laxa* isolate CBS 165.24 was placed in the *M. fructigena* group.

Discriminant analysis was applied to three different combinations of the germ tube characteristics. The following functions were obtained for the combination of total germ tube length and percentage of conidia with more than one germ tube for *M. fructicola*:

$$d_1 = -18.4 + 0.058x_1 - 12.0x_2$$

M. laxa:

$$d_2 = -4.1 + 0.027x_1 - 4.1x_2$$

and *M. fructigena*:

$$d_3 = -14.7 + 0.035x_1 + 15.9x_2$$

where x_1 is the length of germ tube in µm, and x_2 is the arcsine square root transformed proportion of conidia with more than one germ tube.

The cross-validation results showed three misclassified isolates out of the total set of 41 isolates. In *M. fructicola,* isolate JAP 1438 was designated as *M. fructigena*, most likely because mean length of the germ tube was rather low in combination with a high percentage of conidia with double germ tubes (12 %). Isolate CBS 165.24, classified as *M. laxa* on the basis of general colony characteristics, was again assigned to the *M. fructigena* group (confer colony data). Finally, a Dutch *M. fructigena* isolate (PD 4.96m1) could not be classified as such, solely on the basis of the combination of the two characteristics mentioned. This isolate was assigned to the *M. fructicola* group. Error-rate estimates for *M. fructicola*, *M. laxa*, and *M. fructigena* were 0.07, 0.07 and 0.08, respectively.

The combination of the characteristics of germ tube length and percentage of conidia branching prior to 100 µm, turned out to be less useful in delineating the three

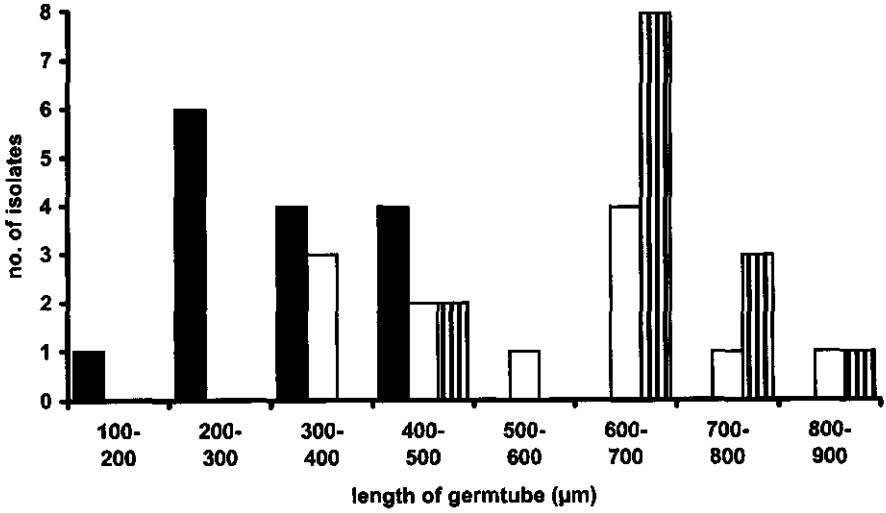

Fig. 4. Frequency distribution of the length of the germ tube of *Monilinia fructicola* (▥), *M. fructigena* (□), and *M. laxa* (■) after 18 h incubation at 22 °C in darkness.

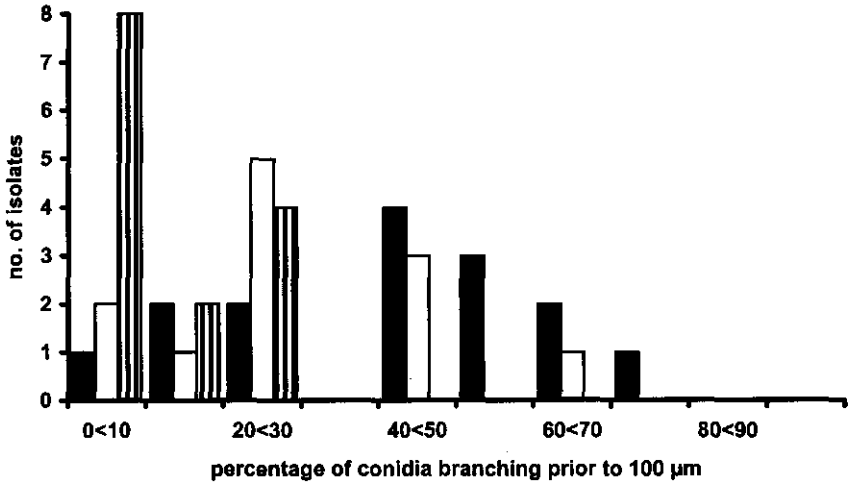

Fig. 5. Frequency distribution of the percentage of conidia showing branching prior to 100 µm of *Monilinia fructicola* (▥), *M. fructigena* (□), and *M. laxa* (■) after 18 h incubation at 22 °C in darkness.

Monilinia spp.. Within *M. fructicola,* 4 isolates out of 14 were misclassified. For *M. laxa*, this was 2 out of 15 isolates, and 6 isolates out of 12 were misclassified in *M. fructigena* (data not shown).

A combination of all three germ tube characteristics did not perform any better than the combination of germ tube length and percentage of conidia with more than one germ tube. As in the analysis for two characteristics (germ tube length/number of germ tubes), 3 misclassifications occurred out of 41 isolates. The isolates JAP 1438 (*M. fructicola)* and PD 4.96m1 (*M. fructigena*) were again designated as *M. fructigena* and *M. fructicola* respectively. Within the group of *M. laxa*, JAP 1390 was misclassified and placed in the *M. fructigena* group. This isolate showed a low percentage of conidia branching prior to 100 µm (18 %), combined with a high percentage of conidia with more than one germ tube (12 %).

The common set of isolates consisted of 11 isolates of *M. fructicola*, and 9 each of *M. laxa* and *M. fructigena*. Data for colony characteristics were only taken from the light/dark regime. The combination of the two characteristics, length of germ tube and growth rate, resulted in two misclassifications. Isolate DAR 27031 was again misclassified, this time the isolate was designated to *M. fructigena*. Isolate PD 8.96 (*M. fructigena*) was put in the *M. laxa* group, most likely because of a short germ tube length. Error-rate estimates for *M. fructicola*, *M. laxa*, and *M. fructigena* were resp. 0.09, 0 and 0.11. The addition of one extra character, sporulation intensity, to this set resulted in only PD 8.96 being misclassified. Addition of the percentage of conidia with more than one germ tube, instead of sporulation intensity, did not improve delineation of the species. The same result was obtained as with only the two characteristics.

Discussion

This paper presents a simple procedure based on quantitative colony and germ tube features only for delineating the three brown rot fungi. The examination of germ tube features only, provides the best means for correct identification. On the basis of the two features 'length of the germ tube' and 'number of germ tubes per conidium', almost all tested isolates could be designated to the right species. Out of a set of 14 *M. fructicola* isolates, 15 *M. laxa* isolates and 12 *M. fructigena* isolates, only one isolate in each species was misclassified on the basis of these two characteristics. Measurement of these two features must be the basis for identification of an unknown isolate. The standardized method of obtaining conidia on PDA under nUV light allowed us to measure the increase in colony diameter from day 3 to 5 (growth rate), before spores were washed off to test germ tube characteristics. With these three parameters we were able to correctly identify nearly all the isolates within the group of common isolates (Table 1). Only 2 misclassifications out of 29 isolates tested were observed. Addition of one extra feature, sporulation intensity, reduced the number of misclassifications to only one. Therefore, the sporulation intensity of the species on agar cultures can be a useful quantitative trait to include in a standard assay. However, the contribution of sporulation intensity, assessed after an incubation period shorter than 14 days, to the performance of a discriminant analysis should be first determined. In general, growth rate and germ tube characteristics can be determined within 7 or 8 days of incubation.

Data on quantitative colony and germ tube features of the three brown rot fungi are scattered throughout the literature and have never been combined to serve as a protocol for delineation. Mostly only two species of the brown rot fungi were studied at the same

time. In our experiments we observed that the growth rate of *M. fructicola* on PDA was consistently higher than that of *M. laxa* and *M. fructigena* (Table 2). In this, *M. laxa* and *M. fructigena* showed overlap to a large extent. However, Batra (1979) stated that *M. laxa* can be distinguished from *M. fructicola* and *M. fructigena* by its slower growth rate. He worked mainly with material from the United States. The author mentions a colony diameter of 8 cm after 6 days for *M. fructicola* on PDA, 8.5 cm after 7 days for *M. fructigena* on YEMEA (yeast extract- malt extract agar), and 4 cm after 6 days for *M. laxa* on PDA (all in 12 h light/12 h dark cycle) (Batra, 1991). In our study mean colony diameters for *M. laxa* were more or less equal to those for *M. fructigena* after 5 days under similar conditions (Table 2). Sonoda & Ogawa (1982), in a study of the growth rate of *M. fructicola* on PDA (isolates from California), found growth rates similar to ours; colony diameter for benomyl-sensitive isolates varied from 35.7 to 46.7 mm, and for benomyl-resistant isolates, from 33.0 to 44.7 mm after 3 days at 20 °C.

Many researchers report 'sparse' sporulation for *M. laxa* and abundant sporulation for *M. fructigena* on PDA in a light/dark regime, especially at temperatures above 20 °C (Jenkins, 1965a; Byrde & Willetts, 1977; Batra, 1991). We did not find a clear difference in sporulation intensity between *M. laxa* and *M. fructigena*. On the contrary, mean sporulation per isolate was even higher for *M. laxa* compared with *M. fructigena*, especially in a light/dark regime. Number of conidia per dish found in our experiments for *M. laxa* agree well with other findings. Ogawa *et al.* (1978) recorded 1×10^7 spores per Petri dish for *M. laxa* on PDA, numbers were comparable with the counts for *M. fructicola* isolates in the same experiment. Tamm & Flückiger (1993) obtained similar spore counts while using a medium based on yeast powder extract and frozen apricot. Data for sporulation in darkness given by Pascual *et al.* (1990) are similar to ours under the same conditions. No quantitative data for *M. fructigena* are known from the literature. A qualitative assessment of the extent of sporulation on the basis of an agar culture, will most probably lead to the conclusion that *M. fructigena* sporulates more abundantly, because of its distinctive concentric rings which rise above the colony's surface on which sporulation occurs.

Germ tube morphology has been mentioned by many authors as a useful characteristic to delineate the brown rot species (Wormald, 1920; Jenkins, 1965a; Gupta & Byrde, 1988). Ezekiel (1924) studied germ tube morphology in drop cultures of potato dextrose decoction at 25 °C. After 18 h of incubation he measured an average length of the germ tube of 769.5 µm for *M. fructicola*, and 140.6 µm for *M. laxa*, using only one isolate for each species. These figures agree well with the range of figures we found for both species. Other authors however, do not give any quantitative data on germ tube morphology, although they stress the fact that branching of the germ tube is a useful characteristic to separate *M. laxa* from *M. fructicola/M. fructigena*. *M. laxa* produces germ tubes that branch at a short distance from the conidium, while in *M. fructicola* and *M. fructigena* germ tubes grow out as a single hypha for some 400-1200 µm before branching (Wormald, 1920). On the contrary, in our experiments we found many *M. fructicola* and *M. fructigena* isolates that also showed branching of the germ tube close to the conidium (prior to 100 µm). One of the problems in comparing our results with those in the literature lies in the fact that branching is not exactly defined. We only considered a ramification of the leading germ tube as a branch, when this side branch was at least 20 µm long. Moreover, different media have been used to study germ tube morphology. Wormald (1920) used a solid medium (prune juice agar), while other researchers used different kinds of liquid media, for example a solution of 0.1 % dextrose + 1 % orange juice (Jenkins, 1965a) or just distilled water (Calavan & Keitt,

1948). Finally, the incubation time varies considerably, from the 18 h we used up to 24-48 h of incubation (Wormald, 1920; Penrose *et al.*, 1976). A typical feature of *M. fructigena* in our experiments was the occurrence of more than one germ tube per conidium. Interestingly, Wormald (1920) mentions the common occurrence of a short germ tube (10-30 μm) in *M. fructigena*, opposite to the end of the conidium where the (long) germ tube develops.

Discriminant analysis on the basis of quantitative colony and germ tube characteristics showed that it was possible to delineate the three brown rot fungi solely with the use of strictly defined, quantitative features. The use of quantitative, unambiguously defined characters to separate these *Monilinia* species is highly desirable, because qualitative features obstruct an unanimous interpretation of characters by different workers in the field of taxonomy. Ogawa & English (1954) described characteristics like 'sclerotia-like bodies' and 'root-like projections of hyphae' to differentiate atypical isolates of *M. laxa* and *M. fructicola*. The interaction of cultures on oatmeal agar was used by Sonoda *et al.* (1982) to distinguish *M. laxa* from *M. fructicola*. The authors considered the formation of 'distinct, sometimes black lines' between isolates as a feature to separate the two species. Our study is the first approach within the taxonomy of the brown rot fungi to delineate the three species solely on the basis of well-defined, quantitative characteristics.

The method described in this paper offers a simple protocol to delineate the brown rot fungi. Only standard laboratory equipment is needed to do the tests for colony and germ tube characteristics. The method will enable diagnostic personnel in harbour and dock sites to accurately identify the three species of the brown rot fungi occurring on imported fruits. As long as other diagnostic tools are not available, such as antibody assays (ELISA) and DNA techniques, the morphological and cultural characteristics of the brown rot fungi will be the key factors for identification. Recently, a species-specific primer for *M. fructicola* has been developed (Fulton & Brown, 1997). In the future, application of diagnostic techniques based on DNA may contribute to an improved and rapid identification of *Monilinia* species.

Chapter 3

Genetic variation among and within *Monilinia* species causing brown rot of stone and pome fruits

Abstract

Nucleotide sequence analysis of the internal transcribed spacer (ITS) regions 1 and 2 of the ribosomal DNA (rDNA) divided the three brown rot pathogens *Monilinia laxa*, *M. fructicola* and *M. fructigena* into four distinct groups. Isolates of *M. fructigena* received from Japan, which varied by 5 base substitutions in the ITS region from the European *M. fructigena* isolates, formed the fourth group. Four of five Japanese isolates of *M. fructicola* tested varied from the New World isolates in that they did not possess a group-I intron in the small subunit (SSU) rDNA. RAPD-PCR data indicated that isolates of *M. laxa* varied but were randomly distributed worldwide; ITS data indicated no apparent distinction between those from *Malus* spp. and those from *Prunus* spp.. *M. fructigena* similarly did not cluster according to geographic origin. In contrast, *M. fructicola* isolates tended to be clustered according to their origin; Japanese isolates of *M. fructicola* clustered together and showed similarity to some of the New Zealand isolates. Isolates from USA and Australia were more variable.

Introduction

Three species of *Monilinia* cause brown rot in *Prunus* spp. (stone fruit) and *Malus* and *Pyrus* spp. (pome fruit) (Byrde & Willetts, 1977). *M. fructigena* is mainly a fruit pathogen, *M. laxa* is considered to be more a pathogen of blossoms and twigs than of fruit and *M. fructicola* is a pathogen of blossom, twigs and fruit but mainly affects stone fruits. In several regions of the world two of the three species coexist. *M. fructigena* is found mainly in the 'Old World', notably Europe and Asia and coexists with *M. laxa*. *M. fructicola* and *M. laxa*, which produce similar disease symptoms and have common hosts, coexist in the 'New World', notably North America and Australia; these species show slight differences in their pathogenicity (Ogawa & English, 1960). Only in Central and Eastern Asia, where *Pyrus*, *Prunus* and *Malus* spp. originate, do all three species of *Monilinia* coexist (Wormald, 1927; Terui & Harada, 1966). It is suggested that the *Monilinia* spp. in an ancestral form first became associated with wild fruit trees in this region and it was postulated by Byrde & Willetts (1977) that an ancestral form of the brown rot fungi was similar to *M. laxa*. They speculated that *M. fructigena* and *M. fructicola* may have evolved from variants of the ancestral form, the latter in North America before the parental form lost the ability to produce apothecia. Phylogenetic evidence supports the view that *M. laxa* was derived first and possibly was more or less similar to the common ancestor of these *Monilinia* species (Holst-Jensen *et al.*, 1997a). Morphological differences between Japanese isolates of *Monilinia* spp. and isolates from elsewhere have been observed and reviewed by Byrde & Willetts (1977) and Batra & Harada (1986). In this paper we describe some genetic variation among isolates of all three species from different geographical regions, but with particular reference to variation in isolates of *M. fructigena* and *M. fructicola* from Japan.

Materials and methods

Fungal cultures
Seventy-one isolates of the three brown rot *Monilinia* species *M. laxa*, *M. fructigena* and *M. fructicola* were used in this study. Many of the isolates were included in a previous study and their origin and identification are given in Fulton & Brown (1997). Isolates cc 954 and ispave 926 previously tabulated were not used in the present study. The origin and identification of the remaining isolates are given in Table 1. *Monilinia* isolates were maintained on potato dextrose agar (PDA) (Oxoid) at 23 °C.

DNA extraction
All fungal isolates were grown over sterile discs of cellophane on PDA plates to facilitate the subsequent removal of the mycelium from the agar. DNA was extracted using a phenol/chloroform extraction method based on that of Raeder and Broda (1985) but with a third additional phenol/chloroform extraction. The genomic DNA pellet was re-suspended in sterile distilled water to give a final concentration of approximately 20-50 ng μl^{-1}. DNA quality was assessed by gel electrophoresis on 1% (w/v) TBE agarose gels stained with ethidium bromide.

PCR amplification and sequencing of ITS regions
The ITS 1, 5.8S and ITS 2 regions of the fungal rDNA were amplified using the primers ITS 1 ext (GTA ACA AGG TTT CCG TAG GTG, an extended primer of ITS1, White *et al.*, 1990) and ITS 4 (White *et al.*, 1990). The reactions were performed on a Perkin Elmer 480 Thermal Cycler using the following programme: 1 min at 94 °C, 1 min 30 s at 53 °C, 2 min at 72 °C, for 30 cycles. The reaction mixtures consisted of 50 ng template DNA, 1.5 mM $MgCl_2$, 1 unit Red Hot *Taq* polymerase (Advanced Biotechnologies), 200 µM dNTPs (Sigma Chemical Co.), 1 X PCR Buffer IV (75 mM Tris-Cl, pH 9.0, 20 mM $(NH_4)_2SO_4$ 0.1% (w/v) Tween) and 0.6 µM of primers ITS 1 ext and ITS 4. DNA in aliquots of the individual PCR reactions were quantified by gel electrophoresis as previously described.

Individual PCR products were purified using the Wizard™ DNA Clean Up kit (Promega) according to the manufacturer's instructions. PCR products were sequenced using double-stranded template (200 ng μl^{-1}) and 1 µM of the ITS1 ext (forward) and ITS4 (reverse) primers, following the protocol supplied with the Prism™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Sequencing was conducted on an Applied Biosystems Model 373A DNA sequencer and the sequence data were compiled and edited using Sequence Navigator™ software (Applied Biosystems). Phylogenetic analyses were conducted using the Molecular Evolutionary Genetics Analysis program (Kumar *et al.*, 1993).

PCR amplification and sequencing of SSU rDNA
The SSU rDNA region from the *M. fructicola* isolates listed in Table 1 was amplified using the universal primers NS1 and NS8 (White *et al.*, 1990) and the PCR products sequenced as previously described (Fulton & Brown, 1997).

RAPD-PCR amplification and data analysis
For RAPD analysis, primers A03 (AGT CAG CCA C), A11 (CAA TCG CCG T), A13 (CAG CAC CCA C), B06 (TGC TCT GCC C), B07 (GGT GAC GCA G) and B10 (CTG CTG GGA C), obtained from Operon Technologies, CA, USA, were used after they were shown to produce clear, reproducible, polymorphic banding patterns with the isolates used in this study. The reactions were again performed on a Perkin Elmer 480 Thermal Cycler using the

Table 1. Seventeen of the 71 isolates of *Monilinia laxa*, *M. fructigena* and *M. fructicola* used in this study (the remaining 54 isolates are listed in Fulton & Brown, 1997)

Isolate no.	Origin	Host	Date
Monilinia laxa			
cc 952	UK	*P. domestica*	1996[B]
PC 239	UK	*Malus* spp.	1996[C]
PC 261	UK	*Malus* spp.	1996[C]
PC 264	UK	*Malus* spp.	1996[C]
PC 271	UK	*Malus* spp.	1996[C]
M. fructigena			
cc 782	UK	*Malus* spp.	1994[B]
jap 1815	Japan	*M. pumila*	1991[A]
jap 2314	Japan	*M. pumila*	1994[A]
jap 2315	Japan	*M. pumila*	1994[A]
jap 2316	Japan	*M. pumila*	1994[A]
pd 27.96	Netherlands	*Pyrus domestica*	1996[A]
M. fructicola			
mf-2	USA	*Prunus* sp.	?[A]
mf-74	USA	*Prunus* sp.	?[A]
jap 1438	Japan	*M. pumila*	1989[A]
jap 1535	Japan	*Prunus* sp.	1990[A]
jap 2636	Japan	*P. cerasus/avium*	1995[A]
jap 2810	Japan	*Prunus* sp.	1996[A]

[A] Cultures supplied by Plantenziektenkundige Dienst (Plant Protection Service), Wageningen, NL.
[B] Cultures supplied by Central Science Laboratory, York, UK.
[C] Cultures supplied by Horticulture Research International, East Malling, UK.

following programme conditions - 1 min 10 s at 94 °C, 2 min at 30 °C and 2 min at 72 °C, with 1 °C s^{-1} ramping to the extension step, for 45 cycles. The reaction mixtures were as above, except that 1 µM of the random primer was used per reaction. Ten µl aliquots of the RAPD- PCR products were run on 1% (w/v) TBE high resolution agarose (Sigma Chemical Co.) and the gel images were scanned using the Grab-It (ver. 2.5, Synoptic Ltd., 1993-97) software package. The banding patterns were analysed using the Phoretix 1D Advanced (ver. 3.01, Non Linear Dynamics Ltd., 1996-97) software package and a similarity matrix produced from simple matching coefficients. Statistical analyses were performed with PHYLIP 3.57 (Felsenstein, 1993) and the trees constructed using the Neighbor-Joining method (Saitou & Nei, 1987).

Results

ITS nucleotide sequence analysis

Nucleotide sequence data were obtained for the ITS 1-5.8S - ITS 2 region of all 71 *Monilinia* isolates and, with the exception of the Japanese isolates of *M. fructigena*, concurred with previously published data (representative EMBL accession nos. *M. laxa*, Z73784; *M. fructigena*, Z73779; *M. fructicola*, Z73777; Holst-Jensen *et al.*, 1997a). The Japanese isolates of *M. fructigena* (EMBL accession no. Y17876) differed from the previously reported sequence for *M. fructigena*, by four transitions within the ITS 1 region and one transition in the ITS 2 region. Both *M. laxa* and *M. fructicola* differed from the Japanese *M. fructigena* sequences by ten variable sites within the ITS1/2 region. Phylogenetic analyses of the ITS nucleotide sequences, including *Sclerotinia sclerotiorum* as an outgroup (EMBL accession no. Z73799), using the Kimura 2-Parameter method (Kimura, 1980) to calculate distance and the Neighbor-Joining method (Saitou & Nei, 1987) to construct the tree revealed that the Japanese *M. fructigena* isolates were more closely related to the non-Japanese *M. fructigena* isolates than to the other two species (Fig. 1).

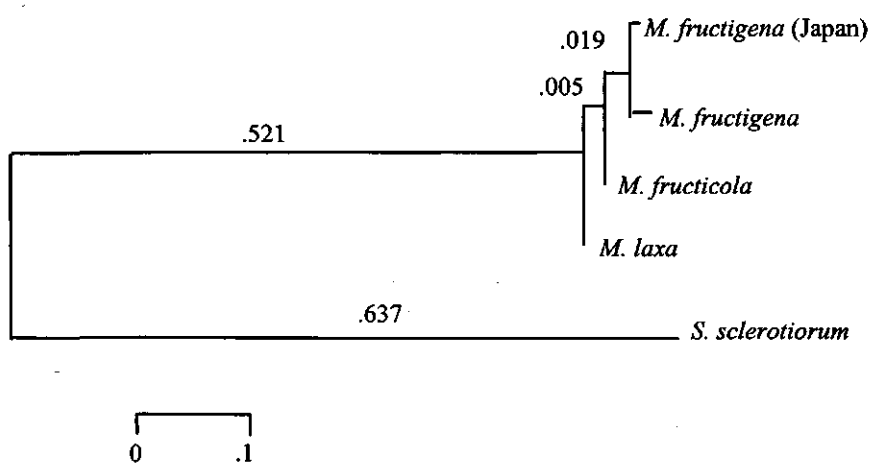

Fig. 1. Neighbor-Joining phylogenetic tree produced from nucleotide sequence analysis of the ITS 1, 5.8S gene and ITS 2 region from isolates of *Monilinia laxa*, *M. fructigena* and *M. fructicola*, using the MEGA statistical package (Kumar *et al.*, 1993). *Sclerotinia sclerotiorum* was included as an outgroup. The pairwise genetic distance values were produced using the Kimura 2-Parameter algorithm.

SSU rDNA sequence analysis

The non-Japanese *M. fructicola* isolates tested revealed the presence of the group-I intron at position 943 in the SSU rDNA (Fulton & Brown, 1997). However, the Japanese *M. fructicola* isolates, with the exception of isolate jap 1829, did not possess an intron in the SSU rDNA.

RAPD-PCR data

Total DNA from 58 *Monilinia* isolates was assessed for polymorphism using 6 random 10-base primers. Each of the primers amplified reproducible fragments with faint or ambiguous bands being excluded from the analyses. A total of 138 bands across 20 *M. laxa* isolates, 139 bands across 20 *M. fructigena* isolates and 90 bands across 18 *M. fructicola* isolates were analysed. *M. laxa* isolates from *Prunus* hosts were randomly distributed throughout the dendrogram (Fig. 2) and there was no suggestion of geographic groupings.

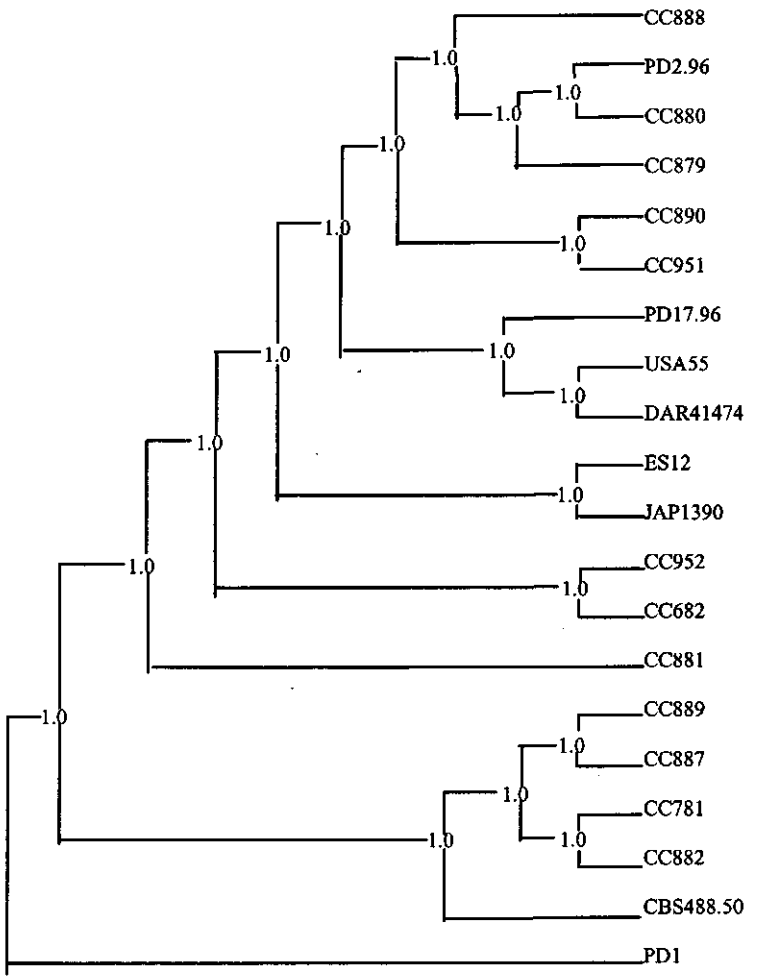

Fig. 2. Clustering of individual isolates of *Monilinia laxa* from pairwise comparison of RAPD-PCR data using PHYLIP (Felsenstein, 1993) (host and origin of isolates are given in Table 1 and Fulton & Brown, 1997).

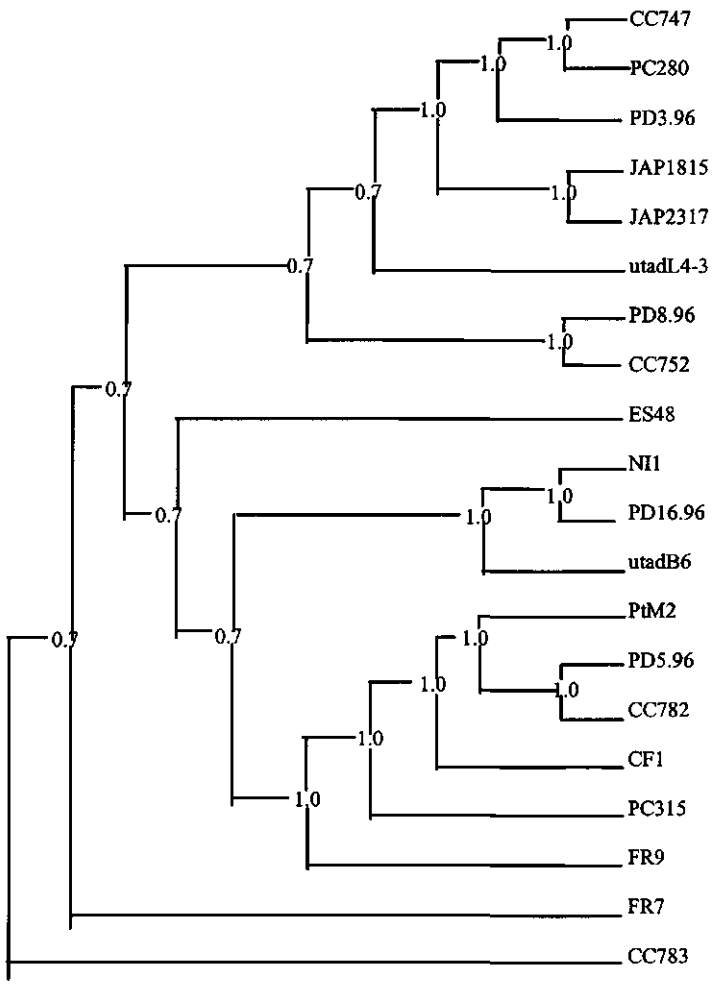

Fig. 3. Clustering of individual isolates of *Monilinia fructigena* from pairwise comparison of RAPD-PCR data using PHYLIP (Felsenstein, 1993) (host and origin of isolates are given in Table 1 and Fulton & Brown, 1997).

Similarly, RAPD-PCR analysis revealed no suggestion of geographic grouping among *M. fructigena* isolates (Fig. 3) despite the ITS sequence differences between European and Japanese isolates. With *M. fructicola*, however, there was clustering of isolates from different geographic regions; the Japanese isolates clustered together but formed two subgroups. The New Zealand isolates also tended to cluster together while isolates from USA and Australia

showed greater variation (Fig. 4). The Japanese isolates appeared more closely related to some of the New Zealand isolates than to the Australian or USA isolates.

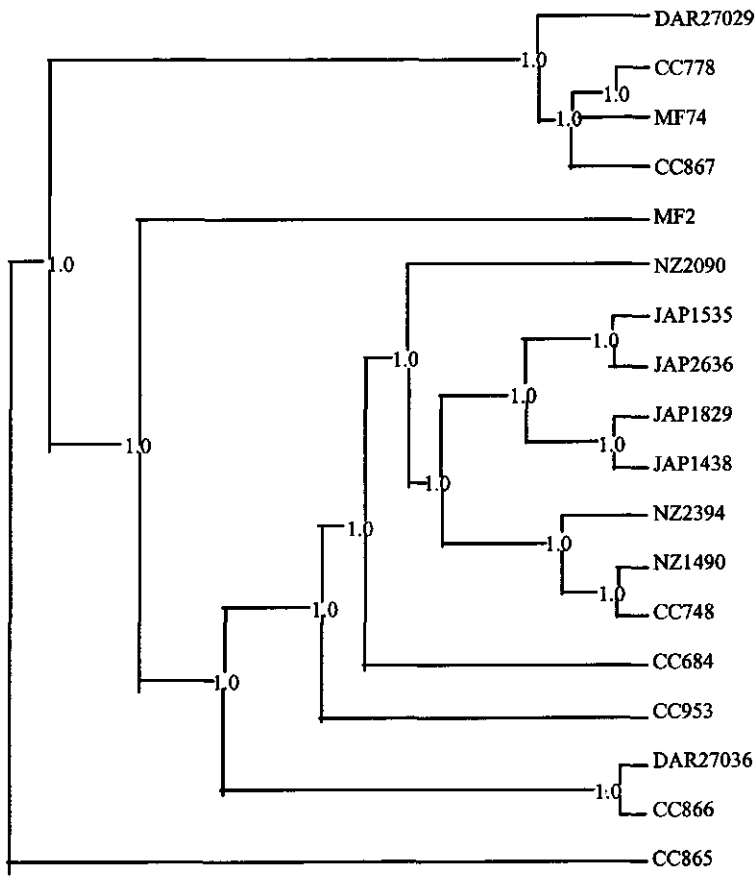

Fig. 4. Clustering of individual isolates of *Monilinia fructicola* from pairwise comparison of RAPD-PCR data using PHYLIP (Felsenstein, 1993) (host and origin of isolates are given in Table 1 and Fulton & Brown, 1997).

Discussion

Genetic information has already been published on *Monilinia* species that cause brown rot of pome and stone fruits (Carbone & Kohn, 1993; Holst-Jensen *et al.*, 1997a, b). In this paper, however, we present some new information on the molecular identification of isolates of *M. fructigena* and *M. fructicola* obtained from Japan. No variation has been observed in the ITS nucleotide sequence of isolates of *M. laxa* from *Malus* and *Prunus* spp. and from worldwide sources (Holst-Jensen *et al.*, 1997a). On the basis of RAPD-PCR data, variation in *M. laxa* appears unrelated to geographic origin. This suggests that *M. laxa* has been randomly distributed worldwide and appears to have readily adapted to its different hosts.

It was suggested that variant forms of a *M. laxa*-like ancestor (Holst-Jensen *et al.*, 1997a) may have evolved in Central or Eastern Asia to give new subspecies or species related to *M. fructigena* that became pathogens of *Malus* spp. (Byrde & Willetts, 1977). The isolates from Japan, designated *M. fructigena*, were however, on the basis of ITS nucleotide sequence data, distinct from European isolates. Isolates with the same ITS sequence as the European *M. fructigena* isolates were not found among *Monilinia* isolates from Japan suggesting that they may not have evolved in Eastern Asia. Although ITS sequence data indicated that the Japanese *M. fructigena* isolates were more similar to the European *M. fructigena* isolates than to either *M. laxa* or *M. fructicola* they were, by comparison with other species of Sclerotiniaceae (Carbone & Kohn, 1993; Holst-Jensen *et al.*, 1997a, b), sufficiently different so as to be possibly regarded as a separate species. The faster growth rate of the Japanese *M. fructigena* isolates and the production of smaller conidia compared with European isolates (van Leeuwen, unpublished, 1998) and morphological variation described by Batra & Harada (1986) could support separation of the distinct ITS rDNA genotypes.

In a previous study (Fulton & Brown, 1997), a group-I intron was located in the SSU rDNA of *M. fructicola* isolates (a 943 SSU group-I intron; Gargas *et al.*, 1995) but not in *M. fructigena* or *M. laxa*. On the basis of this, PCR primers were designed to distinguish *M. fructicola*, which is an EU quarantine pathogen, from the other two brown rot species and for its rapid detection in the host tissues. Since that report further isolates of *M. fructicola* have been analysed and those received from Japan did not contain the intron. This inconsistency renders this rapid detection method somewhat less useful than had been thought previously. *M. fructicola* was first reported in Japan only in 1963 (Terui & Harada, 1966) and could be a relatively recent introduction. Similarities in the RAPD-PCR banding patterns between the Japanese *M. fructicola* isolates and those from New Zealand might suggest that they had a similar origin, Japanese isolates having either lost the intron from the SSU rDNA or developed from an isolate which never possessed one. We do not know whether or not isolate jap 1829, which possessed the group-I intron, was native to Japan or had been isolated from imported fruit. It would be of considerable benefit to investigate further East Asian isolates to obtain a better understanding of the distribution and genetic variation within the brown rot *Monilinia* species.

Chapter 4

Distinction of the Asiatic brown rot fungus *Monilia polystroma* sp. nov. from *Monilia fructigena*

Abstract

Deviating *Monilinia fructigena* strains from Japan were compared with strains from Europe. General colony characteristics, stroma formation, growth rate and conidial dimensions were determined for six isolates each from both groups, as well as sporulation intensity on potato dextrose agar (PDA) and germ tube features. Potential differences in pathogenicity were tested on the pear cultivars Conference and Doyenné du Comice, and on the apple cultivar Cox's Orange Pippin. A marked difference in stroma formation occurred, the area of stromatal plates ranged from 4.11 to 5.19 cm^2 in the Japanese group, and from 0 to 0.85 cm^2 in the European. The mean growth rate was significantly higher for Japanese strains (*t*-test, $P = 0.01$). Length and width of conidia were significantly greater in European strains (*t*-test, $P = 0.01$). Conidia measured on average 19.2 x 11.3 µm in European strains, and 15.9 x 9.8 µm in Japanese ones when grown on cherry agar. On fruits, the difference in conidium size was even more pronounced. Sporulation intensity on PDA and germ tube features did not differ between both groups. No differences were found in latency period, lesion growth rate or sporulation intensity on apple and pear fruits between both groups. Together with previously published differences in the ITS region of ribosomal DNA, our results show that the Japanese isolates belong to a distinct species, *Monilia polystroma* sp. nov.. A description of the anamorph is given, as well as a table summarising key features for all four brown-rot associated *Monilia* species.

Introduction

Since Honey (1928) erected the genus *Monilinia* within the Sclerotiniaceae, the group of brown rot fungi within this genus has always comprised three distinct species: *M. fructicola* (Wint.) Honey, *M. laxa* (Aderh. & Ruhl.) Honey and *M. fructigena* (Aderh. & Ruhl.) Honey. Within *Monilinia*, these three species constitute the section *Junctoriae*. In contrast with the section *Disjunctoriae* they do not possess disjunctors between the mature conidia within the macroconidial chains (Honey, 1936). Identification of the brown rot fungi is based on morphology and general colony characteristics on natural media (Hewitt & Leach, 1939; Byrde & Willetts, 1977; Batra, 1991). Recently, Van Leeuwen & Van Kesteren (1998) developed an identification protocol based on quantitative cultural and morphological features. Lately, delineation of the three species on the basis of sequence divergence within the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) has been given some more attention (Carbone & Kohn, 1993; Holst-Jensen *et al.*, 1997a; Snyder & Jones, 1999). Five basepair substitutions were found between *M. fructicola* and *M. laxa*, while *M. fructigena* showed sequence divergence at 13 positions compared with both other species in the ITS region (Holst-Jensen *et al.*, 1997a; Fulton

et al., 1999). Intraspecific sequence variation was found in *M. fructicola* as well as *M. laxa*, consisting of one to three base substitutions (Holst-Jensen *et al.*, 1997a; Snyder & Jones, 1999). While working with a larger set of *M. fructigena* isolates, Fulton *et al.* (1999) found distinct intraspecific variation in *M. fructigena.* Isolates from Japan had four base substitutions in ITS 1 and one substitution in the ITS 2 region compared with the sequence found in European *M. fructigena* isolates. The sequence for the isolates from Europe concurred with that reported by Holst-Jensen *et al.* (1997a). No further variation was detected within the Japanese nor within the European group.

To what extent genetic variation in the ITS region of rDNA corresponds to morphological and biological differences, varies considerably within the Sclerotiniaceae. Within the section *Disjunctoriae* in *Monilinia, M. gaylussaciae* Batra and *M. vaccinii-corymbosi* (Reade) Honey have identical ITS sequences (Holst-Jensen *et al.*, 1997a), but are distinct morphologically and biologically (Batra, 1991). Phylogenetic analysis on the basis of ITS and partial sequencing of the small (18S) and large (28S) subunits of rDNA, has shown that the section *Junctoriae* forms a monophyletic lineage with three outgroup taxa, *Botryotinia* Whetzel, *Ciboria* Fuckel and *Sclerotinia* Fuckel (Holst-Jensen *et al.*, 1997a). The level of sequence heterogeneity within this lineage is low, while morphologically these are well-delimited genera. For example, *M. fructicola* and *Botryotinia fuckeliana* differ in only four nucleotide sites within the ITS 1 region (Carbone & Kohn, 1993). Given the genetic differences found between Japanese and European *M. fructigena* strains, we expected to find cultural and morphological differences between these two groups. So far, however, a direct comparison of cultural and morphological characteristics between Japanese and European *M. fructigena* strains has not been made.

Morphological as well as biological characteristics may differ between Japanese and European *M. fructigena* strains. Biological characteristics such as host plant range, rate of development and formation of survival structures, form an important aspect in pest risk analysis (OEPP/EPPO, 1997). The host range reported for Japanese *M. fructigena* (Harada, 1998), is similar to that reported for European strains (Byrde & Willetts, 1977), and includes *Malus* spp., *Pyrus* spp., *Cydonia* spp. and *Prunus* spp.. In the life cycle of both Japanese and European *M. fructigena* strains, the teleomorph hardly plays a role. In Europe, Solkina (1931) found apothecia of *M. fructigena* in the field, other workers obtained apothecia only after experimental incubation of mummified fruits (Aderhold & Ruhland, 1905; Johansen, 1945). Also in Japan apothecia are seldom found in the field (Batra & Harada, 1986), but Harada (1977) managed to obtain apothecia *in vitro* with Japanese *M. fructigena* strains.

In this paper we present a comparative study of cultural, morphological and biological characteristics of Japanese and European *M. fructigena* strains. We present evidence that Japanese *M. fructigena* strains should be considered a new species, and discuss the possible evolutionary history of speciation. Finally, potential consequences of our findings for plant health and quarantine aspects will be discussed.

Materials and methods

Set of isolates

Japanese *M. fructigena* isolates were kindly provided by Y. Harada, Faculty of Agriculture and Life Science, Hirosaki University, Japan. A corresponding set of six isolates representative of European *M. fructigena* strains was selected from a culture collection established by the

cooperation of several European institutes and universities. Initially, *M. fructigena* strains were lyophilised and subsequently used for experiments. Later, cultures were maintained on V-8-juice agar slants stored at 10 °C. Vitality of the cultures was guaranteed by stimulating sporulation under near-ultraviolet (nuv) light after every transfer in storage. Host, geographical origin and year of isolation of the cultures are given in Table 1.

Table 1. List of *Monilinia fructigena* isolates used in this study.

Isolate	Host	Origin	Year of isolation
JAP 1145	*Malus pumila*	Japan	1987
JAP 1815	*Malus pumila*	Japan	1991
JAP 2314	*Malus pumila*	Japan	1994
JAP 2315	*Malus pumila*	Japan	1994
JAP 2316	*Malus pumila*	Japan	1994
JAP 2317	*Malus pumila*	Japan	1995
PD 4.96	*Malus pumila*	The Netherlands	1996
PD 8.96	*Prunus persica*	The Netherlands	1996
ES-48	*Prunus domestica*	Spain	1996
CC 752	*Prunus domestica*	Poland	1993
HU 1.96	*Prunus sp.*	Hungary	1996
FR-8	*Prunus armeniaca*	France	199?

Colony morphology, growth rate and conidial dimensions

General colony characteristics commonly used to describe *Monilinia* cultures (Byrde & Willetts, 1977; Batra, 1991) were determined for Japanese and European *M. fructigena* cultures grown on potato dextrose agar (PDA, Oxoid, Basingstoke, UK) in 9 cm diameter, plastic Petri dishes for 10-12 days at 22 °C in 12 h light/12 h dark regime. Light during incubation was provided by two nuv tubes (Philips TLD, 18 W/08) at approximately 15 cm above the Petri dishes.

Formation of stroma on agar plates was quantified by image analysis. For this, isolates were grown on cherry decoction agar (CHA). CHA was prepared by filtering a cherry juice suspension, and adjusting the pH to 4.4 with KOH. After dissolving the agar (Technical Agar no. 3, Oxoid, Basingstoke, UK) in boiling distilled water, one part of cherry juice was mixed with nine parts of the agar medium. The medium was sterilised at 102 °C for 5 min. Dishes were inoculated with *M. fructigena*, and incubated in darkness at 22 °C for six days. Plugs of 4 mm diam were taken from the edge of the colony and placed at the centre of new CHA dishes (six replicates per isolate). Subsequently, plates were incubated at 22 °C in darkness and sealed with parafilm after one day incubation to avoid dehydration of the plates. After 21 days incubation, the area of stroma formation per Petri dish was quantified using an image analyser (Quantimet 570, Cambridge Instruments Ltd., UK). From the bottom side of the plates, the black stromatal plates contrasted well with the yellowish colour of the colony, which facilitated precise selection.

Colony growth rate of Japanese and European *M. fructigena* isolates was compared on PDA. Dishes were inoculated from stock cultures, and incubated at 22 °C in 12 h light/12 h dark regime (nuv). After eight days incubation, plugs taken from the edge of the colony were placed in the centre of new PDA plates. Four replicate plates were incubated at 22 °C in darkness, and four were placed at 22 °C in 12 h light/12 h dark regime (nuv). Mean colony diameter, including the 4 mm plug, was determined after three and six days incubation. Subsequently, the absolute growth rate was calculated in mm day^{-1} between the third and sixth day. The experiment was conducted twice.

The size of conidia obtained from agar cultures and from sporulating fruits was determined for both groups. After 9-13 days incubation at 22 °C in 12 h light/12 h dark regime (nuv), conidia were washed off CHA plates by adding 4-5 ml of distilled water and gently scraping the cultures with a needle. For each isolate, length and width of 35 conidia were measured in distilled water at 400x magnification. Ripe pear fruits (cv. Conference) were inoculated and incubated in closed trays in which the temperature ranged from 14-18 °C. Light intensity was 10.000-12.000 lux in a 14 h light/10 h dark regime. Eight days after inoculation, the lids were removed from the trays to promote dehiscence of conidial chains at lower relative humidity (RH 60-70 %). Conidia were collected in distilled water ten days after inoculation, and per isolate 35 conidia were measured as described above. Experiments *in vitro* as well as *in vivo* were conducted twice.

Mean area of stroma formation, growth rate and conidial dimensions were determined for each isolate, and subsequently the overall means for the Japanese and European group were compared with a *t*-test. For stroma formation, isolates that did not produce any stroma were excluded from the statistical analysis.

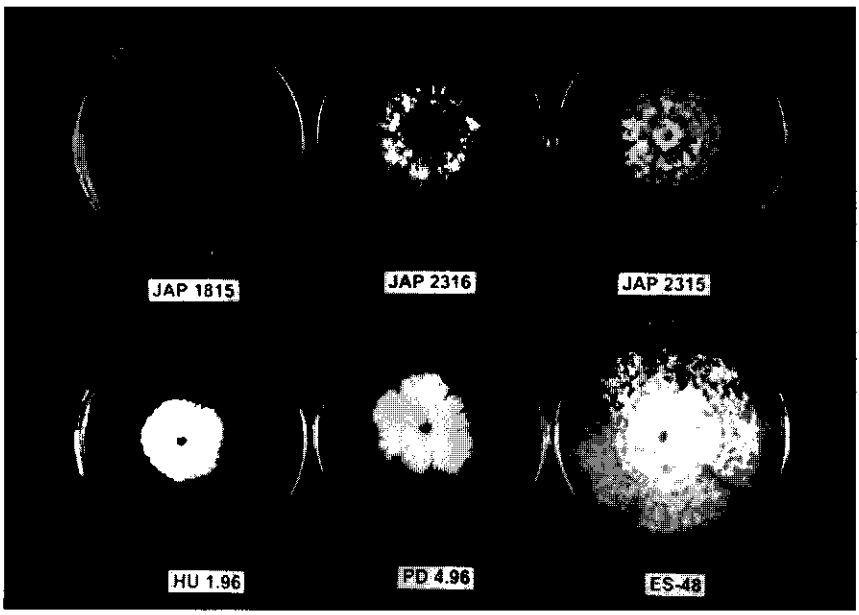

Fig. 1. *M. fructigena* cultures on potato dextrose agar after incubation for ten days at 22 °C in 12 h light/12 h dark regime (nuv). Upper row Japanese strains, lower row European strains.

Increase in colony diameter, sporulation intensity and germ tube characteristics
In a former study, a protocol was developed to delineate *M. fructicola*, *M. laxa* and *M. fructigena* on the basis of quantitative characteristics (Van Leeuwen & Van Kesteren, 1998). Increase in colony diameter, sporulation intensity on PDA, and germ tube characteristics were included in this protocol. To determine whether these characters were also appropriate to distinguish Japanese from European *M. fructigena* isolates, a comparison was made between five isolates of each group. Characters were determined according to the method described in Van Leeuwen & Van Kesteren (1998). Distance from conidium to the first branch in the germ tube was not determined. Means of both groups were compared with a *t*-test, data for sporulation intensity were first log-transformed. Mean percentages of conidia with more than one germ tube were compared with a Wilcoxon-test.

Pathogenicity on fruits
Pathogenicity of Japanese and European *M. fructigena* strains was assessed on pear (cv. Conference, cv. Doyenné du Comice) and apple (cv. Cox's Orange Pippin) in ripe and unripe stage (cv. Doyenné du Comice only in ripe stage). In a small hole punched in the fruit skin (4 mm diam), an agar plug from the edge of an actively growing colony on PDA or CHA was inserted. Subsequently, fruits were incubated in trays with transparent lid (l x w x h= 45 x 30 x 30 cm), with a layer of water underneath the plateau on which the fruits were placed. Six replicate fruits per isolate were used in each experiment, distributed over six trays (blocks). The trays were placed in a climate room. Temperature inside the trays ranged from 14 to 18 °C, RH was 95-98 % and light intensity 10.000-12.000 lux (Philips, TD 32W/84HF) in a 14 h light/10 h dark regime. After inoculation, fruits were examined every day to score the appearance of conidial pustules (latency period). Width and height of the developing lesion was determined after three and six days incubation (ripe Conference, experiment I), or, alternatively, after two and four days (in all other experiments). The lid was removed from the trays after four days in experiments with unripe fruits, and after five days for ripe fruits, to promote dehiscence of conidial chains at lower RH (70-75 %). One day later, conidia were collected by shaking the fruits in 30-40 ml water at 200 rpm for 30 s in an orbital shaker. The suspension was filtered through a double layer of cheesecloth, and subsequently the number of conidia per ml was determined using a haemocytometer. The total number of conidia produced per fruit was calculated by multiplying the number of conidia per ml with the collected volume in ml.

Lesion growth rate in mm day^{-1} was calculated from the difference in lesion size between the two assessment dates. The number of conidia produced per cm^2 lesion was calculated by taking the quotient of the number of conidia produced per fruit and the lesion area on day 4. Sporulation data were log-transformed for statistical analysis. The procedure PROC MIXED of the statistical program SAS (SAS Institute Inc., Cary, NC, USA) was used to test for significant differences between the Japanese and European groups of *M. fructigena* isolates (*F*-test). The factors 'block' and 'isolate' were included as random effects in the model.

Results

Colony morphology, growth rate and conidial dimensions
Small differences were found in general colony morphology between both groups. The development of aerial mycelium on which sporulation occurs, was more profuse in European

M. fructigena strains. In two of the European isolates, aerial mycelium rose 4-5 mm above the colony's surface, while in Japanese isolates this was only 1-2 mm. The colour of sporogenous tissue was distinct buff/pale luteous (Rayner, 1970) in both groups, and clear concentric rings of sporulation appeared in isolates with a high growth rate (e.g. JAP 2315, JAP 2317 and ES-48). After 10-12 days incubation, stroma started to develop in Japanese isolates; especially in isolate JAP 1815 this was very pronounced (Fig. 1). At that time, there was no stroma visible in any of the European isolates.

The mean area of stroma formed after 21 days incubation ranged from 4.11 to 5.19 cm^2 in the Japanese isolates and from 0 to 0.85 cm^2 within the European group (Table 2). In two of the European isolates no stroma was observed at all (CC 752 and HU 1.96). The difference in stroma formation between the two groups was highly significant (*t*-test, $P = 0.001$).

Table 2. Comparison of stroma formation, growth rate and conidial dimensions between European (Eur) and Japanese (Jap) *M. fructigena* isolates

	mean area of stromatal plates (cm^2) [a]	range (cm^2)
Eur	0.35 ± 0.25 (3) a [b]	0.0-0.85
Jap	4.55 ± 0.24 (5) b	4.11-5.19

	12 h light/12 h dark regime		darkness	
	mean colony growth rate (mm day^{-1})	range (mm day^{-1})	mean colony growth rate (mm day^{-1})	range (mm day^{-1})
Eur	5.0 ± 0.87 (6) a [c]	2.0-8.3	3.7 ± 0.66 (6) a [c]	2.6-7.0
Jap	8.4 ± 0.44 (6) b	7.5-10.4	7.0 ± 0.76 (6) b	3.7-9.3

	conidia grown on CHA		conidia obtained from sporulating pear fruit	
	mean length x width (µm) [d]	range (l x w) in µm	mean length x width (µm) [d]	range (l x w) in µm
Eur	19.2 x 11.3 (6) a [c]	17.7-20.6 x 10.3-12.3	21.5 x 13.1 (6) a [b]	20.5-23.4 x 12.6-13.4
Jap	15.9 x 9.8 (6) b	12.8-17.1 x 8.8-10.7	16.1 x 10.6 (6) b	14.6-19.2 x 9.1-11.9

[a] mean ± s.e.m.; number of values used in calculating the mean given in parentheses
[b] values followed by different letters significantly differ at $P = 0.001$ (*t*-test)
[c] values followed by different letters significantly differ at $P = 0.01$ (*t*-test)
[d] 35 conidia were measured per isolate

Mean colony growth rate of Japanese *M. fructigena* isolates was significantly higher (*t*-test, $P = 0.01$) in both light regimes (Table 2). In 12 h light/12 h dark regime, the mean growth rate for Japanese isolates was 8.4 mm day^{-1} and 5.0 mm day^{-1} for European isolates; in continuous darkness this was 7.0 and 3.7 mm day^{-1}, respectively. Between individual isolates, some variation occurred within each group. Within the European group, isolate ES-48 showed a relatively high growth rate (8.3 mm day^{-1} in light/dark regime, 7.0 mm day^{-1} in darkness). Within the Japanese group, limited within-group variation occurred in the light/dark regime compared with the variation observed in continuous darkness (Table 2). In replicate experiments, similar results were obtained, though absolute growth rates were slightly lower than those in the first experiment (data not shown).

Conidial dimensions of Japanese and European isolates were compared *in vitro* as well as *in vivo* (Table 2). Length and width of conidia obtained from CHA were significantly greater in European *M. fructigena* isolates compared with Japanese isolates (*t*-test, $P = 0.01$). The range in mean length and width calculated per individual isolate, showed no overlap between the groups, except for a slight overlap in mean width (Table 2). The difference in conidium size turned out to be even more pronounced when conidia taken from sporulating fruits were measured. Mean length and width for European isolates was 21.5 x 13.1 µm, and 16.1 x 10.6 µm for Japanese isolates. Difference in mean length and width was highly significant (*t*-test, $P = 0.001$).

Increase in colony diameter, sporulation intensity and germ tube characteristics

A comparison of increase in colony diameter, sporulation intensity and germ tube characteristics between European and Japanese *M. fructigena* isolates is given in Table 3. Though the increase in colony diameter of Japanese isolates seemed higher than that of European ones, the difference was not significant (*t*-test, $P = 0.05$). No significant differences could be detected in sporulation intensity, germ tube length and percentage of conidia with more than one germ tube (Table 3).

Table 3. Comparison of characteristics used in a previously defined identification protocol (Van Leeuwen & Van Kesteren, 1998) between European (Eur) and Japanese (Jap) *M. fructigena* strains

	increase in colony diameter day3-day5 (mm) [a]	no. of conidia per Petri dish (log-transformed)	length of germ tube (µm)	percentage of conidia with more than one germ tube
Eur	8.6 ± 1.71 (5)	4.68 ± 0.28 (5)	766 ± 52.2 (5)	72.8 ± 7.9 (5)
Jap	11.8 ± 0.46 (5)	4.48 ± 0.25 (5)	934 ± 80.3 (4)	57.5 ± 16.2 (4)

[a] mean ± s.e.m.; number of values used in calculating the mean given in parentheses

Pathogenicity on fruits

Minimal variation in latency period occurred between individual fruits in the experiments with unripe fruits. Three days after infection, the first pustules appeared in Japanese as well as European strains on cv. Conference and cv. Cox's Orange Pippin. More variation in latency period between strains was found in ripe fruits. In Japanese strains, the latency period ranged from 3 to 4.7 days on cv. Conference in the first experiment, whilst in European strains this was 3 to 3.5 days. In the second experiment with cv. Conference, values ranged from 2.3 to 3.0 days and 2.2 to 3.8 days for Japanese and European isolates, respectively. The difference between both groups was not significant (Wilcoxon-test, $P = 0.05$). Results for lesion growth rate and sporulation intensity on ripe fruits are shown in Table 4. In the first experiment with ripe Conference fruits (Exp I), we found a significantly higher lesion growth rate for Japanese strains compared with European strains (t-test, $P = 0.05$), viz 16.0 and 13.6 mm day^{-1}, respectively. However, in later experiments no significant difference in lesion growth rate was found (Exp II and III, Table 4). No differences were found in the number of conidia produced per cm^2 lesion between Japanese and European strains. In the other pear cultivar, Doyenné du Comice, neither differences in lesion growth rate nor in sporulation intensity were observed between the *M. fructigena* groups (data not shown). Mean lesion growth rate on cv. Cox's Orange Pippin was equal for both groups, as was sporulation intensity. Similar experiments with unripe fruits did not show significant differences in measured characters between the groups (data not shown).

Table 4. Lesion growth rate and sporulation intensity of European (Eur) and Japanese (Jap) *M. fructigena* strains on ripe fruits of apple and pear

		cv. Conference		cv. Cox's Orange Pippin	
		lesion growth rate (mm day^{-1}) [a]	no of conidia cm^{-2} (log-transformed) [b]	lesion growth rate (mm day^{-1})	no of conidia cm^{-2} (log-transformed)
Exp I	Eur	13.6 ± 0.49 (6) a [c]	nd		
	Jap	16.0 ± 0.39 (6) b	nd		
Exp II	Eur	16.4 ± 0.85 (5)	5.05 ± 0.03	13.0 ± 0.59 (5)	3.75 ± 0.09
	Jap	20.7 ± 1.85 (5)	5.08 ± 0.07	12.8 ± 0.78 (5)	3.79 ± 0.18
Exp III	Eur	12.4 ± 1.23 (5)	4.76 ± 0.11	16.7 ± 1.01 (5)	4.24 ± 0.06
	Jap	15.9 ± 0.99 (5)	4.75 ± 0.24	17.8 ± 0.32 (5)	3.91 ± 0.31

[a] mean ± s.e.m.; number of values used in calculating the mean given in parentheses

[b] conidia collected from fruits 6 days after inoculation of the fruits; lesion size data from day 4 used

[c] values followed by different letters significantly differ at $P = 0.05$ (t-test)

Discussion

Our study clearly points out that genetic divergence found within the non-coding rDNA ITS-region between Japanese and European *M. fructigena* strains, is reflected in distinct and reproducible morphological differences between both groups. Each of the Japanese isolates formed more stroma than individual European isolates. In a comparison of *Monilinia* strains from different geographical regions, Wormald (1927) noted that a *M. fructigena* culture sent from Japan produced zones of black stromatal plates in culture, a feature he never observed in European *M. fructigena* strains. In his monograph on Japanese species of *Monilinia*, Harada (1977) confirmed the occurrence of intense stroma formation in Japanese *M. fructigena* isolates. Although we only quantified stroma formation *in vitro*, we found similar differences in stroma formation between both groups on infected fruits. Japanese isolates produced a thick hyphal mantle of (ecto)stroma on the host's cuticle, while European isolates produced none (Van Leeuwen, unpublished).

The mean radial growth rate of Japanese *M. fructigena* strains on PDA was significantly higher than that of European strains. However, when measured between the third and fifth day of growth, the difference was not significant (Table 3). The same occurred in calculating and comparing mean lesion growth rates on Conference fruits (Table 4). It seems that initially, in the first days of colony expansion, no difference in growth rate exists between both groups, but after this period colony/lesion expansion rate is higher in Japanese strains. The fact that less time is needed for Japanese *M. fructigena* strains to colonise cv. Conference fruits completely than for European strains, support this interpretation (Van Leeuwen, unpublished). Harada (1977) found that a Japanese *M. fructigena* isolate had a growth rate even higher than *M. fructicola* on PDA, whereas Van Leeuwen & Van Kesteren (1998) showed that growth rate on PDA under similar conditions was consistently lower in European *M. fructigena* strains than in those of *M. fructicola*. Though overall means, averaged over isolates, significantly differed between both groups, the European isolate ES-48 showed a growth rate similar to Japanese strains (Fig. 1). Obviously, the distributions of both *M. fructigena* groups show some overlap in growth rate.

Clear differences in the size of conidia were found between the geographical groups (Table 2), conidia being smaller in isolates of the Japanese group. Khokhriakova (1978) found differences in conidium size between *M. fructigena* populations from different regions in the former USSR. Mean length and width of conidia for the European population was 19.4 x 11.5 µm; for the Central Asian and the Far Eastern populations this was 17.2 x 11.9 µm and 18.0 x 11.4 µm, respectively. Thus, conidia tended to be smaller in Asian regions. On apple fruits incubated at 15 °C, Batra & Harada (1986) measured a range in conidium length x width of 14-20 x 9.5-14.5 µm for Japanese material, concurring perfectly with our findings. Interestingly, Tanaka & Yamamoto (1964) reported that conidia of the Japanese pear fungus causing scab, were smaller than those of the European scab fungus, *Venturia pirina* Aderh.. On the basis of additional marked differences between both scab fungi, they considered the causal fungus of Japanese pear scab to be a distinct species, *Venturia nashicola* Tanaka & Yamam..

The colony and germ tube characteristics which enabled us to delineate *M. fructicola*, *M. laxa* and (European) *M. fructigena* (Van Leeuwen & Van Kesteren, 1998), were not found useful for separating Japanese and European *M. fructigena* strains (Table 3). Well-discriminating morphological characters between Japanese and European isolates of *M. fructigena* are the intensity of stroma formation and conidium size. An overview of distinguishing characters of the brown rot fungi is given in Table 5. *M. fructicola* and *M. laxa* are distinguished on the basis of

Table 5. Distinguishing *in vitro* characters of the brown rot fungi of fruit crops

	Colonies on PDA (22 °C, 12 h light/12 h dark regime)	
M. fructicola	growth rate high; sporulation intense (also in continuous darkness); colour of sporogenous tissue hazel/isabelline (Rayner, 1970)	
M. laxa	growth rate low, renewed outgrowth of mycelium at the edge of the colony taking place after growth is checked temporarily (rosetting, i.e. mycelium in distinct layers on top of each) (Corazza *et al.*, 1998); sporulation sparse, colour of sporogenous tissue hazel/isabelline (Rayner, 1970)	
M. fructigena	growth rate low-moderate; sporulation sparse, on distinct tufts/rings of aerial mycelium, colour of sporogenous tissue buff/pale luteous (Rayner, 1970)	
M. polystroma	growth rate moderate; sporulation sparse; intense formation of black, stromatal plates initiated after 10-12 days incubation; colour of sporogenous tissue buff/pale luteous (Rayner, 1970)	
	Conidial dimensions (mean and range) [a]	
M. fructicola	13.1 x 9.1 µm (8) [b]	12.4-14.4 x 8.1-9.9 µm
M. laxa	12.0 x 8.7 µm (4)	11.2-12.8 x 8.2-9.3 µm
M. fructigena	19.2 x 11.3 µm (6)	17.7-20.6 x 10.3-12.3 µm
M. polystroma	15.9 x 9.8 µm (6)	12.8-17.1 x 8.8-10.7 µm
	Germ tube morphology [c]	
M. fructicola	long, straigth, 750-900 µm, one germ tube per conidium	
M. laxa	short, twisted, 150-350 µm, one germ tube per conidium	
M. fructigena	long, straigth, 600-900 µm, often two germ tubes per conidium	
M. polystroma	long, straigth, 700-1000 µm, often two germ tubes per conidium	

[a] conidia grown on CHA at 22 °C under nuv light
[b] 35 conidia measured per isolate in distilled water, number of isolates in parentheses
[c] after 18 h at 22 °C on water agar plates

differences in sporulation intensity, shape of the colony and growth rate (Table 5). European and Japanese *M. fructigena* isolates differ in stroma formation and conidial dimensions. Moreover, European and Japanese *M. fructigena* isolates show basepair substitutions in the ITS region in five positions (Fig. 2), equal to the number of substitutions found between *M. fructicola* and

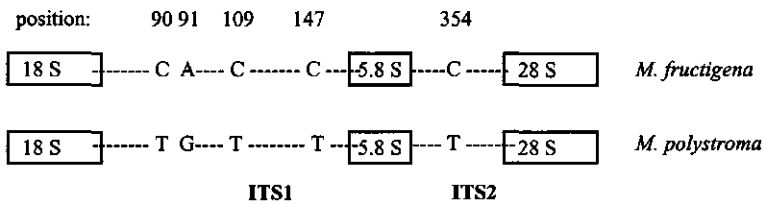

Fig. 2. Sequence divergence in the ITS region of ribosomal DNA (rDNA) between *M. fructigena* and *M. polystroma*.

M. laxa. Therefore, we conclude that isolates from Japan, formerly described as *M. fructigena*, belong to a distinct species, which is hereby designated *Monilia polystroma* sp. nov.. In order to avoid confusion about the place of valid publication of this species, the Latin diagnosis is omitted from the present preprint. A description of the anamorph of a type specimen is presented below.

Monilia polystroma Van Leeuwen sp. nov.

Colonies on potato dextrose agar (PDA) reaching 50-60 mm diam. after 6 days at 22 °C under 12 h light/12 h dark cycle. Colony margin even, sporogenous tissue slightly elevated above the colony surface (1-2 mm), colour buff/pale luteous. Stromatal initials formed 10-12 days after inoculation at 22 °C under 12 h light/12 h dark cycle; mature, black stromatal plates first discrete, later coalescing. Macroconidia globose, ovoid or limoniform, smooth, measuring 12--21 x 8--12 µm, av. 16.4 x 10.1 µm (distilled water) when grown on cherry agar (CHA) at 22 °C under nuv, and 11--20 x 8--11 µm, av. 14.9 x 9.1 µm on pear fruit at 15 °C. On fruit, a thick hyphal layer of stroma appearing after the fruit is colonised; conidial tufts buff to brownish-grey.
Habitat. On fruits of *Malus* spp., *Pyrus* spp. and *Prunus* spp., producing fruit rot. Throughout Japan. Cultures deposited in CBS, Baarn, The Netherlands, as CBS 101504, 102686, 102687, 102688.

Unfortunately, we were not able to induce the formation of apothecia neither in European nor in Japanese material. Following the method described by Willetts & Harada (1984), in Japanese material we only obtained minute apothecial stipe initials measuring 4-5 mm on stromatised pear discs. These initials shriveled before discs could develop. It is very likely that the description of a teleomorph in material from Japan given by Batra & Harada (1986) and designated as

M. fructigena, is an account of the teleomorph of *Monilia polystroma*. The authors describe the intense formation of stroma in the anamorph, and conidial size on sporulating fruits matches with our observations in *M. polystroma*. The description of the teleomorph given, did not differ from those of apothecia of *M. fructigena* found in Europe (Aderhold & Ruhland, 1905; Johansen, 1945). Because of uncertainties and no teleomorphic material being available to serve as type, we only describe here a new anamorph taxon.

Our study gives a clear example of speciation due to geographical isolation, though geographical isolation is particularly important in plants and animals, rather than in fungal evolution (Moore-Landecker, 1996). Byrde & Willetts (1977) postulated that in the past *M. laxa* probably evolved first from an ancestral form of the brown rot fungi. Phylogenetic analysis of rDNA sequence data support this (Holst-Jensen *et al.*, 1997a). According to Leppik (1970), the Far East may be the place where the common ancestor was first associated with fruit plants. The occurrence of substantial genetic variation, not only in *M. fructigena/M. polystroma*, but also in Japanese *M. fructicola* isolates (Fulton *et al.*, 1999), supports this view. A *M. laxa*-like ancestor presumably caused blossom- and twig blight on the wild progenitors of present day fruit crops in this region. When people started to select for cultivars with larger and more juicy fruits, the ancestor of *M. fructigena* and *M. polystroma* might have evolved in the Far East as a specialised fruit pathogen. Subsequently, due to geographical separation, once introduced into their own locality, two distinct forms evolved in Europe and Japan. Differences in host gene pool, climatic conditions, and biotic environment might have played a role in morphological and genetic divergence (Moore-Landecker, 1996). Harada (1998) mentions the occurrence of a mycoparasite, *Lambertella corni-maris* Höhnel, on stroma of *M. fructigena* on apple fruits in Japan. A hostile biotic environment may have triggered defense mechanisms, with intensified stroma formation as a consequence.

Clear morphological (this study) and genetic differences (Fulton *et al.*, 1999) between *M. fructigena* and *M. polystroma* have been shown. Biologically, differences found thus far seem small. Measurements of lesion growth rate and sporulation intensity on the pear cultivars Conference and Doyenné du Comice showed no significant differences. The same held for the apple cultivars Cox's Orange Pippin (Table 4), cv. Golden Delicious and cv. James Grieve (Van Leeuwen, unpublished). However, on cv. Conference we found indications that lesion growth rate was higher in *M. polystroma* when determined later in the process of lesion expansion. Fruits infected in the field might be faster colonised by *M. polystroma* compared with *M. fructigena*. Moreover, the abundant stroma formation by *M. polystroma* is also expressed *in vivo*. The mantle of (ecto)stroma formed on the host's cuticle after conidial pustules have ruptured the cuticle, protects the colonised fruit against degredation and decomposition by abiotic and biotic factors. Because the pathogen survives in dried-out infected fruits (mummified fruits), inhibition of quick decomposition of infected fruits might enhance survival, and subsequently increase the amount of primary inoculum in the next season (Byrde & Willetts, 1977). These aspects deserve attention in future research, in order to determine the risk of introduction of *M. polystroma* into areas outside Japan, using pest risk analysis schemes (OEPP/EPPO, 1997). Furthermore, it would be of particular interest to collect *M. fructigena* isolates in other areas in East Asia (China, Russia), and characterise these morphologically and genetically. This will give a better understanding of the geographical distribution of *M. fructigena* and *M. polystroma*, and may clarify their evolutionary history.

Chapter 5

Yield loss in apple caused by *Monilinia fructigena* (Aderh. & Ruhl.) Honey, and spatio-temporal dynamics of disease development

Abstract

Monilinia fructigena (Aderh. & Ruhl.) Honey causes considerable yield losses in pome fruit culture. During a field study in the Netherlands in 1997 and 1998, the increase in disease incidence in time was assessed and final pre- and post-harvest losses were recorded in the susceptible apple cultivars James Grieve and Cox's Orange Pippin. Each individual tree was considered as a unique quadrat, and the spatial distribution of diseased fruits among fruit trees at every assessment date was characterised by a dispersion index, Lloyd's index of patchiness (LIP). Spatial autocorrelation was applied to detect potential clustering of trees with diseased fruits within rows. In cv. James Grieve, the rate of increase of disease incidence was constant up to harvest time, whereas in cv. Cox's Orange Pippin disease incidence increased markedly 3 weeks before harvest time, which coincided with the harvest of cv. James Grieve in neighbouring rows. Pre-harvest disease incidence was 4.2-4.3 % in cv. James Grieve in both years, in cv. Cox's Orange Pippin this was 4.4 % in 1997 and 2.7 % in 1998. Post-harvest yield losses amounted on average 1.5-2.0 % for both cultivars, no significant differences were found between the cultivars (*t*-test, $P = 0.05$). Both in 1997 and 1998, clustering of diseased fruits among fruit trees was detected; LIP values were significantly higher than 1 ($P = 0.05$ in 1997, $P = 0.01$ in 1998). Clustering of trees with diseased fruits was detected in 1998, when significant ($P = 0.05$) positive correlation coefficients occurred for 2nd, 3rd and 4th lag-order distances in cv. James Grieve, and a significant ($P = 0.05$) positive first-order correlation in cv. Cox's Orange Pippin. Wounding agents, such as insects and birds, may play an important role in the underlying disease dynamics, and crop losses may be minimised by control of these agents.

Introduction

In pome fruit growing areas, *Monilinia fructigena* (Aderh. & Ruhl.) Honey is an important pathogen that causes fruit rot. *M. fructigena* belongs to the group of brown rot fungi, which includes two other species, *M. laxa* (Aderh. & Ruhl.) Honey and the EU-listed quarantine organism *M. fructicola* (Wint.) Honey. The latter two species more commonly affect stone fruits, although *M. laxa* forma *mali* causes blossom wilt and canker of apple trees (Byrde & Willetts, 1977).

Many studies have focussed on the post-harvest stage of fruit rot caused by *M. fructigena* (Berrie, 1989; Snowdon, 1990; Falconi & Mendgen, 1994), in which losses caused by *M. fructigena* are nowadays usually low. Berrie (1989) reported mean post-harvest losses in cultivar (cv.) Cox's Orange Pippin that ranged from 0.1 % to 0.6 % in the seasons 1982-1988. Crop losses are an important criterion to declare an organism to be a quarantine pest

(OEPP/EPPO, 1993). *M. fructigena* is declared a quarantine pest in the USA, Australia and New Zealand (CMI, 1991). Detailed studies of pre-harvest yield losses in pome fruit caused by *M. fructigena* are rare in recent literature. In a study over three consecutive seasons, Moore (1950) recorded an average of about 9 % of apple fruits becoming infected with brown rot. Differences in susceptibility among apple cultivars under field conditions have been reported from Poland (Cimanowski & Pietrzak, 1991). Under Dutch conditions, apple cvs. James Grieve and Cox's Orange Pippin are among the most susceptibles (Anonymous, 1991).

Few studies have been made to characterise temporal and spatial aspects of increase in *Monilinia*-diseased fruits in orchards. As part of a study that dealt with fungicide-resistant strains of *M. fructicola* in New Zealand, Elmer *et al.* (1998) analysed the spatial distribution of peach fruits affected by brown rot at harvest time. They determined brown rot incidence after a short post-harvest period under controlled conditions. No attempt was made to determine disease incidence during the pre-harvest period *in situ*. In stone fruits, disease incidence can increase very rapidly around harvest time, as mature fruits become more readily infected (Corbin, 1963; Zehr, 1982). *M. fructicola* is able to infect uninjured stone fruits even at the pre-pit-hardening stage (Corbin, 1963). Latent infections of *M. fructicola* can also play a role in the rapid disease development observed around harvest time (Jenkins & Reinganum, 1965; Northover & Cerkauskas, 1994). Latent infections occur in immature fruits particularly in a season of severe blossom infection, and after a period of quiescence that may last several weeks, rots start to develop as the fruits ripen. In contrast, *M. fructigena* relies almost exclusively on pre-existing wounds in the fruit skin for penetration, although uninjured, ripe apples have been successfully infected via lenticels (Horne, 1933). Latent infections of immature fruits as described for *M. fructicola* have never been reported for *M. fructigena*.

The objective of this study was to study space-time variation of disease development of *M. fructigena* in apple. More specifically, it focussed on: (i) quantification of pre- and post-harvest yield losses caused by *M. fructigena* in two susceptible apple cultivars; (ii) the increase in disease incidence in time, based on the hypothesis that disease incidence gradually increases up to harvest time, unlike the rapid increase at harvest time frequently observed in stone fruits affected by *M. fructicola* or *M. laxa*; (iii) determination of spatial pattern of diseased fruits among fruit trees in time; and (iv) determination of the extent of clustering of trees with diseased fruits within the field. Spatial analysis allows for the development of hypotheses to account for observed associations (Campbell & Madden, 1990), thus giving information on the population dynamics of the pathogen. Based on the results, we present some hypotheses in relation to the underlying disease dynamics, and make a comparison with those postulated for *M. laxa* and *M. fructicola* in stone fruits.

Materials and methods

Study area

The study was done in an experimental orchard (IPM) situated in the Rhine basin in the centre of the Netherlands during two consecutive growing seasons in 1997 and 1998. The orchard contained three rows of cv. James Grieve alternating with two rows of cv. Cox's Orange Pippin (Fig. 3). The trees were planted in 1984, at a tree spacing of 3 x 1.25 m. In both years cv. James Grieve bore a moderate fruit crop, with a mean number of 70 fruits per tree in 1997 and 55 in 1998; mean numbers of cv. Cox's Orange Pippin were 58 and 50, respectively. Regular spraying against apple scab (*Venturia inaequalis*) followed normal commercial practice from bud break until the end of June in both years. Insecticide was applied after

blossoming against apple sawfly (*Hoplocampa testudinea*) and early codling moth (*Pammene rhediella*) after blossoming to prevent early dropping of fruit.

Evaluations

Disease incidence was assessed by counting the number of fruits per tree which showed sporulation of *M. fructigena*. Fruits showing sporulation of *M. fructigena* in the tree canopy were not removed, whereas those which had dropped to the ground were removed to the grass pathway between the rows to avoid double countings. Observations were made at intervals of 7-9 days until harvest time, starting on 16 July in 1997, and 1 week earlier in 1998. For cv. Cox's Orange Pippin, observations were less frequent at the beginning of the epidemic because of a minimal increase in disease incidence. Harvest of cv. James Grieve took place on 22 August (day 234) in 1997 and 12 August (day 224) in 1998; the equivalent dates for cv. Cox's Orange Pippin were 12 (day 255) and 2 September (day 245), respectively. At harvest time, the number of fruits per box was determined per cultivar for five randomly chosen boxes. The number of harvested fruits was estimated by multiplying the mean number of fruits per box by the total number of boxes. Percentage disease incidence was defined as the quotient of the (cumulative) number of *Monilinia*-diseased apples at a certain assessment date and the number of harvested fruits, multiplied by 100.

After harvest, the boxes with fruits were stored in an open shed, protected from rain but not wind. Post-harvest disease assessments were made 4 days after harvest in 1997, and 7 days after harvest in 1998. Mean daily temperatures registered in the post-harvest period ranged from 15 to 20 °C in both years. Disease incidence in storage was calculated as the number of fruits showing sporulation of *M. fructigena* relative to the total number of fruits per box. For each cultivar, mean disease incidence of five boxes was determined, and means of both cultivars were compared with a *t*-test.

Spatial statistics

The spatial pattern of diseased fruits among fruit trees was determined for each cultivar. The objects of study were single trees, and each week the (cumulative) number of *Monilinia*-diseased apples per tree was determined. In this study 'clustering of fruits' is defined as the spatial condition in which the number of diseased fruits per tree is more locally condensed than at random (Madden, 1989), i.e. the situation where there are trees with a considerable number of diseased fruits whilst others have none. Furthermore, it was our aim to study to what extent 'clustering of trees with diseased fruits' appeared within the field.

Lloyd's index of patchiness (LIP) was calculated to determine clustering of diseased fruits. LIP was calculated on the basis of the (cumulative) number of diseased fruits per tree. Per cultivar, the rows were considered as one, and the analysis performed. LIP constitutes the ratio of mean crowding (m^*) and mean density (m), where mean crowding is defined as the mean number of other diseased fruits per tree per diseased fruit, and mean density is the mean number of diseased fruits per tree (Lloyd, 1967).

Mean crowding was calculated as

$$m^* = m + \left(\frac{s^2}{m} - 1 \right),$$

where m is the estimated mean number of diseased fruits per tree and s^2 the estimated variance. Unlike mean density (m), m^* is not affected by trees without diseased fruits. The data set with the number of diseased fruits per tree at each assessment date, was considered as a sample of the total population of orchards where cv. James Grieve and cv. Cox's Orange

Pippin are grown in an alternating one-row system. To calculate confidence bounds for LIP we applied a bootstrap procedure (Efron & Tibshirani, 1993). Numbers were randomly selected with replacement from the original data set to create a new data set of the same size, for which the LIP value was calculated. This was done 10.000 times, and subsequently mean LIP and a 95 % and 99 % confidence interval for the population mean were determined. Clustering was considered to be more pronounced when significant at $P = 0.01$ (99 % confidence interval) compared with significance at $P = 0.05$ (95 % confidence interval). Mean LIP values not significantly different from 1 indicate a random distribution, whereas clustering was indicated by values significantly greater than 1. For cv. Cox's Orange Pippin, LIP was not calculated for the first assessment dates in both years, because there were very few diseased fruits present.

Spatial autocorrelation analysis was used to determine the extent of clustering of trees with diseased fruits within the field (Cliff & Ord, 1981). The number of diseased fruits per tree was compared to the values in the proximal trees. Only within-row comparisons were made. First-order spatial correlations were determined by relating the number of diseased fruits in each tree to that in the adjacent trees. Higher lag-order correlations were based on trees separated by one or more trees. In the analysis, the rows were combined for each cultivar, with an adjustment for unequal row length by input of missing values. Within-row comparisons up to 10 lags were made for every assessment date.

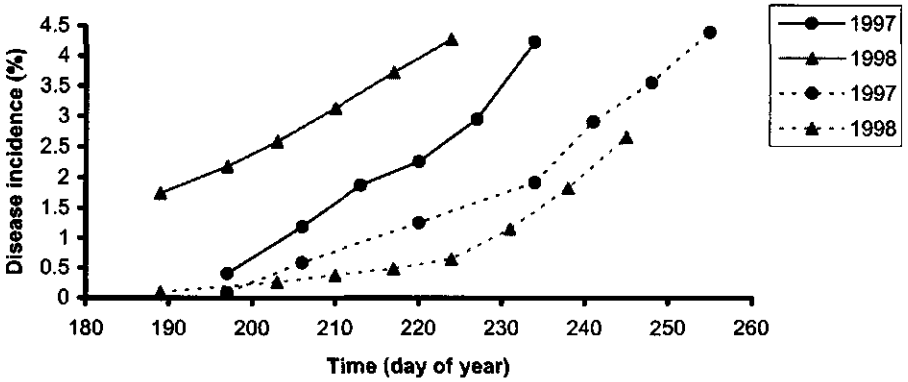

Fig. 1. The (cumulative) percentage of apples showing sporulation of *M. fructigena* in the field during two consecutive seasons in cv. James Grieve (drawn line) and cv. Cox's Orange Pippin (dotted line).

The covariance function between the two values of a pair is defined as

$$C(h) = E[x(s).\, x(s+h)] - \mu_x^2 \ ,$$

where s and $s+h$ are positions along a row, separated by distance h, $x(.)$ is the number of diseased fruits, and E denotes the mathematical expectation. The covariance is calculated as

$$\hat{C}(h) = \frac{1}{N(h)} \sum_{i=1}^{N(h)} x_i x_{i+h} - m_{-h} m_{+h} \ ,$$

where $N(h)$ is the number of pairs (x_i, x_{i+h}), m_{-h} is the mean of the tail values (first attribute value of each pair, x_i), and m_{+h} is the mean of the head values (second attribute value of each pair, x_{i+h}). The correlation function is defined as the covariance standardised by the respective tail and head standard deviations

$$\rho(h) = \frac{C(h)}{\sigma_{-h}\sigma_{+h}},$$

where σ_{-h} and σ_{+h} are the standard deviations of the tail and head values respectively (Deutsch & Journel, 1998). For $\rho(h) > 0$, a positive spatial autocorrelation exists, indicating similar numbers of diseased fruits in nearby trees. Negative coefficients indicate dissimilar values. The correlation function is estimated by dividing $\hat{C}(h)$ by estimated $\hat{\sigma}_{-h}$ and $\hat{\sigma}_{+h}$ values, respectively. Calculations were made with the geostatistical program GSLIB (Deutsch & Journel, 1998). The significance of the obtained correlation coefficients ($\rho(h) \neq 0$) was tested ($P = 0.05$) against the hypothesis of no autocorrelation ($\rho(h) = 0$).

Results

The increase in disease incidence during both growing seasons is shown in Fig. 1. In cv. James Grieve, the rate of increase was approximately constant throughout the season, although there was a slight increase in the last week before harvest in 1997 (Fig. 1). In 1998 disease incidence in cv. James Grieve amounted already to 1.7 % on the first assessment date. Initially, in cv. Cox's Orange Pippin a slow, constant increase in disease incidence occurred (0.050 % per day in 1997 and 0.015 % in 1998). Three weeks before harvest time, which coincided in both years with the harvest of cv. James Grieve, a sudden increase in the rate of increase of disease incidence was observed (Fig. 1). In both years, final pre-harvest disease incidence was equal to 4.2-4.3 % for cv. James Grieve; for cv. Cox's Orange Pippin this was 4.4 % in 1997 and 2.7 % in 1998. In the post-harvest phase, disease incidence was 1.3 % in cv. James Grieve, and 1.7 % in cv. Cox's Orange Pippin in 1997. Means for both cultivars were not significantly different (t-test, $P = 0.05$). In 1998, post-harvest disease incidence was 2.0 % in cv. James Grieve, and 1.2 % in cv. Cox's Orange Pippin, being an insignificant difference.

Mean values of Lloyd's index of patchiness (LIP) with time are shown in Fig. 2. For cv. James Grieve, diseased fruits were clearly clustered (aggregated) in 1998. At every assessment date, the LIP value was significantly greater than 1 ($P = 0.01$). Some trees had a considerable number of diseased fruits whilst others had none (Fig. 3). At the first assessment date in 1998

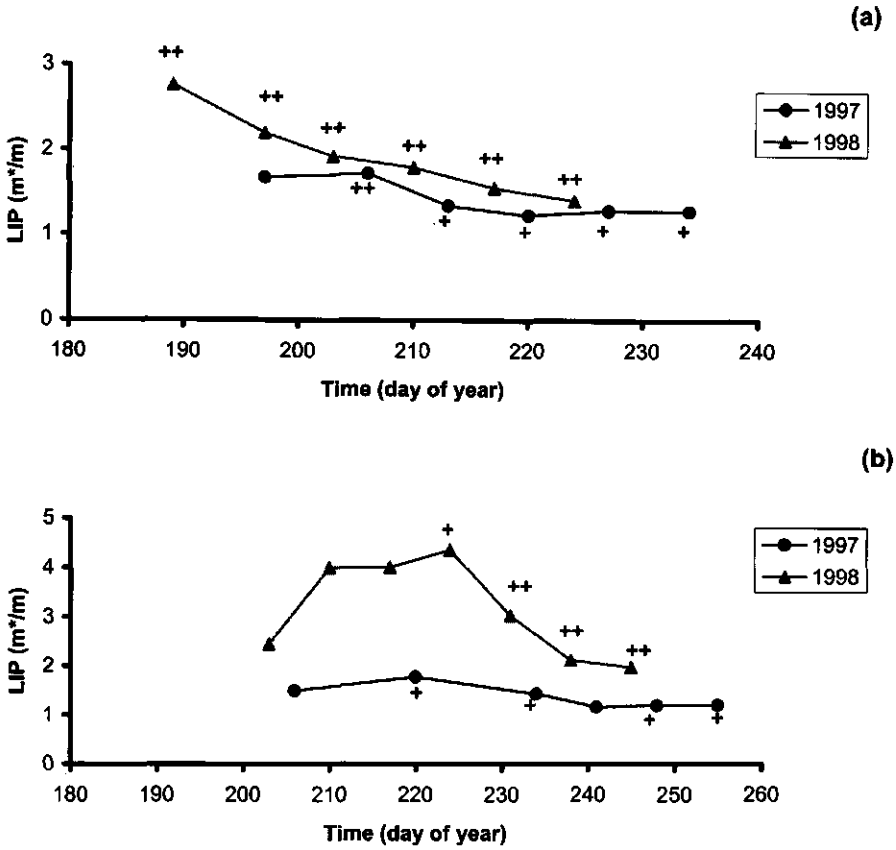

Fig. 2. Lloyd's index of patchiness (LIP) during the season in (a) cv. James Grieve and (b) cv. Cox's Orange Pippin (+ = LIP value significantly different from 1, $P = 0.05$; ++ = idem, $P = 0.01$).

the maximum number of diseased fruits per tree was 12, whilst half of the James Grieve trees were without any diseased fruit. In 1997, clustering of diseased fruits was less pronounced in cv. James Grieve, though significant ($P = 0.05$). A similar pattern was observed in cv. Cox's Orange Pippin, clustering of fruits tended to be more pronounced in 1998 than in 1997 (Fig. 2). Initially, in 1998 high mean values of LIP occurred (2.4-4.4) due to a few trees bearing 2-3 diseased fruits, whilst most of the trees had none. Those LIP values, however, were not significantly different from 1 ($P = 0.05$).

Clustering of trees with diseased fruits was analysed by within-row autocorrelation. In 1997 no significant autocorrelations were found for either cultivar (data not shown), but in 1998 significant autocorrelation was detected in cv. James Grieve as well as cv. Cox's Orange

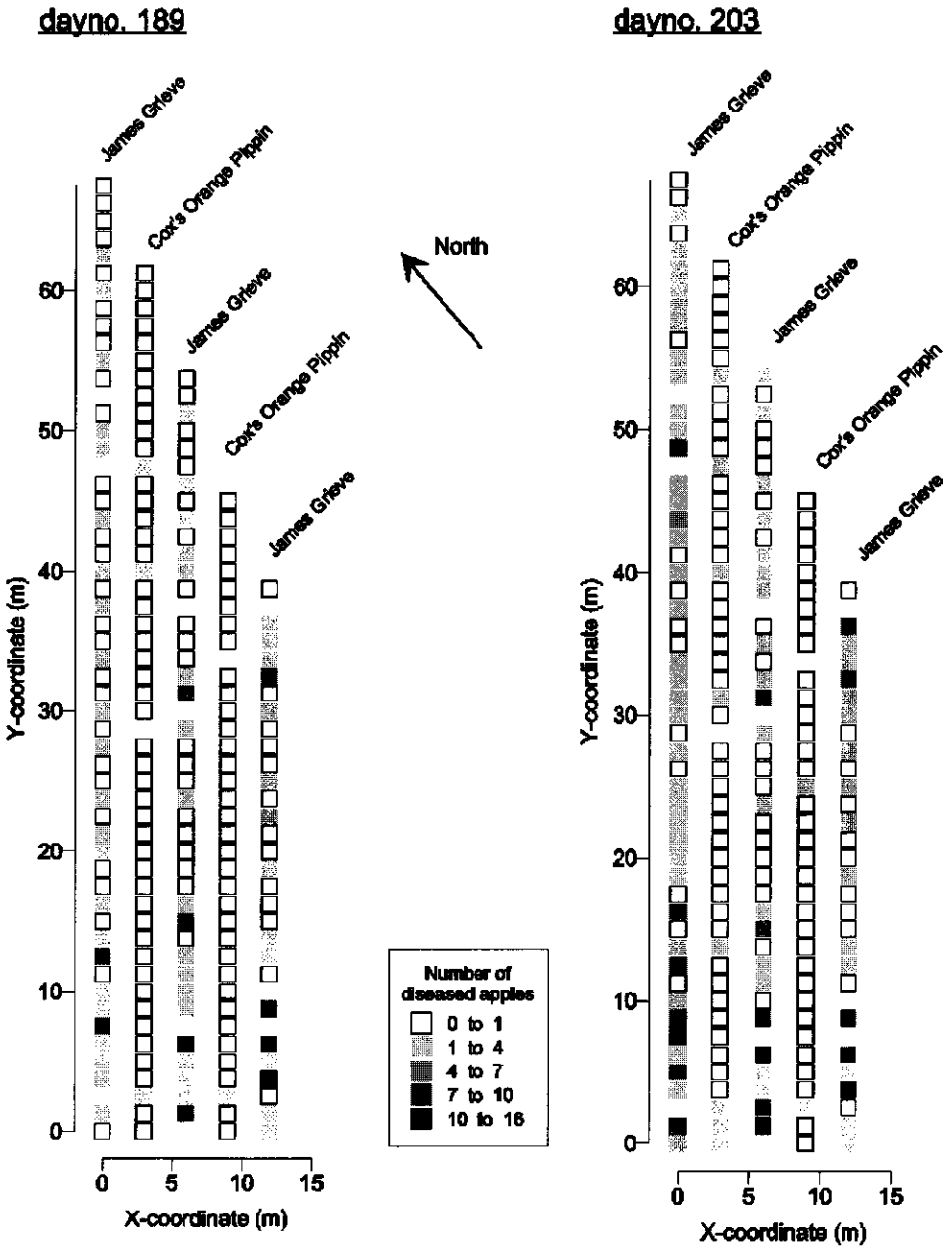

Fig. 3. Spatial map of the number of *Monilinia*-diseased fruits per tree at two different assessment dates in 1998. Number of diseased fruits grouped only for presentation not for analysis.

Pippin. Significant, positive correlation coefficients for 2nd, 3rd and 4th lag-order distances were found in cv. James Grieve (P = 0.05). The patterns for each of four consecutive assessment dates turned out to be similar (Fig. 4). Correlation coefficients ranged from 0.2-0.3 for nearby trees (2nd to 4th lag order), and approached zero or became negative at lag-orders 8 up to 10. Typically, no significant positive correlation was found for the adjacent quadrat (first-order, P = 0.05). In cv. Cox's Orange Pippin, significant first-order autocorrelation occurred in 1998 at the moment when the total number of diseased fruits started to increase considerably (Fig. 5, P = 0.05). Significant higher lag-order correlations were not found in cv. Cox's Orange Pippin.

Discussion

Our results support the hypothesis that disease incidence in apple caused by *M. fructigena* increases gradually up to harvest time. In cv. James Grieve, we observed no marked increase around harvest time, as often reported for *M. fructicola* (Hutton & Leigh, 1956; Zehr, 1982; Northover & Cerkauskas, 1994). However, in cv. Cox's Orange Pippin a distinct increase in disease incidence occurred in the last 3 weeks before harvest maturity (Fig. 1). This increase occurred in both years after cv. James Grieve was harvested. Disease incidence in the late cultivar increased as soon as the early cultivar had been harvested. The latent period is only 3-4 days at temperatures occurring in summertime; at the next assessment date after cv. James Grieve was harvested (7 days later), a distinct increase in disease incidence was observed in cv. Cox's Orange Pippin. Most probably, in cv. Cox's Orange Pippin, a build-up of inoculum on the fruits could have taken place during the weeks that cv. James Grieve was ripening, as reported for early and late maturing cultivars in stone fruits (Kable, 1971). The build-up of inoculum in cv. Cox's Orange Pippin may have taken place in the period before harvest of James Grieve, as the aerial spore content dropped sharply after harvest of James Grieve (G.C.M. van Leeuwen, unpublished). The increase in disease incidence in cv. Cox's Orange Pippin cannot be explained by increased susceptibility of ripening fruits. In a laboratory test, ripe Cox's Orange Pippin apples without injuries did not become infected when sprayed with a highly concentrated conidial suspension of *M. fructigena*, and incubated at 16-18 °C at 90-100 % RH for 7-10 days (G.C.M. van Leeuwen, unpublished). It is possible that wounding agents (insects, birds), formerly aggregating in cv. James Grieve, shifted to cv. Cox's Orange Pippin after cv. James Grieve was harvested. For birds, it has been shown that they have a preference for early maturing apple cultivars (Mitterling, 1965; Tobin *et al.*, 1989), such as James Grieve.

Final pre-harvest yield losses in both apple cultivars ranged from 2.7 % to 4.4 % over both years. Over three seasons, Moore (1950) recorded about 9 % incidence of brown rot of apples in trees under a complete spray schedule against apple scab, sawfly and codling moth. Probably, the pesticides used in those days were not as effective as current pesticides and alternative control measures. For example, codling moth damage amounted in one year as much as 20 % in the complete spray treatment in spite of a lead arsenate spraying in June (Moore, 1950). Pre-harvest losses in stone fruits caused by *M. fructicola* are on average higher compared with those caused by *M. fructigena* in pome fruits. Seasons with high yield losses, amounting up to 30-40 %, alternate with those of low to moderate losses of 5-15 % (Morschel, 1956; Kable, 1969; Hong *et al.*, 1997). Post-harvest losses observed in both cultivars exceeded those reported by Berrie (1989) during the storage season in cv. Cox's Orange Pippin and cv. Bramley's Seedling. However, in the latter case fruits had received a

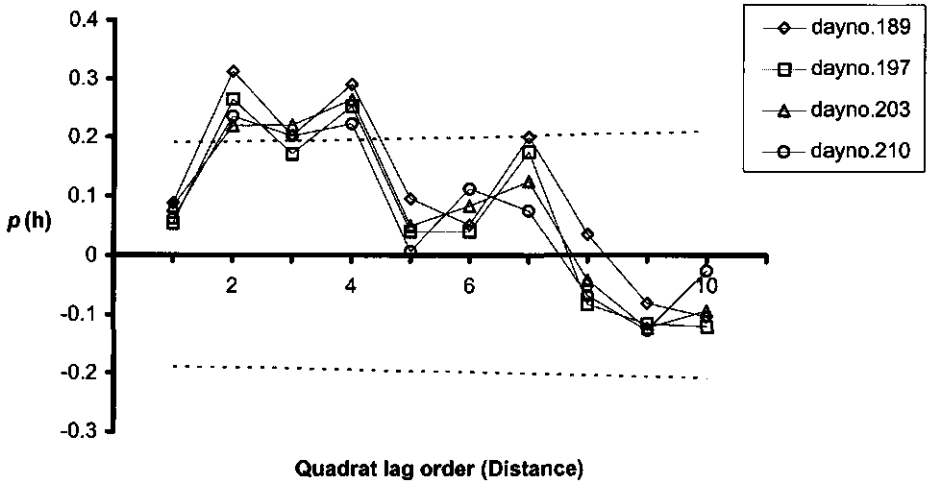

Fig. 4. Spatial autocorrelation for 1-10 lags distance at four consecutive assessment dates in cv. James Grieve in 1998. Dotted lines represent critical values of the Pearson product-moment correlation coefficient, $P = 0.05$; p(h) values exceeding these lines are significantly different from 0.

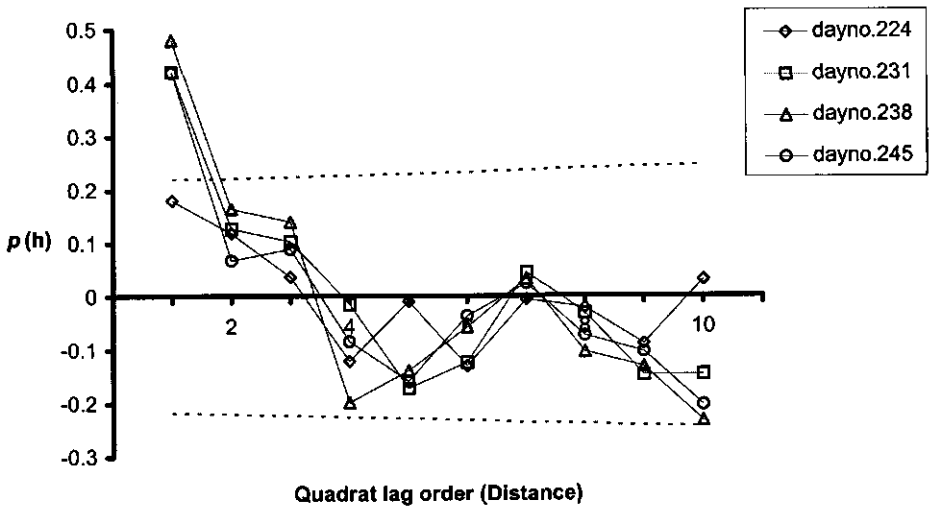

Fig. 5. Spatial autocorrelation for 1-10 lags distance at four consecutive assessment dates in cv. Cox's Orange Pippin in 1998. Dotted lines represent critical values of the Pearson product-moment correlation coefficient, $P = 0.05$; p(h) values exceeding these lines are significantly different from 0.

post-harvest fungicide treatment and were stored under controlled atmosphere conditions, before disease incidence was assessed.

In 1998, the spatial distribution of diseased fruits among the trees was clearly clustered, indicated by highly significant LIP values ($P = 0.01$). In time, the mean LIP values decreased as diseased fruits started to appear in trees previously devoid of diseased fruits. The initially high LIP values, especially in cv. Cox's Orange Pippin in 1998 (Fig. 2), resulted from the accumulation of diseased fruits within trees where the first diseased fruits were recorded, whilst the majority of trees harboured no diseased fruits at that time. For the brown rot fungi, fruit-to-fruit contact is one of the main mechanisms for spread of disease within a tree, and for which injuries are not necessary (Michailides & Morgan, 1997). Splash dispersal of conidia is one of the other mechanisms for spread of disease within a tree. Pauvert *et al.* (1969) demonstrated that conidia of *M. fructigena* are easily splash dispersed over short distances; this process facilitates spread of propagules within a tree, though final infection depends on the presence of injuries in this case. LIP values similar to our results, were calculated by Elmer *et al.* (1998) for *M. fructicola*-diseased fruits in peach and nectarine orchards at harvest time, but the authors did not determine confidence intervals. In our study, mean LIP values gradually decreased towards harvest time for both cultivars, but remained statistically different from 1 (Fig. 2).

Clustering of fruits was more apparent in 1998, and so was clustering of trees with diseased fruits. Significant autocorrelations ($P = 0.05$) were found in both cultivars in the second year. For cv. James Grieve, autocorrelation occurred at the 2nd- to 4th-order lag whereas a first-order correlation was absent (Fig. 4). The fact that first-order correlations were not significant, rules out the importance of inoculum concentration *per se* in the immediate environment to cause infections. Thus, the observed spatial distribution may depend more on behavioural characteristics of wounding agents than on inoculum concentration in the environment; this is supported by observations in cv. Cox's Orange Pippin, where we observed a distinct increase in the rate of increase of disease incidence after cv. James Grieve was harvested (see above). Remarkably, in 1998 (but not 1997) the intensified increase in disease incidence was accompanied by a significant first-order autocorrelation in cv. Cox's Orange Pippin (Fig. 5). Clustering of trees with diseased fruits became apparent, though limited to the direct neighbour trees. Elmer *et al.* (1998) also found significant correlations in only one out of two seasons in a similar study with *M. fructicola*-diseased fruits.

Based on the results of our study, we have indications that the control of wounding agents during fruit development is an essential part of disease management of brown rot in pome fruits caused by *M. fructigena*. Although we did not examine closely which wounding agents were responsible for injuries, blackbirds (*Turdus merula*) were often observed pecking at fruits. Bird-scaring methods are an option to limit bird damage (Hasey & Salmon, 1993), but these will also interfere with the activity of predatory birds (e.g. *Parus major*) that feed on noxious Lepidoptera species (Kristin & Patocka, 1997). An interesting approach to limit damage caused by American robins (*Turdus migratorius*) in small fruits, such as blueberries and cherries, was reported by Brugger & Nelms (1991), who suggested that modification of the relative sugar content in fruits might raise an aversion to the food. Some fruit-eating bird pests lack the enzyme sucrase which is necessary to digest the disaccharide sucrose. Direct control of wounding agents in pome fruits would certainly decrease disease incidence caused by *M. fructigena*. Moreover, less easily controllable abiotic factors, such as rain around harvest time, do not cause injuries to pome fruits to the same extent as experienced in stone fruits.

In conclusion, yield loss caused by *M. fructigena* did not exceed 5 % in the pre-harvest stage, and losses in the post-harvest stage were comparable with those reported in the

literature. In time, disease incidence gradually increased in the orchard, and no sharp increase was observed around harvest maturity, though rainy, damp weather conditions prevailed during harvest in 1998. In this, disease dynamics of *M. fructigena* in apple differ sharply from that of *M. fructicola* in stone fruits. A statistical analysis in space and time, using the LIP index, to which we added bootstrap confidence intervals, proved to be useful to distinguish differences between cultivars and between years. Distinct clustering of diseased fruits among fruit trees occurred in both years, but was more pronounced in 1998. Cluster size did not extend beyond tree boundaries, although first-order autocorrelation was detected in cv. Cox's Orange Pippin in the second year. Clearly, spread of disease within a tree occurred more readily with *M. fructigena*, than spread of disease to adjacent trees.

Chapter 6

Air dispersal of conidia of *Monilinia fructigena* in an apple orchard in relation to environmental factors

Abstract

The aerial concentration of *Monilinia fructigena* conidia, monitored during two consecutive years in 1997 and 1998 in an apple orchard, was related to environmental conditions. The highest hourly concentration measured in 1997 was 233 conidia/m^3 and occurred during afternoon hours, in 1998 concentrations were lower than in 1997 throughout the season. In the second year the viability of dispersed conidia was assessed using fluorescein diacetate (FDA), and 60 % of the conidia caught were viable. Relationships between spore catches and (derived) environmental variables were tested in order to identify the most important explanatory variables. Univariately, relative humidity and mean temperature prior to spore catch, best explained the variation in hourly spore counts in both years, these were followed by wind speed. Temperature and wind speed were positively related to spore catches, relative humidity negatively. Multivariate models were constructed by forward selection, i.e. first the variable which best explained the variation in spore catches univariately was added to the model, subsequently the next best as long as additions were worthwhile (χ^2-test, $P = 0.05$). In 1997 the best fitting model included the variables relative humidity, temperature, wind speed and wind direction, whereas in 1998 the best model consisted of only the former two. Spore concentrations observed in the present study were compared with observations done in stone fruit orchards where *M. laxa* and *M. fructicola* are the predominant species, and differences in epidemiology between these systems are discussed.

Introduction

The brown rot fungi of fruit crops (*Monilinia* spp.) cause blossom blight and fruit rot in rosaceous fruit crops in the temperate regions of the world (Byrde & Willetts, 1977). The group consists of three species: *M. fructicola*, *M. laxa* and *M. fructigena*. Sporodochia produced on blighted blossoms and infected fruits consist of numerous chains of conidia. Wind, water, insects, birds and man are responsible for the dispersal of conidia in pome and stone fruit orchards (Byrde & Willetts, 1977). Splash dispersal is important for short range dispersal within a tree (Pauvert *et al.*, 1969), while vectorborne (Lack, 1989) and airborne conidia (Kable, 1965a) ensure a wide dispersal of conidia within an orchard. The viability of dispersed conidia has generally often been overlooked. The viability of aerially dispersed spores can be reduced by low air humidity and u.v. radiation (Rotem *et al.*, 1985).

Most of the work on aerial dispersal of *Monilinia* conidia has been done with *M. fructicola* in stone fruits. Kable (1965a) found one major dispersal period in peach orchards, commencing one month before harvest and continuing up till two months after harvest. Jenkins (1965b) detected only small numbers of conidia in the air in a peach orchard, whereas large numbers were found in traps which collected rain water. In apricot affected by *M. laxa*, Corbin *et al.* (1968) found that the aerial spore content rapidly increased 10-14 days before the harvest ripe stage. In pome fruits *M. fructigena* is the predominant species causing fruit

rot, and dispersal of conidia by water and insects has been studied (Pauvert *et al.*, 1969; Lack, 1989). Although airborne conidia have been trapped on exposed plates and vaseline slides (Horne, 1933; Bucksteeg, 1939), no quantitative study of the aerial spore content in *M. fructigena* infested orchards has been made to date.

In addition to seasonal patterns, distinct diurnal patterns in aerial spore content have been observed and these have been related to environmental conditions. Peak concentrations of *Monilinia* conidia occurred during the afternoon when relatively low air humidities and high wind speeds prevailed (Kable, 1965a; Corbin *et al.*, 1968); this concurs with the characteristics of the group of dry-air spore types, to which *Cladosporium, Ustilago* and the group of powdery mildews belong (Hirst, 1953; Sutton & Jones, 1979; Willocquet & Clerjeau, 1998). Relationships between environmental factors and aerial spore concentration have been studied extensively, but in most cases analysis is restricted to simple regression analysis (Corbin *et al.*, 1968; Sutton & Jones, 1979; Sanderson & Jeffers, 1992). However, environmental variables can be strongly correlated, and multiple regression techniques offer the possibility to test the relative importances of explanatory variables in relation to the dependent variable (Draper & Smith, 1998). Moreover, possible interactions between variables can be tested by introducing product terms as independent variables. Workers have related the aerial spore concentration with instantaneous environmental conditions in most studies, whilst for some variables a certain lag period may be more appropriate. For example, in *Monilinia* it is known that dehiscence of conidial chains is stimulated by a decrease in the ambient relative humidity (Byrde & Willetts, 1977), but liberation and final take-off of conidia obviously do not happen instantaneously.

In this paper we present a two-years study of the aerial concentration of *M. fructigena* conidia in an apple orchard during the growing season. It was our objective to monitor the aerial spore content and determine the viability of trapped conidia. In an exploratory study we determined the most important environmental variables explaining the variation in spore catches observed. We focus more on model building than on formal hypothesis testing. A generalised linear model (GLM) was used to relate spore catch data with recorded environmental variables univariately as well as multivariately.

Materials and methods

Orchard site

The study was conducted in an experimental orchard (IPM) situated in the Rhine basin in the centre of the Netherlands during two consecutive growing seasons in 1997 and 1998. A Burkard 7-day recording volumetric spore trap (Burkard Manufacturing Co., Rickmansworth, Hertfordshire, UK) was placed in the centre of a plot which consisted of three rows of the apple cultivar (cv.) James Grieve alternating with two rows of cv. Cox's Orange Pippin, both planted in 1984. The plot was flanked by a block with the apple cultivars Golden Delicious and Lombarts Calville at the north-west side, and by a block with old bush trees (apple) at the south-west side. The remaining sides were flanked by high windbreaks. The spore trap was placed within a row where a tree was missing, and operated continuously with the orifice 2 m above ground level from the beginning of May until mid-October in 1997, and from half July until mid-September in 1998.

Aerial spore concentration, environmental variables

In 1997, the spore trap was operated at a flow rate of approximately 5 L/min due to a malfunctioning flow meter. In 1998, the flow rate was 10 L/min as is commonly used in similar studies (Gottwald & Bertrand, 1982; Aylor, 1993; Willocquet & Clerjeau, 1998).

Conidia were trapped on a strip of Melinex tape wrapped around a rotating drum. The strip was coated with a thin layer of Gelvatol (Monsanto Chemical Co., St. Louis, USA), on which, 24 h later, a layer of vaseline was smeared. The strip was changed every 7 days, and on removal the strip was marked with a needle at distances of 2 mm, corresponding with hourly time intervals. Subsequently, the strip was cut into pieces covering 24 h, after which these were mounted in glycerol on 76 x 26 mm glass slides. Hourly spore counts were made during the main dispersal period from mid-July until the end of August. *M. fructigena* conidia vary in size and shape, ranging from elongate-ellipsoid to ovoid and limoniform. Only limoniform-shaped conidia with a length greater than 20 µm could be identified definitely as *M. fructigena* conidia among the miscellaneous variety of trapped particles, and therefore only these were counted. The number of conidia occurring on traverses 200 µm wide, perpendicular to the direction of movement of the drum, was determined. Counts were first multiplied by 10, in order to estimate the total number of conidia caught in 1 h. Then, the estimated number of conidia per m^3 air was calculated according to the measured flow rate (5 L/min in 1997, and 10 L/min in 1998).

In 1998, a second Burkard trap was operated for 10 days in order to determine the viability of trapped conidia. Every 3 days the strip was changed, cut into sections corresponding to one day periods, and subsequently submerged in a 1:10 solution of fluorescein diacetate (FDA; Sigma Chemical Co., St. Louis, USA), of which the stock solution contained 1 mg FDA/ml aceton. After incubation for 20 min at 25 °C in darkness, the strip was mounted in glycerol on a glass slide, and subsequently examined for living and dead *M. fructigena* conidia. Viable conidia stained bright yellow with FDA when viewed under a Zeiss Axioskop equipped with an incident-light fluorescence illuminator, a 450-490 nm excitation filter, a 510-nm dichroic mirror, and a 520-nm barrier filter.

Meteorological data were obtained from a weather station, located at a distance of approximately 15 m from the spore trap. Temperature was measured in a Stevenson screen at 1.5 m height, wind speed and wind direction with an A100r switching anemometer, and rainfall with a standard pluviometer. Relative humidity data were obtained from a weather station 10 km from the experimental orchard, as the hygrometer inside the orchard did not function properly during a part of the season in both years. Relative humidity data from the weather station 10 km away showed a strong correlation with those from inside the orchard. All variables were recorded every 30 min, except for relative humidity which was recorded on an hourly basis. Wind direction was measured in degrees, and for analysis converted into a qualitative variable with four levels corresponding to the quadrants of a wind rose.

Statistical analysis

Environmental factors were related with spore catch data during the period mid-July until the end of August in both years. In the regression models the number of conidia counted in the traverses 200 µm wide was used as response variable. Standard linear regression analysis could not be applied, because residuals were not normally distributed (Draper & Smith, 1998). The probability distribution that is generally used to describe count data is the Poisson distribution, and this distribution was used. A generalised linear model (GLM) was constructed, relating the response variable to a set of predictor variables with a logarithmic link function (Dobson, 1990). The method of maximum likelihood was used to estimate parameter values (Dobson, 1990; Draper & Smith, 1998). The adequacy of a given model was determined by comparing the log-likelihood function under this model with that under the saturated model. The saturated model provides a complete description of the data, the number of parameters being equal to the total number of observations. Twice the difference between the log-likelihoods of the saturated model and the given model is called the deviance *D*. *D* follows approximately a χ^2_{N-p} distribution, where *N* is the number of observations and *p* the

number of parameters. Models were constructed which explained a significant part of the variation using only a few parameters. The deviance was used to compare models. The difference between two deviances, corresponding to two nested models to be compared, follows approximately a χ^2_{p-q} distribution, with p the number of parameters in the larger model and q in the smaller model.

The weather variables were first related univariately to hourly spore catches, all models initially including a quadratic component. The quadratic component was omitted from the model where it did not significantly reduce D (χ^2-test, $P = 0.05$). Temperature influences the rate of conidial development, and thus mean temperature over the preceding 0, 3, 6, .., 27 and 30 h was related to hourly spore catches. Relative humidity affects dehiscence of conidial chains, and a possible delayed response was studied by relating lagged relative humidity data to hourly spore catches (lag period was 0, 1, 2, .., 7 and 8 h). Total mm of rainfall in the preceding 0, 1, 2, .., 5 and 6 h was associated with spore catches. For each weather variable, models with the lowest D value were selected and subsequently used to construct multivariate models (see below). Mean hourly wind speed was related to spore catches in the same hour, and the same held for wind direction.

A forward selection procedure was applied to construct the best multivariate model in both years (Draper & Smith, 1998). The first variable entered in the model was that with lowest D in the univariate procedure, followed by the second best variable and interactions terms among variables etc., as long as additions were worthwhile, i.e. lowered D significantly (χ^2-test, $P = 0.05$). The best model for 1997 was also applied to the data of 1998.

Results

From May until mid-July in 1997, *M. fructigena* conidia were trapped only occasionally, which held also for the period after mid-September. The first fruits bearing pustules of *M. fructigena* were detected in trees of cv. James Grieve in the first week of July, and subsequently aerial conidia were detected regularly (Fig. 1).

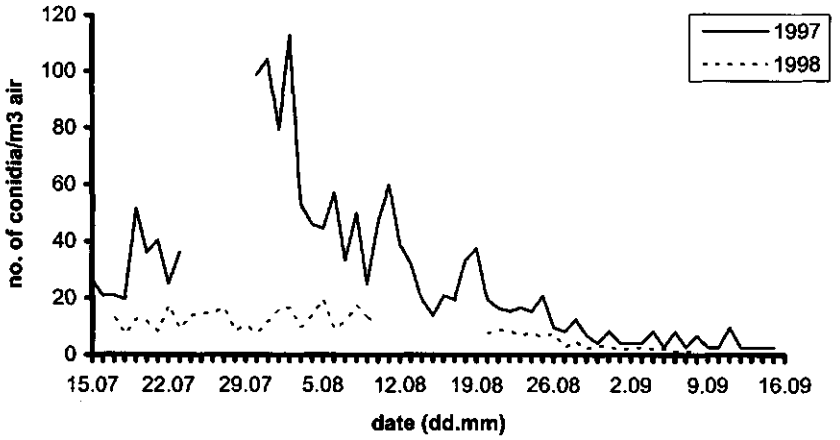

Fig. 1. Mean daily concentration of *M. fructigena* conidia in the air measured in an apple orchard from mid-July until mid-September (gaps in graphs due to missing data).

A distinct peak in spore concentration was found in the beginning of August in 1997, when a rainy period ended and a period of hot, dry weather commenced. In 1998, aerial spore concentrations were much lower, and no peak was observed (Fig. 1). The highest hourly concentration recorded was 233 conidia/m^3 in 1997, and 83 conidia/m^3 in 1998, both occurring in afternoon hours. For 1997, the frequency of occurrence of *M. fructigena* conidia in hourly intervals throughout the day was determined (Fig. 2). Frequency was highest during the afternoon hours, though conidia were also detected between 4 and 5 a.m. in almost 50 % of the days in the chosen period.

The second trap to determine the viability of aerial dispersed conidia, was operated during a moderately dry period in the beginning of August 1998. The number of conidia detected on the strips was 164 of which 98 turned out to be living (60 %).

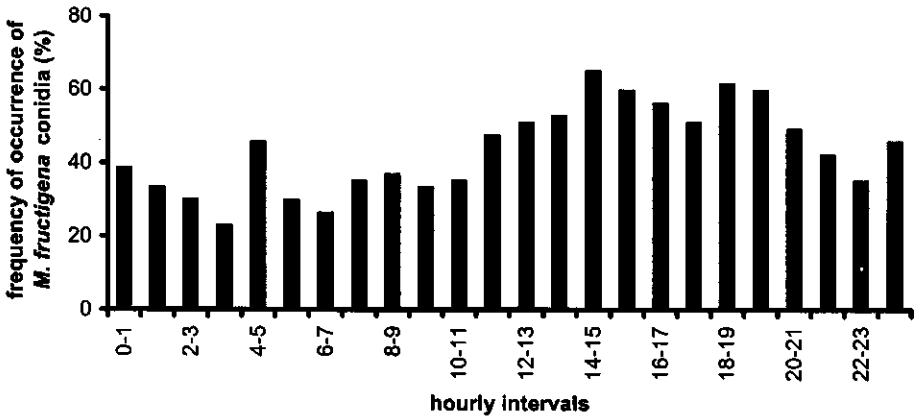

Fig. 2. Frequency of occurrence of *M. fructigena* conidia in sampled air during hourly time intervals in the period from mid-July until mid-September 1997

The results of simple regression analysis are shown in Table 1. In 1997, all variables caused a significant drop in deviance (D) when included in the model, whereas in 1998 only wind direction did not (χ^2-test, $P = 0.05$). Regression between relative humidity and spore catches gave the lowest D when lagged for 2 h in 1997, similarly to that in 1998 (1 h lag period). Relative humidity was negatively related to spore catches in 1998, and similarly in 1997. In 1997 the relation between spore catches and relative humidity showed an optimum at 40 % relative humidity. Mean temperature was positively related to spore counts (Table 1). Similar equations for wind speed were found in both years (Table 1). Wind speed ranged from 0.09 to 2.84 m/s in the observation period in 1997, and from 0.09 to 3.79 m/s in 1998. For both years, the fitted model showed a linear increase in expected spore counts with increasing wind speed, which started to level off at a wind speed of approximately 2 m/s due to the quadratic component in the equation. In 1997, a significant negative association appeared to exist between spore counts and winds blowing from eastern/southern directions (Table 1). This was to be expected because there was only a relatively small number of trees in the respective quadrant. Total mm of rainfall in preceding hours was related to spore catches, and in 1997 no significant drop in D was found up to rainfall in the four preceding hours. A significant negative relation was found between rainfall in the five preceding hours and spore counts (Table 1), in which the amount fallen ranged from 0 to 9.2 mm. Similarly, in 1998 a negative relation was found with total mm rainfall in the two preceding hours, and total mm of rainfall in this period ranged from 0 to 11.4 mm.

Table 1. Univariate models for the different environmental variables with smallest deviance (D) in 1997 and 1998

Variable [a]	Deviance	Regression model [b]
1997		
Rh, 2 h (rh2)	1494	$\log Y = -0.18 + 0.034*rh2 - 0.0004*(rh2)^2$
Mean T, 3 h (mtp3)	1570	$\log Y = -2.99 + 0.23*mtp3 - 0.0038*(mtp3)^2$
Wind speed (ws)	1579	$\log Y = -0.51 + 0.84*ws - 0.16*(ws)^2$
Wind direction (wd)	1648	$\log Y = 0.65 - 0.35*wd_1$
Rain, 5 h (rn5)	1664	$\log Y = 0.089 - 0.099*rn5$
1998		
Mean T, 0 h (mtp0)	1095	$\log Y = -1.77 + 0.076*mtp0$
Rh, 1 h (rh1)	1109	$\log Y = 1.08 - 0.02*rh1$
Wind speed (ws)	1163	$\log Y = -0.75 + 0.54*ws - 0.15*(ws)^2$
Rain, 2 h (rn2)	1170	$\log Y = -0.40 - 0.15*rn2$

[a] Relative humidity (rh) with lag period in h, mean temperature in preceding hours, total mm rainfall in preceding hours

[b] Y = expected number of conidia (present on 200 µm wide traverses)

Table 2. Multivariate models for 1997 and 1998 resulting from the procedure of forward selection

Variable [a)]	Deviance	DF	ChiSquare	*P*-value
1997				
Intercept	1650.87	0	.	.
Rh2	1512.10	1	138.77	0.0001
Rh2*rh2	1493.69	1	18.42	0.0001
Mtp24	1447.57	1	46.12	0.0001
Mtp24*mtp24	1443.54	1	4.02	0.0449
Rh2*mtp24	1439.00	1	4.54	0.0331
Rh2*rh2*mtp24	1431.33	1	7.67	0.0056
Ws	1419.46	1	11.88	0.0006
Wd	1392.16	3	27.30	0.0001
1998				
a.				
Intercept	1176.23	0	.	.
Mtp0	1095.40	1	80.83	0.0001
Rh4	1091.38	1	4.02	0.0449
b.				
Intercept	1176.23	0	.	.
Rh1	1109.49	1	66.74	0.0001
Mtp3	1086.44	1	23.05	0.0001

[a)] for explanation, see Table 1

The best fitting multivariate model for 1997 is given in Table 2. It appeared that addition of mean temperature over the preceding 3 h after relative humidity in the model (Table 1), did not significantly lower D (χ^2-test, $P = 0.05$). From analysis, it appeared that both variables were highly correlated (data not shown). However, addition of mean temperature over a more extended period did reduce D in the presence of relative humidity in the model. The average temperature over the preceding 24 h gave the most significant drop in D (Table 2). Significant interaction terms between relative humidity and temperature lowered D even more. Wind speed and especially wind direction significantly contributed to the model fit (Table 2). In 1998, the number of variables in the final model was much smaller. Starting with a model with only mean temperature (mtp0), only the addition of relative humidity lagged over 4 h further decreased D significantly (Table 2). When relative humidity was first entered in the model as in 1997, it was shown that addition of mean temperature over the preceding 3 h significantly reduced D, and that this model had a lower D than when temperature was entered first (Table 2). Typically, while in 1997 addition of the mean temperature over the preceding 3 h caused no drop in D, in 1998 it did. The correlation between relative humidity and mean temperature was much weaker in 1998 than in 1997.

When the model of 1997 was applied to the 1998 data, it turned out that wind speed, wind direction and the interaction between relative humidity and temperature caused no significant drop in D (χ^2-test, $P = 0.05$). Thus, the final model for 1998 contained only two variables, relative humidity and temperature, as with the 1998 models shown in Table 2.

Discussion

The concentration of *M. fructigena* conidia in the air increased considerably when the first diseased fruits appeared in the beginning of July. In 1997, before diseased fruits appeared, conidia were trapped only occasionally, probably originating from sporulating mummies at that time. Bucksteeg (1939) showed that the aerial concentration of *Monilinia* conidia in a pome fruit orchard peaked during June and July, but he did not relate this to possible sources. Similar to our observations with *M. fructigena*, Corbin *et al.* (1968) found that *M. laxa* conidia were only detected regularly when approximately 1 % of the fruits in the trees had *M. laxa* sporulation visible. Though the aerial concentration of *M. fructigena* conidia in the orchard plot was distinctively higher in 1997 compared with 1998 (Fig. 1), final disease incidence in cv. James Grieve was equal in both years (Van Leeuwen *et al.*, 2000). However, in cv. Cox's Orange Pippin fruit loss caused by *M. fructigena* was higher in 1997 compared with 1998 (4.4 % and 2.7 % respectively). The maximum hourly concentration detected in our study was approximately 200-250 conidia/m^3, which was much lower than concentrations measured in stone fruit orchards. Kable (1965a) reported that in a peach orchard with approximately 5 % of the fruits infected by *M. fructicola*, a maximum concentration of 5000 conidia/m^3 was measured. Corbin *et al.* (1968) found a maximum concentration of 1260 conidia/m^3 in an apricot orchard affected by *M. laxa*. The gradual increase in disease incidence in pome fruits compared with that in stone fruits (Corbin, 1963; Zehr, 1982; Van Leeuwen *et al.*, 2000), might explain the lower peak concentration observed in our study.

The clear peak in aerial spore content observed in 1997 in the beginning of August may have been due to a distinct change in weather. A period of rainy, dull weather was followed by 4 weeks of hot, dry weather during August 1997. In 1998 only a short period of hot, dry weather occurred (8-15 August), but unfortunately we missed part of this period due to problems with the trap (Fig. 1). It is hard to judge how the difference in flow rate of the trap between both years influenced the results. Hirst (1952) found a flow rate of 10 L/min the most satisfactory, he assumed that a lowering of the suction rate would reduce the velocity of the air stream close to the sticky surface, resulting in fewer spores impacted. The diurnal pattern of spore concentration observed during the major dispersal period in 1997 (Fig. 2) approached the typical pattern for the group of dry-air spore types (Hirst, 1953). Although on average, a slight peak in spore concentration was observed during the afternoon hours, absolute differences between afternoon hours and night hours were small. In a similar study with powdery mildew (*Podosphaera leucotricha*) in apple, Sutton & Jones (1979) distinguished days with highest wind speed and lowest relative humidity during midday associated with a typical diurnal pattern of spore catches, but also days with similar environmental conditions and no discernable periodicity.

The regression models obtained by simple regression were markedly similar in both years. Univariately, relative humidity and temperature caused the highest drop in deviance D, followed by wind speed (Table 1). Our hypothesis concerning a lagged effect of relative humidity on spore catches was supported. Relative humidity lagged over 2 h and 1 h in respectively 1997 and 1998, explained best the variation in spore catches observed. Corbin *et al.* (1968) found windspeed to be the most important (single) variable, followed by temperature and relative humidity, in explaining the variation in hourly spore catches in an

apricot orchard affected by *M. laxa*. A strong increase in afternoon wind speed was peculiar for the region where the study took place (Sacramento Valley, CA, USA), and this might have strengthened the explanatory value of wind speed. The regression models for wind speed (Table 1) predicted a linear increase in the number of trapped spores up to 2 m/s, after which the effect levelled off (due to the quadratic component). Similarly, Sutton & Jones (1979) reported a linear increase in spore release with increasing wind speed in the range of 0.7 to 2.2 m/s in *Podosphaeara leucotricha*. In both years, total rainfall caused the least significant drop in *D* univariately, and played no role in the multivariate models. We found a significant negative correlation between spore catches and rainfall when summed over preceding hours. The concentration of spores in the air can be directly influenced by rainfall due to scrubbing. Hirst (1953) noted that the concentration of conidia of *Ustilago* and *Erysiphe* sharply decreased after a heavy shower (7-8 mm within 1 h).

In the multivariate models for 1997 and 1998 both relative humidity and temperature played a major role. Probably due to higher spore catches in 1997 during a prolonged dry period, the best model in 1997 showed distinct effects of relative humidity and mean temperature. The effect of relative humidity was expressed over a period of hours (short-term), whereas temperature exerted a significant effect when averaged over a 24 h period (long-term). This agreed with studies on spore formation and maturation influenced by temperature (Hall, 1963), and spore liberation influenced by ambient relative humidity (Byrde & Willetts, 1977). However, the best model for 1998 did not include the long-term temperature effect, but instead both temperature and relative humidity showed a short-term effect. The data set of 1998 contained many zero values, and there might have been too little variation in the absolute numbers caught per hour to discern the effects of certain variables.

In conclusion, we found rather low concentrations of *Monilinia* conidia in the air in a pome fruit orchard in comparison with those determined in stone fruits. However, it should be noted that high concentrations in stone fruit orchards only occurred when disease incidence increased rapidly around harvest time. This latter phenomenon does not occur in pome fruits, where disease incidence increases gradually during the season (Van Leeuwen *et al.*, 2000). The level of contamination of healthy fruits by aerially dispersed conidia might also gradually increase during the season. However, the presence of wounds as a prerequisite for infection in *M. fructigena* renders the concentration of conidia on the fruit skin less important. In stone fruits *M. fructicola* is able to infect non-injured fruits as fruits mature, and the density of conidia on the fruit's surface is very important in this (Corbin, 1963). Thus, the percentage of aerially dispersed conidia causing infection in stone fruit culture might well be far higher than that in pome fruit culture, and in this way the epidemiology of *Monilinia* fruit rot disease in pome fruits might differ from that in stone fruits.

Chapter 7

Mummification and sporulation of pome fruits infected by *Monilinia fructigena* (Aderh. & Ruhl.) Honey

Abstract

A two-years field experiment (1997/1998, 1998/1999) was conducted to study mummification and subsequent sporulation in spring of pome fruits infected by *Monilinia fructigena*. Fruits of apple cultivars James Grieve and Golden Delicious and pear cultivar Conference were artificially infected at 9, 5, 3, and 0 weeks before estimated harvest maturity, and left in the tree canopy until the next year. After overwintering 12 fruits were collected per infection date in April, and the extent of mummification, percentage sporulating and sporulation intensity per sporulating fruit were determined. A second batch of equal size was collected at the moment of sporulation of individual fruits in the field (May-June). Sporulation intensity was measured after incubation for 7 days at 18-20 °C and RH 95-98 %. Under controlled conditions the effect of different post-infection regimes on mummification and sporulation intensity after a cold period was studied. In the field experiment most mummified fruits were found in James Grieve and Conference, whereas especially late infected Golden Delicious fruits were still soft when examined in April. In the first year these fruits had a significantly higher sporulation intensity per sporulating fruit (Kruskal-Wallis test, $P = 0.05$) compared with Golden Delicious fruits infected 9 and 5 weeks before harvest maturity, which were partly mummified. We conclude that early as well as late in the season infected fruits contributed to primary inoculum in the next season. In a post-infection regime of 25 °C and RH 65-75 % the percentage of fruits sporulating decreased rapidly, after 12 weeks incubation sporulation had completely ceased. After 8 weeks incubation sporulation intensity in the post-infection regime at 10 °C was significantly higher than that at 20 and 25 °C in the first experiment (Kruskal-Wallis test, $P = 0.05$). Results of the second experiment were less clear. The results are discussed in relation to disease management and potential future control options.

Introduction

The brown rot fungi of fruit crops (*Monilinia* spp.) cause considerable fruit losses worldwide (Byrde & Willetts, 1977). *M. fructicola* (Wint.) Honey is the predominant species in stone fruit culture, whereas *M. fructigena* (Aderh. & Ruhl.) Honey is predominant in pome fruits. Usually, the first infected fruitlets in pome fruit orchards appear approximately 5-6 weeks after full bloom, and subsequently infection of healthy fruits occurs continuously up to harvest time (Van Leeuwen *et al.*, 2000). Infection takes place via cracks and wounds in the fruit skin, and also via fruit-to-fruit contact (Michailides & Morgan, 1997). Disease incidence can be controlled by avoiding fruit injury caused by biotic (insects, birds, man) and abiotic (frost, hail) factors. However, in practice these factors commonly occur in pome fruit orchards. Another control option is the reduction of disease pressure in orchards by minimising the spore load in the environment. In this, special attention should be given to reduction of the amount of primary inoculum in spring. Primary inoculum of *M. fructigena* consists of conidia

which are produced on so-called mummified fruits or 'mummies' that have remained in the orchard from the previous season to spring. Mummified fruits develop due to dehydration after infection in the previous season, and consist of a mixture of fungal and plant tissue (Honey, 1928; Willetts, 1968). A combination of humid weather followed by temperatures of 15-20 °C in springtime, induces the formation of a fresh crop of conidia on the surface of mummified fruits (Byrde, 1954a).

In the past, winter sprayings with eradicant fungicides have been applied to inhibit the production of conidia on mummified fruits in springtime. Byrde (1954b) found a marked inhibition of sporulation on mummified apple fruits after trees were treated with pentachlorophenol in February. At the beginning of the season significantly less brown rot infection was found on trees which were treated with pentachlorophenol. Phenyl mercury chloride and sodium arsenite preparations have also been used in eradicant sprays (Wilson, 1950), but the use of this type of fungicides is no longer permitted, and it is necessary to develop other means of minimising the amount of primary inoculum. Workers have observed that not all mummified fruits sporulate in spring, even after incubation under optimal conditions for sporulation (Biggs & Northover, 1985; Van Leeuwen, unpublished).

The overall aim of this study was to shed more light on the process of mummification and subsequent sporulation after overwintering. The major objective was to determine the extent of mummification and spring sporulation of pome fruits infected by *M. fructigena* in the previous year. We hypothesised that the pathogen would survive winter conditions best in mummified fruits. Subsequently, mummified fruits would contribute most to renewed sporulation in spring. Fruits infected late in the season (September-October) were expected not to become mummified due to inappropriate environmental conditions, and consequently not contribute to primary inoculum in the next season. Once a suitable cultivar had been selected from the field experiment, additional experiments were done under controlled conditions to study the influence of different climatological conditions in the post-infection period on mummification and sporulation.

Materials and methods

Field experiment

The study was conducted in an experimental orchard situated in the Rhine basin in the centre of the Netherlands during two consecutive seasons (1997/1998 and 1998/1999) using two apple cultivars and one pear cultivar. For apple the early maturing cv. James Grieve and the late maturing cv. Golden Delicious were chosen, for pear cv. Conference. Healthy fruits, still attached to the tree, were artificially infected by inserting an agar plug taken from an actively growing *M. fructigena* colony in a small puncture made in the fruit. Fungal strain PD 4.96 isolated from apple (*Malus pumila*) in the Netherlands in 1996 was used in all field and climate room experiments. Fruits of James Grieve were infected approximately 9 (treatment J1), 5 (J2), 3 (J3) and 0 (J4) weeks before estimated harvest maturity, the same held for the other two cultivars (encoded with 'G' for Golden Delicious, 'C' for Conference). The estimated harvest maturity for James Grieve was approximately mid August, for Golden Delicious the beginning of October, and for Conference the beginning of September. Individual fruits were removed from the trees when a clear lesion had developed, and placed in string bags (size 18 x 24 cm). Thus, for each cultivar 24 fruits were collected per infection date and subsequently the string bags were hung at random in the tree canopy of a row of apple trees (12 trees, 2 bags per infection date per cultivar in each tree). The following year the bags were collected at two sampling moments (2 x 12 fruits). A first batch was collected mid-April before natural sporulation occurred in the field, and the second batch consisted of

the remaining 12 fruits which were collected as soon as the first conidial pustules were detected on individual fruits in the field. After collection the fruits were immediately taken to the laboratory, where the extent of mummification (only in April) and sporulation intensity were determined.

A mummification index (m.i.) was used to determine the extent of mummification: 0 = decomposed (fruit falls apart with little pressure); 1 = soft (fruit deformed by pressure); 2 = mummified (firm, not deformed by pressure). To determine the percentage of fruits sporulating and the sporulation intensity per sporulating fruit, fruits were incubated at 18-20 °C at a relative humidity (RH) of 95-98 % for 7 days in closed trays (standard conditions). Light intensity was low (100-150 lux), due to diffuse light from other parts of the climate room (16 h light/8 h darkness). Before incubation fruit structures were lightly brushed in running tap water to remove conidia from the surface. For the sample taken in April, fruits coming from one tree were collected in a single tray (12 trays in total). After 7 days lids were removed from the trays and the presence or absence of conidial pustules of *M. fructigena* on each fruit was determined. Trays were left open to promote dehiscence of conidial chains at a lower relative humidity (RH 75-85 %), and 24 h later conidia were collected by washing the fruits in 30-40 ml water in glass jars in an orbital shaker at 300 rpm for 30 s. The conidial concentration of the suspensions obtained after filtering through double cheesecloth was determined using a haemocytometer.

The number of fruits sporulating differed between infection dates, and for this means of sporulation intensity per sporulating fruit per infection date were compared by Kruskal-Wallis test for each cultivar in the sample taken in April. Mean sporulation intensity of the sample taken in April and that of the sample taken at the time of natural sporulation were compared with a *t*-test after log-transformation.

Controlled environment experiments

Conference appeared to be the most appropriate for further experiments under controlled conditions, as fruits of this cultivar mummified well and showed less variation in sporulation intensity between fruits. A more practical reason was that Conference fruits are available throughout the year. In experiment I unripe fruits were used, in experiment II ripe Conference fruits (size class 45-55 mm). Fruits were infected by inserting an agar plug taken from an actively growing colony, and incubated in closed trays at 20 °C and RH 95-98 % for 9-10 days. Subsequently, those fruits which were almost completely covered with a lesion were selected, and fruits with a sporulation area of similar size were equally distributed over the treatments. Treatments consisted of four different post-infection regimes: (1) 9-10 °C, RH 85-95 %, (2) 14-15 °C, RH 75-85 %, (3) 19-20 °C, RH 75-85 %, and (4) 24-25 °C, RH 65-75 %. Light intensity during incubation was low (100-150 lux), due to diffuse light from other parts of the climate room (16 h light/8 h darkness). Ten and eight replicates were used in experiment I and II respectively. After 4, 8 and 12 weeks incubation individual fruits were removed from the climate rooms, placed in small plastic pots (l x w x h = 6.5 x 6.5 x 8 cm), and incubated for 3 months at 2-3 °C followed by a 25-days period at -20 °C to mimic winter conditions. After being taken from the -20 °C freezer, fruits were left for one day, and then tested for extent of mummification as described for the field experiment. The percentage of fruits sporulating after 6 days incubation at 18-20 °C and RH 95-98 % was determined, as well as sporulation intensity per fruit after 7 days (same procedure followed as in field experiment). For each incubation period, means of sporulation intensity per treatment were compared with a Kruskal-Wallis test.

Results

Field experiment

In James Grieve most fruits infected early (treatments J1, J2 and J3) were found to be mummified (m.i.= 2) when examined after overwintering. However, in the first year none of the fruits infected at harvest maturity (J4) mummified, and two fruits were found to be decomposed (m.i.= 0) in April 1998. In the second year, eight out of 12 fruits were classified as mummified in treatment J4, and no decomposed fruits were found. As expected, mummification of infected fruits in the late apple cultivar Golden Delicious was poor (Table 1). In the first year only one mummified fruit was found at each of the two early infection dates (G1, G2), whereas fruits infected 3 and 0 weeks before harvest maturity remained soft and still had a high water content after overwintering. Results were similar in the second year, although more mummified fruits were found (Table 1). Results for Conference were similar to those obtained for James Grieve. All fruits infected 9 weeks before harvest maturity developed into mummified fruits in both years. After infection of Conference fruits at harvest maturity (C4) in the second year, only a lesion developed on the infected fruits and hardly any sporulation occurred. Other fungi like *Botrytis cinerea* and *Rhizopus* spp. rapidly colonised the fruits. Eight out of the 12 fruits decomposed after overwintering.

Table 1. Extent of mummification of fruits of cv. Golden Delicious infected by *M. fructigena* at different moments before harvest maturity (numbers per class, $n = 12$ per treatment) [a)]

	1998				1999			
mummification index	G1 [b)]	G2	G3	G4	G1	G2	G3	G4
0 (= decomposed)	0	0	0	0	0	0	0	2
1 (= soft)	11	11	12	12	7	9	11	10
2 (= mummified)	1	1	0	0	5	3	1	0

[a)] fruits infected in 1997 and 1998, mummification determined after overwintering mid-April 1998 and 1999 respectively

[b)] fruits infected 9 (G1), 5 (G2), 3 (G3) and 0 (G4) weeks before harvest maturity (harvest maturity beginning of October)

The percentage of fruits sporulating and mean sporulation intensity per sporulating fruit structure sampled mid-April, are given in Fig. 1. Most remarkably, the non-mummified fruits of late infected Golden Delicious fruits (treatments G3, G4) sporulated profusely (Fig. 1c, d). In 1998, all fruits in treatments G3 and G4 showed sporulation, compared with only 40 % of the fruits in treatments G1 and G2. Moreover, sporulation intensity per sporulating fruit was significantly higher in treatment G4 compared with treatments G1 and G2 (Kruskal-Wallis test, $P = 0.05$; Fig. 1d). By contrast, in 1999 treatments G1 and G2 showed the highest sporulation percentages, and differences in sporulation intensity were not found. In James Grieve the pattern of percentage of fruits sporulating also differed considerably between the two years (Fig. 1a). In Conference the percentage of fruits sporulating decreased as fruits were

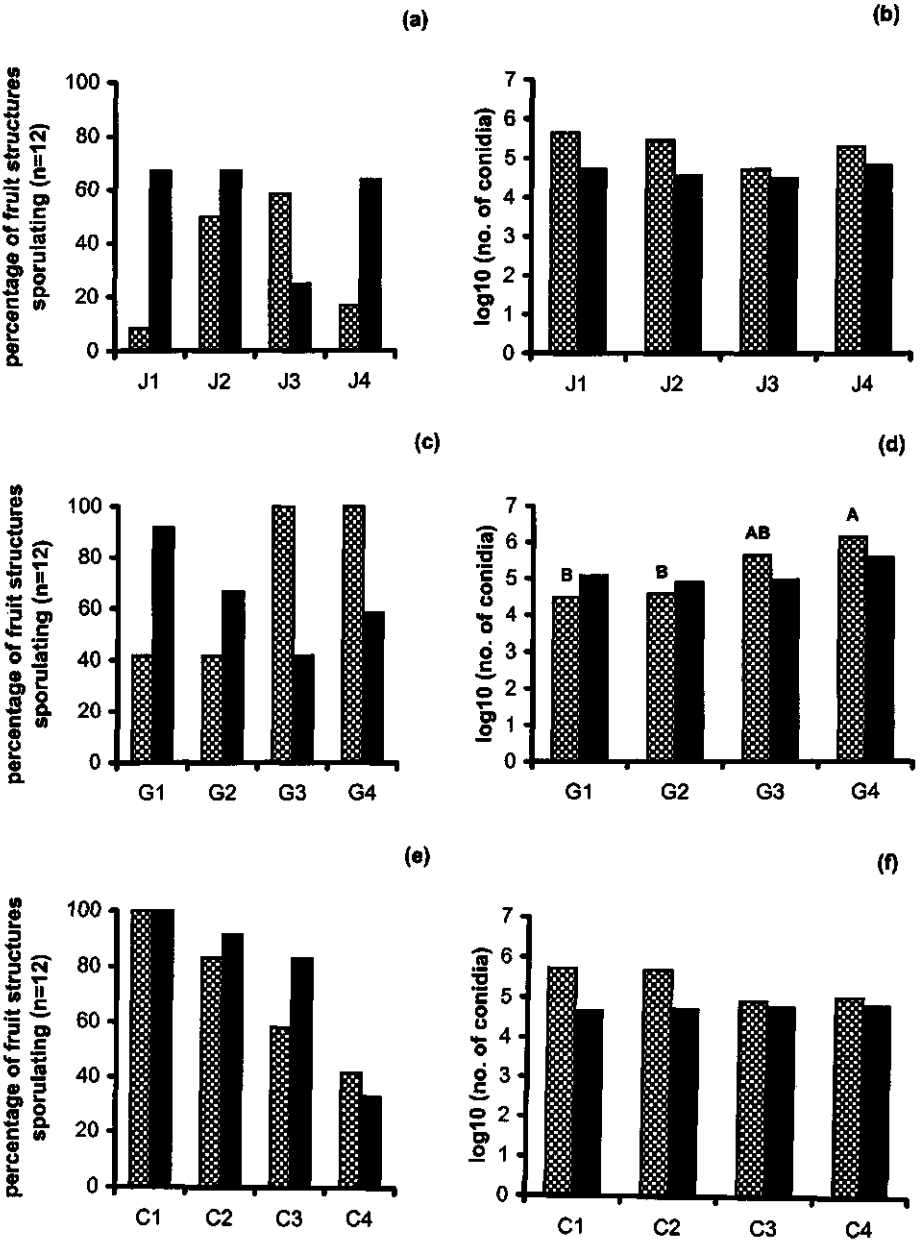

Fig. 1. Percentage of fruit structures sporulating, and the number of *M. fructigena* conidia produced per sporulating fruit structure (log10-transformed) after incubation for 7 days at 18-20 °C and RH 95-98 %. Sampling mid-April 1998 (▒) and 1999 (▓). J = cv. James Grieve, G = cv. Golden Delicious, C = cv. Conference. For further explanation of codes, see footnote Table 1. Bars indicated by different letters are significantly different (Kruskal-Wallis test, $P = 0.05$).

infected later in the previous season (Fig. 1e), and results were similar for both years. In the second year, only those fruits which had not decomposed sporulated in treatment C4 (see above). No significant differences were found in sporulation intensity between treatments (Fig. 1f).

In 1998, first sporulation of the fruits left in the field was observed on 26 May. Subsequently, sporulating fruits were collected regularly and taken to the laboratory to determine sporulation intensity, and on 16 July all remaining fruits, which had still not shown any sporulation, were collected. Of these, only one out of 19 fruits sporulated after incubation at standard conditions in the laboratory. In 1999, first sporulation was detected on 8 May, and at the final collection date on 1 July there were still 33 non-sporulating fruits remaining. None of these sporulated after subsequent incubation under standard conditions. The percentage of fruits sporulating under field conditions in 1998, was higher than that in the sample taken mid-April for all cultivars in all treatments. For example, in James Grieve the percentage sporulating rose from 8 % in April to 58 % in the field sampling in treatment J1, and also in treatment J4 a considerable increase was observed (from 17 % to 58 %). A similar pattern was observed in the second year.

Differences in sporulation intensity of sporulating fruits between both sampling moments are shown in Fig. 2. Due to considerable variation in sporulation intensity, few significant differences were found. In some treatments, sporulation intensity of fruits at the second sampling occasion was significantly higher than that in mid-April (*t*-test, $P = 0.05$). However, in Golden Delicious a significant decrease was observed in 1998 in treatment G4 (Fig. 2c). Whereas in mid-April sporulation had occurred over the whole surface of the soft fruit structure, at the moment of sporulation in the field fruits had shrunk considerably and sporulation intensity per fruit had dropped considerably.

Controlled environment experiments

Very few mummified fruits were formed under the given climate room conditions, probably because of the generally high relative humidity. After 12 weeks incubation in the driest climate (24-25 °C, RH 65-75 %), only two out of 10 fruits became mummified in experiment I, and none in experiment II. The percentage of fruits sporulating sharply decreased with exposure to the 24-25 °C and RH 65-75 % regime (Fig. 3). After 8 weeks incubation the percentage of fruits sporulating was 20 % and 0 % in experiment I and II respectively, and no sporulating structures were found after 12 weeks incubation. Percentage sporulating was highest in the 10 °C and RH 85-95 % regime, though in experiment II an unexpected increase in percentage sporulating was observed from 8 to 12 weeks incubation at 15 °C (Fig. 3). Sporulation intensity per sporulating fruit for the different treatments is shown in Fig. 4. In experiment I no significant differences in sporulation intensity were found after 4 weeks incubation. After 8 weeks incubation sporulation intensity was significantly lower in the post-infection regimes of 20 and 25 °C compared with that at 10 °C (Kruskal-Wallis test, $P = 0.05$); similarly after 12 weeks incubation. In experiment II where ripe Conference fruits were used, no clear differences in sporulation intensity were found between treatments. After 4 weeks incubation sporulation intensity in the 15 °C regime was significantly higher than that at 20 and 25 °C, but after 8 weeks the lowest value was found at the 15 °C regime (Fig. 4).

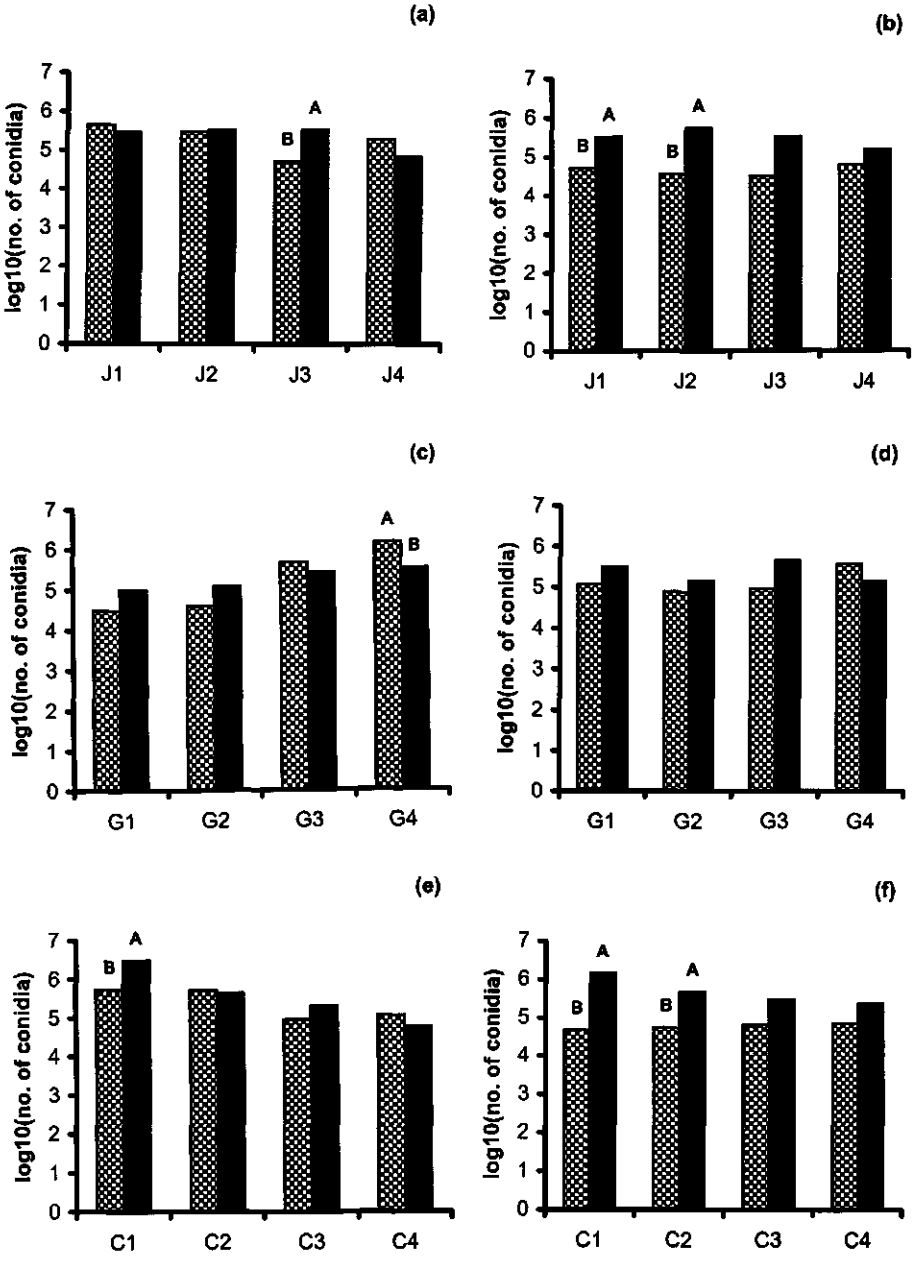

Fig. 2. Mean sporulation intensity per sporulating fruit structure (log10-transformed) after incubation for 7 days at 18-20 °C and RH 95-98 %, in 1998 (left) and 1999 (right). Sampling mid-April (▒) and at the moment of first sporulation in the field (■). J = cv. James Grieve, G = cv. Golden Delicious, C = cv. Conference. For further explanation of codes, see footnote Table 1. Bars indicated by different letters are significantly different (*t*-test, $P = 0.05$).

Discussion

The hypothesis that only mummified fruits would sporulate after overwintering was rejected. Fruits of Golden Delicious infected at the beginning of October had not mummified after overwintering, but nevertheless sporulated profusely when brought under optimal conditions. The percentage of fruits sporulating was higher in treatment G4 compared with treatments G1 and G2 in the first year, as also was the sporulation intensity per fruit. The results from the two-years field experiments show that, in the cultivars tested, early as well as late infected fruits contribute to the production of primary inoculum in the next season.

Sporulation in the field occurred relatively late in 1998. Usually, in western Europe climatic conditions do not favour the production of a fresh crop of conidia on *M. fructigena*-infected fruits before the beginning of May. Sporulation depends on the combination of moist conditions immediately followed by temperatures approaching 20 °C (Byrde, 1954a). Differences in percentage sporulating between the sample taken mid-April and that observed in the field was sometimes considerable. In the first year the percentage of fruits sporulating in the field was higher in all treatments for each cultivar. Probably, the standard incubation period of 7 days used to stimulate sporulation in the sample taken in April, was too short. However, in the second year an extra batch of overwintered fruits was incubated for a period of in total 21 days, but only two out of 39 fruits which did not sporulate after 7 days, finally sporulated after 21 days. We conclude that the incubation method used before sporulation in the field occurred underestimated the percentage of fruits sporulating later in the field. Sporulation intensity per sporulating fruit determined after standard incubation increased significantly at the second sampling date in some treatments (Fig. 2). This only occurred in treatments with a high percentage of mummified fruits, in James Grieve and Conference. The higher sporulation intensity observed at the second sampling date was to be expected, because at that moment the fruits already showed initial sporulation in the field (pre-conditioning).

Results obtained in the controlled environment experiments clearly expressed the effect of climatological conditions in the post-infection period on subsequent sporulation after a cold period. Under relatively cold and wet conditions (10 °C, RH 85-95 %) the percentage of fruits sporulating decreased only after 12 weeks incubation, whereas sporulation intensity per sporulating fruit remained constant (Figs. 3 and 4). In the field similar conditions would be experienced by fruits infected late in the season, thus the intense sporulation found for Golden Delicious infected in September and October the previous year (treatments G3 and G4), is not surprising. The warmer and (relatively) drier the post-infection conditions, the faster the percentage of fruits sporulating decreased (Fig. 3). Furthermore, in experiment I sporulation intensity per sporulating fruit dropped significantly in the 20 and 25 °C regime compared with 10 °C. Interestingly, at a constant temperature of 25 °C and a relative humidity of 65-75 %, infected fruits soon lost their ability to sporulate. Infected fruits subjected to these conditions in the field, would hardly contribute to primary inoculum in the next season. However, under Dutch conditions high temperatures in summer usually accompany much lower relative humidities during the day (approximately 35-50 %). These conditions stimulate rapid drying of infected fruits which results in the formation of mummified fruits. Mummified fruits were hardly found when infected fruits were incubated at RH 65-75 % at 25 °C for 12 weeks.

In the controlled climate experiments it was shown that infected fruits which were still soft (high water content) before being subjected to -20 °C, sporulated profusely after the cold treatment. Thus, mummification is not essential to resist a period of low temperatures. It was noted that the new conidia on the surface of these fruits originated from sporodochial cushions formed immediately after infection. Thus, regeneration of conidia occurred from the same sporulating area, as reported for *M. fructicola* (Corbin & Cruickshank, 1963). Very likely,

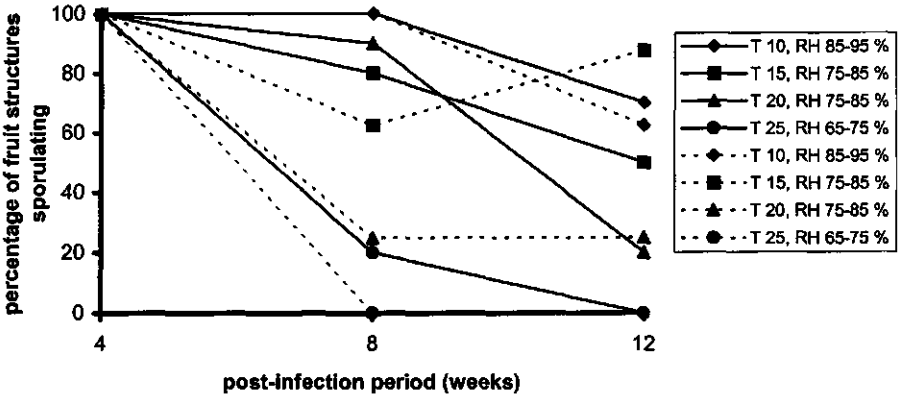

Fig. 3. Effect of different climatological conditions in the period after infection on the percentage of fruit structures sporulating after a subsequent cold period (3 months at 2-3 °C, 25 days at -20 °C). Sporulation determined after incubation for 6 days at 18-20 °C and RH 95-98 %. Experiment I (—), experiment 2 (-----), both cv. Conference.

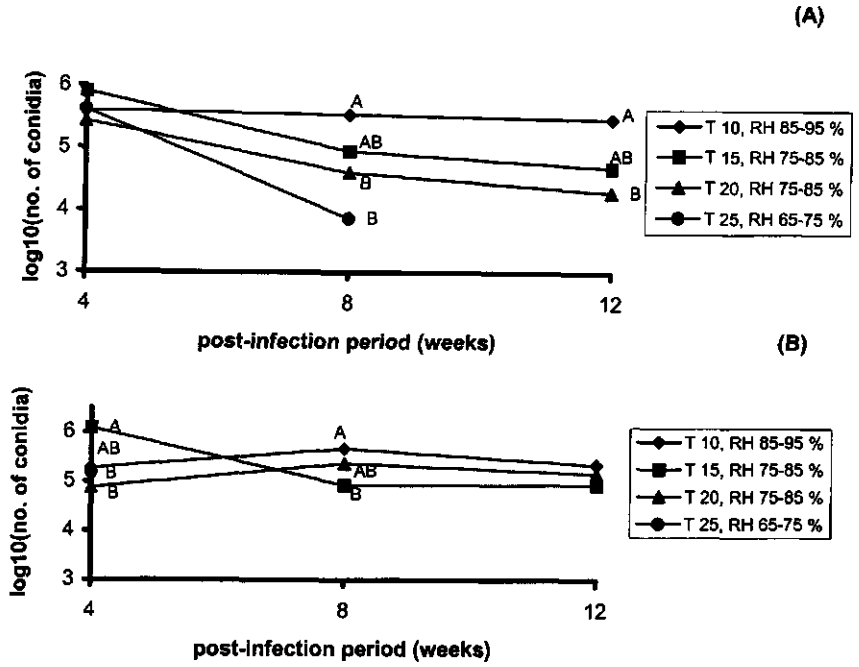

Fig. 4. Effect of different climatological conditions in the period after infection on sporulation intensity per sporulating fruit structure after a subsequent cold period (3 months at 2-3 °C, 25 days at -20 °C). Sporulation determined after incubation for 6/7 days at 18-20 °C and RH 95-98 %. (A) experiment I, (B) experiment II, both cv. Conference. Per incubation period, means indicated by different letters are significantly different according to Kruskal-Wallis test ($P = 0.05$).

there is a strong correlation between initial sporulation intensity immediately after infection, and later (re)sporulation on the same fruit. This may have affected our results in the field as well as controlled climate experiments. In the latter, the sporulating area per infected fruit was quite uniform in experiment I, and consequently fruits with similar sporulation areas could easily be distributed over the different treatments. In experiment II where ripe fruits were used, a considerable variation in sporulating area per fruit was observed after infection, and an equal distribution over treatments was difficult. This affected the results considerably (Figs. 3 and 4). The high variation in sporulation intensity found within treatments in the field experiment could also have been caused partly by differences in initial sporulation after infection, but this was not determined.

Corbin & Cruickshank (1963) showed that apricot fruitlets, collected in the field after a brown rot epidemic caused by *M. fructicola*, were able to produce several crops of conidia. They showed that in short incubation periods of 12 h at 20 °C and RH 98 %, fruitlets produced up to 10 crops of conidia before regeneration of conidia ceased. The number of conidia produced per crop diminished quickly in successive crops. Thus, if environmental conditions favour the rapid production of several conidial crops, fruits might quickly loose the ability to resporulate. This process might also have influenced the results in our experiments. For example, under controlled conditions several crops of conidia might have been produced at 25 °C and RH 65-75 %, and consequently the percentage of fruits sporulating after 8 and 12 weeks incubation decreased rapidly. Knowledge about the effect of environmental factors on regeneration of conidia is very limited (Corbin & Cruickshank, 1963), especially the effect of temperature has not been studied.

Alternatively, environmental conditions might directly affect the viability of sporodochial cushions and conidiophores from which sporulation occurs. This might have played a role in the field where a marked drop in sporulation intensity per fruit was observed in Golden Delicious (treatment G4) at the moment of sporulation in the field in 1998 compared with the sample taken mid-April (Fig. 2c). At the beginning of May, a prolonged period with temperatures ranging from 25 to 30 °C during daytime and high radiation preceded first sporulation in the field at the end of May. The viability of sporodochial cushions and conidiophores at the surface of the fruit structure might have been seriously affected by these environmental conditions. The detrimental effect of u.v. radiation on the viability of conidia in *M. fructicola* is well known (Thanos, 1951; Shepherd, 1968).

In conclusion, we have clearly shown that fruits infected by *M. fructigena* early in the season as well as those infected late, contribute to the production of primary inoculum in spring. For practical control measures this implies that not only should mummified fruits be removed and destroyed prior to sporulation in spring, but all fruits infected by *M. fructigena* in the previous season. However, it is hard to recognise those fruits infected but not mummified. Alternatively, we should aim at minimising the number of conidia produced per overwintering fruit. The controlled environment experiments clearly indicated that regeneration of new crops of conidia strongly diminished under certain conditions. Unfortunately, in the field we are hardly able to manipulate climatological conditions. Nevertheless, it is worthwhile to study in more detail the process of multiple sporulation from the same sporulating area. Possibly, there is an opportunity to interfere with the process of regeneration of conidia by application of selected micro-organisms. It has been shown that *Penicillium frequentans* produces antifungal substances which inhibit germination and germ tube growth in *Monilinia laxa* (De Cal *et al.*, 1988). Pusey *et al.* (1986) showed that *Bacillus subtilis* produced an antibiotic substance toxic to *M. fructicola*. Further research effort to unravel the mechanism of (re)sporulation in *M. fructigena* might lead to future control measures which

lower the amount of primary inoculum. Together with adequate control measures during the season this would result in lower fruit losses due to *M. fructigena*.

Chapter 8

Pest Risk Analysis (PRA) for the countries of the European Union (as PRA area) on *Monilinia fructicola*

Abstract

A pest risk analysis (PRA) was performed to determine the probability of introduction of *Monilinia fructicola* (Winter) Honey in the countries of the European Union (EU), and its potential economic impact. Data on the biology of the pathogen were combined with trade pathways and information on use of commodities, in order to quantify risk. On the basis of the Guidelines on Pest Risk Analysis, we concluded that there is a serious risk that *M. fructicola* becomes established in the EU. This merits the phytosanitary measures previously taken.

Introduction

Monilinia fructicola (Winter) Honey is a serious fungal pathogen of stone fruit crops in North and South America, South Africa, Japan and Australia, and is listed on the EPPO A1 list of quarantine organisms for Europe (CABI/EPPO, 1997). Two closely related species are endemic in Europe, *M. fructigena* (Aderh. & Ruhl.) Honey and *M. laxa* (Aderh. & Ruhl.) Honey. The three species are commonly referred to as "the brown rot fungi of fruit crops". They cause blossom and twig blight, and fruit rot in rosaceous crops. Recently, a collaborative effort was made to improve identification and (quick) detection methods for *M. fructicola* in a project financed by the European Union (Corazza *et al.*, 1999). We consider there is a need to assemble and update information on *M. fructicola* related to the probability of introduction of the pathogen into the European Union (EU). Introduction is defined here as the entry of a pest resulting in its establishment. We present a full pest risk analysis (PRA) on *M. fructicola* according to the guidelines previously published (OEPP/EPPO, 1997).

Stage 1. Initiation

Identify pest, PRA area

Name: *Monilinia fructicola* (Winter) Honey
Synonyms: *Sclerotinia fructicola* (Winter) Rehm
Taxonomic position: Fungi, Ascomycota, Discomycetes, Helotiales
Common names: brown rot fungus, brown rot disease (English);
pourriture brune des fruits (French);
Braunfäule der Früchte (German);
la podredumbre morena (Spanish);

As the PRA area we defined the countries of the European Union (Austria, Ireland, Portugal, Belgium, Italy, Spain, Denmark, Sweden, Luxemburg, France, Germany, Greece, The Netherlands, United Kingdom and Finland).

Stage 2. Pest risk assessment
Section A. Qualitative criteria of a quarantine pest

Geographical criteria
The pathogen does not occur in the PRA area, though it has been reported from nearby countries. In Egypt, the pathogen was found on apple fruits (Ali & Morsy, 1972). It is hard to verify this record on the basis of the information given in the publication. The pathogen is known to occur in North and South America, the Far East (Korea, Taiwan, Japan), Australia, Yemen, Zimbabwe and South Africa (CABI/EPPO, 1997).

Potential for establishment
The brown rot fungi of fruit crops have a wide host range, comprising fruit and ornamental crops of the family of Rosaceae. *M. fructicola* is widely reported to occur in stone fruit crops (peach, apricot, etc.). Vast cultivated areas of stone fruit are found in South Europe, and an overview of areas and production is given in Table 1.

Table 1. Area and production of stone fruit crops of EU-countries in South Europe in 1996 [a)]

	(x 1000 ha)				
	peach/nectarine	apricot	cherry	almond [b)]	plum
France	32	18	16	n.a.	n.a.
Greece	45	5	10	25	1
Italy	107	16	29	95	14
Portugal	11	1	4	42	n.a.
Spain	75	24	28	765	20
	(x 1000 ton)				
	peach/nectarine	apricot	cherry	almond	plum
France	463	176	74	n.a.	270
Greece	935	47	42	n.a.	5
Italy	1743	126	145	93	189
Portugal	73	5	10	7.5	n.a.
Spain	892	194	76	227	145

n.a. = not available
[a)] source: Eurostat, European Union, Brussels
[b)] data of 1995

Italy and Spain had the highest production of stone fruits within the EU in 1995/1996. Peach and nectarine are the most important crops. The climate of South Europe is comparable with that of California (USA), where *M. fructicola* is widely spread (Anonymous, 1996a). There is no reason to assume that climatological conditions might restrict the establishment of *M. fructicola* in Europe. *M. fructicola* does not depend on specific vectors for propagule dispersal. Conidia are readily dispersed by wind, water, all kind of vectors (insects, birds) and man (Kable, 1965a; Ogawa *et al.*, 1975).

Potential economic importance
Moderate to high yield losses are caused by *M. fructicola* in the USA and Australia. In addition, introduction of *M. fructicola* in one of the EU countries will have serious consequences for export (intra-EU trade, trade with third countries). A detailed account of the economic importance is given in Section B.

Section B. Quantitative evaluation

1. *Probability of introduction*

Entry
We distinguished two pathways that the pest can be carried on : imported (stone) fruits, and nursery stock.

Fruits :
The greatest bulk of material on which the pathogen could be carried, is represented by imported fruits. The amount of fresh stone fruits imported from regions where *M. fructicola* is known to occur, is given in Table 2.

Table 2. Import of fresh stone fruits (apricot, cherry, peach/nectarine, plum and almond) in EU-countries from regions where *M. fructicola* is endemic (in tons; data for 1998) [a]

	N-America	Argentina	South Africa	Australia [b]	others [c]	total
France	474	118	1278	88	0	1958
Greece	0	0	0	0	0	0
Italy	119	153	453	15	0	740
Portugal	0	0	93	0	17	110
Spain	37	323	1987	0	8	2355
Belgium+ Luxemburg	164	595	6634	9	0	7402
Netherlands	14	387	6751	24	62	7238
UK	6546	64	13201	179	51	20041
Germany	142	26	1958	18	4	2148
Sweden	932	2	0	0	0	934
Finland	203	1	0	0	0	204
Denmark	74	0	0	0	0	74
Ireland	0	0	0	0	0	0
Austria	0	0	0	0	0	0

[a] source: Eurostat Data Shop, Voorburg, The Netherlands
[b] including New Zealand
[c] Zimbabwe, Brazil, Uruguay

It is clear that most of the imported produce arrives in ports of West-European countries (UK, Belgium, The Netherlands), and a relatively small amount is imported directly by the main producers of stone fruits in South Europe. North America (Canada, USA) as well as South Africa are the main exporting countries (Table 2). According to the Inter-African Phytosanitary Council (IAPSC, 1985) *M. fructicola* is present in South Africa, although it did not occur on the distribution maps of quarantine pests recently published by CABI/EPPO (1998). However, recently *M. fructicola* was intercepted by plant protection services in Germany and the UK on material coming from South Africa (Anonymous, 1996b; OEPP/EPPO, 1999).

That the pathogen is able to survive transit is clear. Wormald (1954) reported that *M. fructicola* was found on more than one occasion on fruit imported into England from the USA. Recent interceptions have been reported from Germany, where the pathogen was found on *Prunus* fruit from South Africa (Anonymous, 1996b), and from the UK in 1999 (OEPP/EPPO, 1999). The pathogen is easy to detect, when sporulating on fruits, but culturing is necessary to determine which of the three *Monilinia* species is involved. When only lesions are visible, other fruit pathogens can also be involved (*Botrytis* Pers.: Fr., *Penicillium* Link). Currently, an ELISA-based detection kit is being optimised by the Central Science Laboratory (CSL) in York (UK), in order to quickly detect *M. fructicola* on imported fruits (C. Lane, pers. comm.). *M. fructicola*-specific primers were developed by Fulton & Brown (1997) for *in situ* detection of *M. fructicola* on imported produce. However, intraspecific genetic variation in *M. fructicola* renders these primers less appropriate (Fulton *et al.*, 1999; Förster & Adaskaveg, 2000). A set of newly developed primers directed to other regions in fungal DNA, can probably serve as an adequate tool for quick detection of *M. fructicola* (Förster & Adaskaveg, 2000).

After entry, fruits are considered to be widely distributed in the PRA area. Because the product is aimed for consumption, there is a small chance that (infected) fruits end up in the vicinity of suitable hosts. It is evident from Table 2, that most shipments arrive at entry points far from the main areas of stone fruit production in Europe. Moreover, the bulk of imports arrive in Europe during winter time (period November-March), when hardly any susceptible host tissue is available in orchards for infection.

Nursery stock :

Import of nursery stock of *Prunus*, *Malus*, *Pyrus*, *Cydonia* species and other rosaceous plants, also forms a potential risk. Specific data on import of rosaceous crops were not available, more general data on import of trees and shrubs are given in Table 3. Spain imported a considerable volume of trees and shrubs from risk areas in 1998. The greatest bulk came from Yemen (577 tons), but it is not clear what kind of material was exactly involved. Other countries which exported nursery stock to EU countries were Korea, Japan, South Africa and the USA. *M. fructicola* can survive in twig lesions, and these are usually hard to detect. *M. laxa* and other pathogens cause similar symptoms. Isolation of the pathogen(s) involved is necessary before these can be identified (Corazza *et al.*, 1998; Van Leeuwen & Van Kesteren, 1998). The imported material is not likely to be distributed widely, material will be confined to nurseries initially. On nurseries, the pathogen has an excellent opportunity to infect other trees. For this, strict care should be taken with the import of nursery stock of rosaceous plants.

Table 3. Import of trees and shrubs for the growing of edible fruits in EU-countries from regions where *M. fructicola* is endemic (in tons; data for 1998) [a)]

France	62	UK	12
Greece	0	Germany	0
Italy	0	Sweden	0
Portugal	0	Finland	2
Spain	672	Denmark	0
Belgium+		Ireland	0
Luxemburg	0	Austria	0
Netherlands	23		

[a)] source: Eurostat Data Shop, Voorburg, The Netherlands

Establishment

Host plant preference, ecological niche

M. fructicola shares common features with *M. laxa*; this species is widely established in European stone fruit orchards. However, differences in host plant preference and ecological niche are reported from areas where both species coexist. Most striking in the numerous publications which describe the occurrence of *M. fructicola* and *M. laxa* in the field, is that *M. fructicola* is mostly found on fruits (Ogawa *et al.*, 1954; Boesewinkel & Corbin, 1970; Ogawa *et al.*, 1975). In a survey in the major stone fruit growing areas of California (USA), Ogawa *et al.* (1954) found that 72 % of the isolations of *M. fructicola* came from diseased fruits. *M. laxa* appeared to be more a blossom and twig pathogen, 83 % of the isolations of *M. laxa* came from blighted blossoms and twigs. This confirmed earlier findings by Huber & Baur (1941). Similarly, recent records from Australia state that *M. laxa* causes blossom blight in peach and apricot, but rarely causes fruit rot (Penrose, 1998).

Among the many host plant species present in the PRA area, we expect that peach/nectarine and apricot will be most affected by introduction of *M. fructicola*. Ogawa *et al.* (1954) found 92 % of the collected samples in peach to be *M. fructicola*, in regions where *M. laxa* was dominantly present but caused no harm in peach orchards. Zehr (1982) also stated that *M. laxa* is less important on peaches than on other stone fruits in the USA. However, in Europe *M. laxa* is an important pathogen in peach, but damage is only serious in the blossom- and twig blight phase of the disease (M.-Sagasta, 1977; Melgarejo *et al.*, 1986). An increase in pre- and post-harvest fruit losses in peach/nectarine and apricot is likely to occur once *M. fructicola* establishes itself in Europe. Moderate fruit losses in plum caused by *M. fructicola* have been reported from Ontario, Canada (Northover & Cerkauskas, 1994), but workers in Australia stated that the pathogen is of lesser importance in plum (Kable, 1969).

It is not very likely that *M. fructicola* will enhance damage in the blossom- and twig blight phase of the disease. Though *M. fructicola* causes wilting of blossoms, Kable & Parker (1963) found that subsequent invasion of twigs in sour cherry was limited compared with *M. laxa*. In peach, the severity of twig blight caused by *M. fructicola* seems to vary per region. Willison (1937) and Biggs & Northover (1985) reported the occurrence of perennial cankers in peach in Canada, whereas Kable (1965b) did not find these in Australia. It is likely that the extent of

damage to twigs and branches depends on environmental conditions. This is supported by data from Europe related to *M. laxa* (M.-Sagasta, 1977; Madrigal *et al.*, 1994).

Survival and establishment

Survival mechanisms play an important role in determining whether following initial entry, a pathogen becomes established. *M. fructicola* survives as mycelium in mummified fruits, on which new conidial pustules appear in springtime. Kable (1965b) mentioned the fruit peduncle as an important overwintering site in peach. *M. fructicola* can also survive in twigs and branches, and subsequently produce conidia on these plant surfaces in springtime (Jehle, 1913; Mix, 1930; Biggs & Northover, 1985). In addition, apothecia of *M. fructicola* are regularly found in the field, though not in all regions. Apothecia are found in South Carolina and California, USA (Landgraf & Zehr, 1982; Holtz *et al.*, 1998), also in South America (Uruquay; P. Mondino, pers. comm.), but infrequently in Australia (Jenkins, 1965a; Kable, 1969). By contrast, for the two *Monilinia* species occurring in Europe, the sexual cycle plays no role in their life cycle. Sexual reproduction generates more genetic variation in populations, and this may have quickened the development of fungicide resistance in *M. fructicola*.

Current disease management practice in stone fruits to control *Monilinia* consists of 2-3 fungicide sprayings around blossoming, followed by 1-2 sprayings when fruits start to ripen (Rueegg *et al.*, 1997; Zehr *et al.*, 1999). In the past, regular use of benomyl and dicarboximides in spray programs has led to the development of fungicide resistance in *M. fructicola* in the USA, Australia and Korea (Jones & Ehret, 1976; Penrose *et al.*, 1979; Osorio *et al.*, 1994; Lim *et al.*, 1998). The establishment of fungicide-resistant *M. fructicola* strains in Europe, might aggravate problems in disease control due to lowered efficiency of spray programs. Also in *M. laxa* fungicide resistance has been found in the USA (Ogawa *et al.*, 1984; Osorio *et al.*, 1994), as well as in Europe. Guizzardi *et al.* (1995) studied the sensitivity of *M. laxa* isolates from Italian stone fruit orchards to benomyl and dicarboximides, and found cultures on agar growing even at a hundredfold of normal fungicide concentrations. Development of resistance in *M. fructicola* to new demethylation-inhibiting (DMI) fungicides has been shown by Zehr *et al.* (1999). It is possible that *M. fructicola* develops resistance more quickly than *M. laxa,* which would make resistance management more difficult.

An important aspect of the probability of successful establishment, is the potential of an initially (very) small *M. fructicola* population to establish itself in orchards where *M. laxa* is endemic. A clear ecological disadvantage for *M. fructicola* compared with *M. laxa* is that sporulation only starts in springtime when temperature reaches 15-25 °C, whereas *M. laxa* sporulates at 5-10 °C (Byrde & Willetts, 1977). Thus, the proportion of *M. fructicola* conidia in the environment early in the season would be very low, thus minimising the probability of successful infection of host tissue. Nevertheless, when *M. fructicola* conidia infect fruits later in the season, rapid lesion development and profuse sporulation will enhance dispersal and, ultimately, establishment of the pathogen.

During the last century *M. fructicola* has become established in new regions where it was formerly not known to occur. Among these regions are Japan, Zimbabwe, South Africa and Yemen (Terui & Harada, 1966; CABI/EPPO, 1998). No information is available about eradication measures taken after the pathogen was first detected in these countries. That it is possible to eradicate an exotic *Monilinia* species from a restricted area, has been shown by Batra (1979) in the USA. *M. fructigena* was found on 'Kieffer' pears in an experimental orchard in Beltsville, Maryland in 1974-1975. This orchard was subsequently destroyed, and since then no reports of occurrence of *M. fructigena* in the USA have been made.

2. *Economic impact assessment*

It is difficult to find exact yield loss data for *M. fructicola* in the literature. Losses in stone fruits vary per year and strongly depend on weather conditions around harvest time. In plum, Northover & Cerkauskas (1994) estimated that total pre-harvest fruit loss was 15-30 %, despite a rigid spray programme. In nectarine orchards, Hong *et al.* (1997) recorded 8-10 % fruit loss at harvest time due to *M. fructicola* in 1995, whereas this was only 0.5 % in 1996. Similar variation in yield loss is reported by other workers (Morschel, 1956; Kable, 1969). It is instructive to compare present yield losses in European orchards caused by *M. laxa* and *M. fructigena* with those reported for *M. fructicola*. Usually, *M. fructigena* causes as much fruit rot as *M. laxa* in European stone fruit orchards. In Switzerland, Rüegg & Siegfried (1993) assessed fruit losses caused by *Monilinia* in three sweet cherry orchards treated with regular fungicide sprayings, and found a low yield loss in two orchards (3-5 %), and a moderate loss at the third site (15 %). No other quantitative assessments of yield loss could be found for Europe. According to Byrde & Willetts (1977), *M. fructigena* is less damaging than *M. fructicola* in stone fruits. Thus, it is likely that the introduction of *M. fructicola* will result in higher fruit losses in stone fruit orchards in Europe.

The endangered area within the EU is concentrated in the South (Table 1), and it is not only direct losses which should be considered. It is likely that phytosanitary measures will need to be taken to avoid further spread of the pathogen from a region where it is initially detected (see below). Export markets might get seriously affected. For a country like Spain or Italy, where intra-EU export trade in peach and nectarines amounted up to 239 and 291 million ECU respectively in 1998 (Anonymous, 1999b), detection and spread would be disastrous.

It is hard to predict the extent of subsequent spread of *M. fructicola* once introduced in a certain location. Certainly, when export of fruits and nursery stock is not restricted from a contaminated area or strong regulations are not applied, the pathogen may quickly spread to other fruit growing regions within the EU. Though the pathogen can be dispersed over large distances by natural means (wind, insects, birds), it is likely that such spread will occur only slowly compared with spread by trade pathways. Thus, containment measures are desirable after detection of *M. fructicola* in a restricted part of the PRA area. An indication of how quickly *M. fructicola* spreads, can be deduced from surveys worked out in California (USA). Hewitt & Leach (1939) reported that *M. laxa* was widespread in all stone fruit growing areas, whereas *M. fructicola* was more localised in the peach producing areas. A survey in prune and apricot growing areas during 1982 and 1983, showed that both *M. fructicola* and *M. laxa* were widespread (Michailides *et al.*, 1987). Thus, *M. fructicola* had partly displaced *M. laxa* in those crops, probably encouraged by disease management practice: more than 50 % of the prune and apricot orchards harboured *M. fructicola* isolates resistant to benomyl, whereas all *M. laxa* isolates found were sensitive to benomyl. In Europe, a similar situation might develop over time.

3. *Final evaluation*

The pest risk assessment given above is based on reliable sources from the literature and statistical databases. Nevertheless, detailed information was not always available for our purposes. We clearly indicate the high risk of import of nursery stock from rosaceous crops, but exact import data for these could not be found. We find it surprising that so few interceptions have been reported in recent years. It might be worthwhile for EU member states to re-evaluate intensity of sampling and the quality of the inspection regime. Well-defined instructions with respect to recognition of disease symptoms should be given to inspectors working at dock sites and harbours.

In summary, the probability of introduction of *M. fructicola* into EU countries is high. Firstly, there is a massive import of stone fruits from areas where the pathogen exists, and *M. fructicola*-infected fruits might slip through inspection at entry points. However, chances for establishment are low as the products are meant for direct consumption, and susceptible host tissue is not available in the main period of imports. The last point does not hold however, for produce imported by South European countries early in the year (February/March). Secondly, import of nursery stock forms a serious threat. Once entry has been achieved, the pathogen will encounter ideal conditions at nursery sites for spread and establishment. The economic impact of establishment of *M. fructicola* will be considerable. Not only will direct fruit losses increase in especially peach/nectarine and apricot, but also export markets will be affected. Cost of control will increase, and control measures might become less efficient because of the development of fungicide resistance.

Phytosanitary measures have already been taken to prevent introduction of *M. fructicola* (Anonymous, 1993); our conclusions support these measures which will limit the risk that *M. fructicola* becomes established in Europe. Nevertheless, if in future the pathogen is introduced in European orchards, we believe that eradication or containment measures provide the best means to minimise the economic impact for the EU as a whole.

Chapter 9

General discussion

This thesis gives an overview of the current state of taxonomy in the brown rot fungi of fruit crops (*Monilinia* spp.). As well as the requirement for correct diagnosis and identification, epidemiological knowledge is indispensable for rational disease manage-ment. This thesis also highlights the epidemiology of *M. fructigena* (Aderh. & Ruhl.) Honey, the most important of the brown rot fungi on pome fruit. The results of our study are discussed, and placed in a broader perspective. Recommendations for future basic and applied research are given.

Taxonomy, identification, detection of the brown rot fungi of fruit crops

Taxonomic expertise is essential for the support of biological disciplines, such as ecological research, nature conservation and plant pathology (Berry, 1991). The communication of scientific results depends on correct identification of species involved, and thus, on taxonomy. The essence of international conformity in nomenclature has long been neglected (Hawksworth, 1991). This has caused much confusion in the group of brown rot fungi (see Chapter 1). Identification of the three brown rot fungi has always relied heavily on cultural characteristics, and this method works well for skilled, experienced diagnostic workers, but does not comply with the needs of less specialised workers. A protocol was developed to distinguish the three brown rot fungi on the basis of clearly defined quantitative characteristics (Chapter 2). A combination of growth rate, sporulation intensity and germ tube length data resulted in correct identification of approximately 95 % of the isolates tested. The protocol can be used in all laboratories which have standard mycological equipment. A major drawback of identification methods based on culturing of specimens, is the delay before a definite conclusion can be drawn. Currently, determination of growth rate takes 5 days, and sporulation intensity 14 days according to the protocol developed. It is worthwhile to study if determination of sporulation intensity in an earlier stage affects the performance of the protocol.

Quick identification and detection is essential in *M. fructicola* which is listed as a quarantine organism for Europe (CABI/EPPO, 1997). Suspect freights of imported (perishable) fruits demand a quick diagnosis in order to determine whether *M. fructicola* is present (Chapter 8). Recently, species-specific monoclonal antibodies and molecular tools for quick identification have been developed in an EU-funded project (Corazza *et al.*, 1999). Molecular data on the three brown rot species have considerably accumulated with important consequences for identification, detection and classification (Chapter 3 and 4). Fulton & Brown (1997) developed a set of *M. fructicola*-specific primers based on the occurrence of a 418-bp group-I intron in the small subunit (SSU) rDNA, which was absent from *M. laxa* and *M. fructigena*. However, further studies in *M. fructicola* strains worldwide occurring revealed that Japanese strains partly lacked the intron (Chapter 3), and Förster & Adaskaveg (2000) showed that only seven out of 21 *M. fructicola* isolates collected in California (USA) produced a band of the expected size while using the primer set mentioned. Other primer sets have been developed based on subtle differences in the ITS 1 and ITS 2 region of rDNA (Förster & Adaskaveg, 2000; Hughes *et al.*, 2000; Ioos & Frey, 2000). *M. fructicola*-specific primers designed from the 3'end of the ITS 1 region were able to detect the pathogen in fruit

tissue with quiescent infections (Förster & Adaskaveg, 2000). This offers possibilities to test the quality of batches before being put on long-distance transport.

Application of molecular tools will hopefully result in a quicker handling of shipments suspected of being infected by *M. fructicola* (Chapter 8). The result of diagnosis can be given within one or two days. To what extent the primers directed to differences in ITS sequence detect *M. fructicola* isolates universally, is a matter of concern. However, our study did not reveal any intraspecific variation in the ITS region of rDNA in *M. fructicola* (Chapter 3), concurring with findings of Holst-Jensen *et al.* (1997a). Snyder & Jones (1999) reported intraspecific variation in the ITS 1 sequence of a group of *M. fructicola* isolates from Michigan (USA), but variation did not occur in the critical region from which primers, developed by Förster & Adaskaveg (2000), were designed. Thus, in contrast to variation in the occurrence of a group-I intron in SSU rDNA in *M. fructicola*, variation in the ITS region is limited so that primers designed from this part of the genome are the most reliable.

M. fructigena-specific primers are of interest for countries like Australia and the USA where this pathogen is considered a quarantine pest (Anonymous, 1984; Chang, 1986). In a preliminary report Hughes *et al.* (2000) and Ioos & Frey (2000) mentioned the successful development of such primers, but gave no sequence data.

The species concept in the brown rot fungi

For long the group of brown rot fungi consisted of three species, *M. fructicola*, *M. laxa* and *M. fructigena*. We found distinct genetic variation in the ITS region of rDNA within *M. fructigena*, strains from Europe differed from those originating from Japan (Chapter 3). It was shown that Japanese strains produced significantly more stroma and had smaller conidia than European strains. On the basis of genetic and morphological differences we proposed a new *Monilia* anamorph, *Monilia polystroma*, in which the Japanese strains were included (Chapter 4). A survey in East Asia to determine the distribution of *M. fructigena* and *M. polystroma* genotypes would be very interesting. Possibly, the occurrence of *M. polystroma* is not restricted to Japan, and moreover, strains with the *M. fructigena* genotype could also be present in Japan. It is also worthwhile to study the genetic constitution and morphological characteristics of the *M. fructigena* population known to occur in South America (Byrde & Willetts, 1977). Wormald (1954) did not report the occurrence of *M. fructigena* in South America, so it might have been recently introduced. From a plant health point of view questions arise regarding the phytosanitary status of *M. polystroma* (cf. *M. fructicola*, Chapter 8). The intense formation of stroma which was observed *in vitro* as well as *in vivo*, might enhance survival of the pathogen. More research needs to be done regarding pathogenicity and survival ability before conclusions regarding the phytosanitary status can be drawn.

Systematics within the kingdom Fungi has been based strongly on the morphological species concept. In this, the degree of morphological difference is the criterion of species status (Mayr, 1957). The enormous increase in molecular data becoming available in the last two decades, has stimulated a more molecular approach to systematics (Hillis, 1987). Often genetic analysis has confirmed a formerly determined (morphological) classification, or it helped solve a questionable morphological distinction (Baayen *et al.*, 1997; Man in 't Veld *et al.*, 1998). Another concept employed in biology is the biological species concept, which is based on the claim that species are reproductive communities (Mayr, 1942). For the brown rot fungi it would be very interesting to study to what extent geographical isolation impedes exchange of genetic material between populations. Though sexual reproduction hardly occurs in the brown rot fungi, except for *M. fructicola*, vegetative heterokaryon formation occurs frequently (Hoffmann, 1972, 1974). A study of the ability of different strains to form a heterokaryon, probably followed by parasexual exchange of genetic material (Leslie, 1993),

would give more insight in the relatedness of different populations. It would be most interesting to study heterokaryon formation between *M. fructigena* strains from different geographical regions, including strains from the closely related *M. polystroma* occurring in Japan (Chapter 4).

Epidemiology and disease management of *M. fructigena*

Epidemiology as a science leads to disease management as a technology (Zadoks & Schein, 1979). Although much data on the epidemiology of the brown rot fungi have been gathered during the last century (Wormald, 1954; Byrde & Willetts, 1977), disease management strategies nowadays may still not prevent the occurrence of severe epidemics in some years. Especially in stone fruits severe epidemics occur under adverse weather conditions during flowering or fruit ripening (Weaver, 1950; Zehr, 1982; Wilcox, 1989). In stone fruit culture fungicides are regularly applied against brown rot, primarily caused by *M. fructicola* and *M. laxa*, during blossoming and the fruit ripening period (Hogmire & Biggs, 1994; Penrose, 1998). In pome fruits only fruit infection is important, and no fungicidal sprays are applied specifically against brown rot in Europe. The flowering period constitutes a relatively short period in which susceptible tissue is available, in contrast with fruits which are susceptible to infection from 5-6 weeks after blossoming up to harvest time. Reducing the amount of primary inoculum in the direct environment to minimise flower infection, has been emphasised in research during the 1940s and 1950s. Several eradicant fungicides were tested for their ability to destroy conidial tufts of *M. laxa* appearing on twigs in late winter, in order to prevent blossom infection later (Wilson & Baker, 1946; Wilson, 1950). As flowers are susceptible for only 1-2 weeks at ordinary temperatures (Calavan & Keitt, 1948), secondary inoculum hardly plays a role in flower infection (Ogawa *et al.*, 1967). In fruit infection however, primary as well as secondary inoculum plays a role, and this requires a different disease management strategy. A summary of knowledge about the epidemiology of *M. fructigena* in pome fruits is given below, current control measures are discussed, and ideas for future research are presented.

Fruit injury

In pome fruits avoidance of fruit injury is very effective in the control of brown rot caused by *M. fructigena*. Xu & Robinson (2000) showed that no brown rot developed after inoculation of non-wounded apple fruits with a concentration of 7.5×10^5 conidia/ml. However, infection via fruit-to-fruit contact does not require wounds, and is partly responsible for distinct clustering of diseased fruits in the tree canopy (Chapter 5). Fruit injury is caused by abiotic (frost, hail, wind) as well as biotic factors. In the latter, birds (Tobin, 1989; Van 't Westeinde, 1999) and insects (Moore, 1950; Croxall *et al.*, 1951) are regarded the most important wounding agents. When these wounding agents at the same time act as vectors, as reported for dried fruit beetles in peach by Kable (1969), immediate infection of freshly wounded fruits occurs. As infection by aerial and water dispersed inoculum depends on the availability of (fresh) wounds and the probability of reaching them, the vectorborne mechanism is far more effective and probably the most important. The seasonal concentrations of *M. fructigena* conidia in the air in a pome fruit orchard was monitored for the first time (Chapter 6), and appeared to be relatively low compared with studies done in stone fruit orchards where *M. fructicola* and *M. laxa* occurred (Kable, 1965a; Corbin *et al.*, 1968). The highest spore concentrations were registered at low relative humidities in the afternoon. In order to evaluate the significance of aerial dispersed inoculum in the development of an epidemic, more knowledge about the longevity of *M. fructigena* conidia under field conditions is essential. As

long as conidia deposited on non-wounded fruits remain viable, subsequent wounding may still lead to successful infection.

It has been easier to demonstrate the essential role of fruit injury in the epidemiology of *M. fructigena* in pome fruits, than to find adequate means to prevent injury. In the field we have no control over the abiotic factors leading to fruit injury. Hellmann (1998) advised immediate fungicide sprayings to diminish a strong increase in brown rot after heavy hail showers, but it is doubtful whether this is economically feasible, as badly damaged fruits are of no marketable value. Bird damage is especially severe in orchards near wooded areas and brushlands, and with control methods such as shooting, gas canons and kites, growers try to combat the problem (Hasey & Salmon, 1993; Van 't Westeinde, 1999). Conflicting situations sometimes occur. For example, the common earwig (*Forficula auricularia*) is an important natural enemy of the woolly apple aphid *Eriosoma lanigerum* in IPM systems (Helsen *et al.*, 1998), but earwigs have also been reported to cause small, shallow wounds in apple fruits which resulted in high brown rot incidence (Croxall *et al.*, 1951).

Reduction of inoculum in the environment

In addition to the prevention of fruit injury, reduction of the amount of inoculum in the environment is another important aspect in disease management of *M. fructigena*. Primary inoculum in springtime originates mainly from overwintered fruits infected the previous season, though sporulation on spurs and twigs also occurs (Byrde & Willetts, 1977). Infected fruits which remain in the tree canopy, contribute to the pool of primary inoculum in the following year (Chapter 7). It has always been recommended to remove and destroy firm, mummified fruits during pruning in the dormant season (Kennel, 1968; Byrde & Willetts, 1977), but our study showed that also fruits not mummified but previously infected by *M. fructigena* should be removed and subsequently destroyed. Fruits which drop to the ground soon after infection usually decompose quickly and thus are unlikely to contribute to primary inoculum in the next year (Willetts, 1971; Van Leeuwen, unpublished). One of the best control measures is to take fruits out of the tree as soon as the first brown rot symptoms appear, and to leave these on the grass sod between the rows, where regular mowing practice will destroy most of the fruits. This (i) prevents further spread of the disease within a fruit cluster, (ii) prevents the fungus from growing into spurs/twigs, (iii) minimises the amount of secondary inoculum produced, and (iv) stimulates quick decomposition of the fruit (reduces primary inoculum for next year). However, in practice few pome fruit growers spend time and money on this method (Van 't Westeinde, 1999), and it is rarely feasible on nurseries exceeding 15-20 ha.

Given that it is difficult to remove all infected fruit structures, an alternative approach is to minimise the amount of inoculum produced per infected fruit. After infection, the pathogen rapidly colonises the fruit and produces numerous conidia at the surface. After this first crop of conidia, the fungus can produce subsequent conidial crops from the same sporulating area (Corbin & Cruickshank, 1963; Chapter 7). If this process of regeneration of conidia was blocked, no more conidia would be produced during the rest of the season, neither after overwintering. The occurrence of many different micro-organisms on fruits previously infected by brown rot fungi, has been associated with a reduction in subsequent regeneration of conidia (Byrde & Willetts, 1977; Hong *et al.*, 2000). Hong *et al.* (2000) made an inventary of the mycoflora occurring on stone fruit mummies, and found *Penicillium*, *Cladosporium* and *Mucor* as predominant species. Typical antagonistic fungi such as *Aureobasidium pullulans*, *Epicoccum purpurascens*, and *Trichoderma* spp. were found less frequently. Under field conditions, Jenkins (1968) found germ tubes of conidia of *M. fructicola* intensively covered by bacteria, and lysis was observed. The bacteria were tentatively identified as belonging to *Bacillus cereus*. *B. subtilis*, which produces an antibiotic, has been used in post-harvest

control of stone fruit brown rot (Pusey & Wilson, 1984). Perhaps it is possible to enhance the occurrence of antagonistic fungi and bacteria on fruits previously infected by brown rot. The optimal moment for application might be just after the first *Monilinia* pustules have ruptured the fruit skin. A major supply of nutrients becomes available then, which hopefully favours a quick establishment of the antagonists.

Conclusion

Successful control of brown rot in pome fruit orchards depends on the extent to which fruit injury can be avoided. In comparison with stone fruits, the skin of pome fruits is firmer and cracks less easily, so that adverse weather conditions (rain) during fruit ripening are not critical in disease development (Chapter 5). Reduction of the amount of primary inoculum in pome fruit orchards should slow down the initial rate of disease increase as shown by Byrde (1954b). However, it is essential that measures are being taken during the growing season to curtail the production of secondary inoculum on newly infected fruits, otherwise efforts to reduce the amount of primary inoculum may be ineffective.

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Summary

The brown rot fungi of fruit crops (*Monilinia* spp.) cause blossom blight, twig blight and fruit rot in rosaceous fruit crops in the temperate regions of the world. The genus *Monilinia* belongs to the Discomycetes (Ascomycota) and is closely related to the genera *Sclerotinia*, *Botryotinia* and *Ciboria*. The group of brown rot fungi comprises three species: *M. fructicola* (Wint.) Honey, *M. laxa* (Aderh. & Ruhl.) Honey and *M. fructigena* (Aderh. & Ruhl.) Honey. The latter predominantly occurs in pome fruits (apple, pear) where it causes fruit rot, *M. fructicola* and *M. laxa* are mostly confined to stone fruits (peach, apricot, plum). *M. fructicola* is listed as a quarantine organism for Europe, and reliable identification methods are indispensable to distinguish this species from the endemic *M. laxa* and *M. fructigena*.

The three brown rot species are usually distinguished on the basis of qualitative colony characteristics, such as shape of the colony margin and colour of sporogenous tissue. An identification protocol was developed to delineate the three species on the basis of quantitative colony and germ tube characteristics (Chapter 2). Increase in colony diameter and sporulation intensity on potato dextrose agar (PDA), as well as three germ tube characteristics were determined for a set of isolates originating from different geographical regions (Europe, Australia, USA, Japan). The increase in colony diameter was distinctly higher for *M. fructicola* compared with the other two species, as was sporulation intensity, though some *M. laxa* isolates sporulated as profusely as *M. fructicola*. The length of the germ tube of germinating conidia was highest in *M. fructicola* and *M. fructigena* compared with *M. laxa*, and the occurrence of more than one germ tube per conidium was most common in *M. fructigena*. Different combinations of measured variables were tested to find out which combination gave the best species delineation. Discriminant analysis based on a combination of 'increase in colony diameter' and 'length of germ tube' resulted in two misclassifications out of 29 isolates tested. Addition of sporulation intensity to this set resulted in only one isolate being misclassified. The method described is a simple method requiring only standard laboratory equipment, and is an important method to help less experienced diagnostic personnel in correct identification of brown rot specimens.

Genetic analysis of the ITS 1-5.8S-ITS 2 region of ribosomal DNA (rDNA) in a broad range of isolates largely confirmed species delineation on the basis of morphology (Chapter 3). However, four distinct nucleotide sequences were found, of which that of Japanese *M. fructigena* isolates was not previously reported. Japanese *M. fructigena* isolates differed from the sequence found for European *M. fructigena* isolates by four transitions within the ITS 1 region and one transition in the ITS 2 region. Phylogenetic analysis of the ITS nucleotide sequences revealed that the Japanese *M. fructigena* isolates were more closely related to the European *M. fructigena* isolates than to the other two species. Analysis of banding patterns obtained by RAPD-PCR using six random 10-base primers showed no clear geographic groupings in *M. laxa* nor *M. fructigena*. However, in *M. fructicola* isolates from Japan clustered together, and these isolates were closely related to some of the New Zealand isolates. A group-I intron formerly detected in the small subunit (SSU) rDNA in non-Japanese *M. fructicola* isolates, appeared to be present in only one out of five Japanese strains.

The genetic variation found within *M. fructigena* provided a stimulus to compare morphological, cultural and biological characteristics between a group of six Japanese *M. fructigena* isolates and a group of European ones of equal size (Chapter 4). Growth rate on PDA, stroma formation on cherry decoction agar (CHA) and conidial dimensions *in vitro* as well as *in vivo* were determined. A marked difference in stroma formation was observed, the area of stromatal plates ranged from 4.11 to 5.19 cm^2 in the Japanese group, and from 0 to

0.85 cm^2 in the European. Conidia produced by Japanese strains were significantly smaller than those of European strains (*t*-test, $P = 0.01$). Mean growth rate, averaged over isolates, was significantly higher for the Japanese group (*t*-test, $P = 0.01$), but individual European strains showed similar growth rates as Japanese strains. There were no differences in latency period, lesion growth rate or sporulation intensity on apple and pear fruits between both groups. In our opinion, distinct genetic and morphological differences found between both groups merit the erection of a new *Monilia* anamorph, *Monilia polystroma* Van Leeuwen, in which the former Japanese *M. fructigena* strains are included.

Spatio-temporal aspects of disease development of *M. fructigena* in apple were studied during a two-year field experiment (Chapter 5). The early maturing apple cultivar James Grieve and cv. Cox's Orange Pippin were grown in an alternating one-row system. Disease incidence increased at a fairly constant rate in cv. James Grieve, final pre-harvest fruit loss was 4.2-4.3 % in both years; for cv. Cox's Orange Pippin this was 4.4 % in 1997 and 2.7 % in 1998. Initially, the rate of disease increase in cv. Cox's Orange Pippin was slow, but increased markedly after cv. James Grieve had been harvested. Post-harvest yield losses amounted on average 1.5-2.0 % for both cultivars, and no significant differences were found between the cultivars (*t*-test, $P = 0.05$). Spatially, the degree of clustering of diseased fruits among fruit trees, and that of trees with diseased fruits was determined using Lloyd's index of patchiness (LIP) and spatial autocorrelation respectively. Significant clustering of diseased fruits among trees occurred in both cultivars ($P = 0.05$), and was more pronounced in the second year ($P = 0.01$). Within-row clustering of trees with diseased fruits showed less clear results. In 1998 significant ($P = 0.05$) positive correlation coefficients occurred for 2nd, 3rd and 4th lag-order distances in cv. James Grieve, but typically no significant positive correlation was found for the adjacent quadrat (tree). In cv. Cox's Orange Pippin a significant ($P = 0.05$) positive first-order correlation was found in the second year.

During two consecutive seasons a Burkard spore trap was operated to monitor the aerial concentration of *M. fructigena* conidia in an apple orchard. The number of conidia trapped was related to environmental conditions (Chapter 6). The highest hourly concentration measured in 1997 was 233 conidia/m^3, in 1998 concentrations were lower than in 1997. A generalised linear model (GLM) was constructed in which the number of conidia counted was related to ambient relative humidity, temperature, rainfall, wind speed and wind direction in the field using a logarithmic link function. Univariately, relative humidity and temperature explained best the number of conidia caught in both years. Forward selection was used to construct a multivariate model. In 1997, the model which best described variation in spore catches contained all weather variables except rainfall, in 1998 only relative humidity and temperature were included.

Fruits infected by *M. fructigena* gradually dry out and may become mummified. A fresh crop of conidia is produced on mummified structures in the following season (primary inoculum). Mummification and sporulation of pome fruits infected by *M. fructigena* was studied in a field experiment and also under controlled environment conditions (Chapter 7). Fruits of cv. Golden Delicious infected in September and October did not mummify but sporulated profusely after overwintering. We conclude that early- as well as late-infected fruits contribute to primary inoculum in the next season; and that for reduction of primary inoculum, infected but non-mummified fruits should also be removed during winter. In a study of the effect of different post-infection regimes on mummification and sporulation after a cold period, it was shown that the percentage of fruits sporulating sharply dropped at 20 °C and RH 75-85 % and at 25 °C and RH 65-75 %. After 12 weeks incubation at 25 °C none of the fruits sporulated. After 8 weeks incubation, sporulation intensity per sporulating fruit was

significantly higher at a regime of 10 °C and RH 85-95 % compared with that in the post-infection regimes of 20 and 25 °C (Kruskal-Wallis test, $P = 0.05$).

A pest risk analysis on *M. fructicola* was made for the countries of the European Union (Chapter 8). The most important pathways for entry of the pathogen are (stone) fruit imports and imports of nursery stock of rosaceous plants. Though fruit import represents by far the greatest bulk of material, the probability of establishment of the pathogen when present on nursery stock is much greater. Introduction of *M. fructicola* in Europe would affect especially the stone fruit culture of southern Europe, and would probably increase pre- and post-harvest fruit losses. The occurrence of fungicide resistance in *M. fructicola* might aggrevate problems. Recently developed molecular tools to quickly detect *M. fructicola* in imported produce would be an important tool in attempts to keep this quarantine organism out of Europe.

Finally, in Chapter 9 the results of our study and the implications for future research are discussed. The phytosanitary status of the newly described anamorph *M. polystroma* and the distribution of this new species warrants more research. Epidemiologically, the study of mummification and sporulation of fruits infected by *M. fructigena* should lead to further research to unravel the process of repeated sporulation on previously infected fruits. If this process can successfully prevented, the amount of primary inoculum in spring will substantially be reduced.

Samenvatting

Wereldwijd komen er drie *Monilinia* soorten voor die tak- en bloesemsterfte en vruchtrot veroorzaken in fruitgewassen behorende tot de familie van Rosaceae. De drie soorten zijn *M. fructicola* (Wint.) Honey, *M. laxa* (Aderh. & Ruhl.) Honey en *M. fructigena* (Aderh. & Ruhl.) Honey. Het geslacht *Monilinia* behoort tot de klasse der Discomycetes, afdeling Ascomycota, en is nauw verwant aan de geslachten *Sclerotinia*, *Botryotinia* en *Ciboria*. *M. fructigena* komt voornamelijk voor in pitvruchten (appel, peer) waar deze vruchtrot veroorzaakt, *M. fructicola* en *M. laxa* komen vooral voor in de teelt van steenvruchten (perzik, abrikoos, pruim). *M. fructicola* staat op de lijst van quarantaine organismen voor Europa, en adequate diagnostische methoden zijn essentieel om dit quarantaine organisme te onderscheiden van de endemische soorten *M. laxa* en *M. fructigena*.

De drie *Monilinia* soorten worden meestal van elkaar onderscheiden op basis van kwalitatieve koloniekenmerken, zoals vorm van de rand van de kolonie en de kleur van sporulerend weefsel. Een identificatieprotocol werd ontwikkeld om de soorten te onderscheiden op basis van kwantitatieve kolonie- en kiembuiskenmerken (Hoofdstuk 2). De toename in koloniediameter en intensiteit van sporulatie op een medium van aardappel dextrose agar (PDA), en drie kenmerken van de ontwikkeling van de kiembuis werden gemeten voor een collectie isolaten afkomstig uit verschillende regio's (Europa, Australië, VS, Japan). De toename in koloniediameter was het grootst in *M. fructicola* vergeleken met de andere twee soorten, evenals het sporulerend vermogen ('sporulation intensity'), hoewel sommige *M. laxa* isolaten even intens sporuleerden als *M. fructicola*. *M. fructicola* en *M. fructigena* hadden de grootste kiembuislengte vergeleken met *M. laxa*, en uitgroei van meerdere kiembuizen per conidium kwam het meest voor bij *M. fructigena*. Verschillende combinaties van kenmerken werden getest om te bepalen welke combinatie resulteerde in de beste soortsscheiding. Discriminantanalyse op basis van de combinatie van 'toename in koloniediameter' en 'kiembuislengte' resulteerde in twee verkeerde classificaties op een groep van 29 geteste isolaten. Na toevoeging van het kenmerk 'sporulerend vermogen' aan deze set werd er slechts één isolaat verkeerd geclassificeerd. De beschreven methode is een eenvoudige methode die alleen standaard laboratoriumbenodigdheden vereist. Het is een belangrijke methode voor minder ervaren diagnostische medewerkers om te komen tot een correcte identificatie van *Monilinia* soorten.

Analyse van het ITS 1-5.8S-ITS 2 gebied van ribosomaal DNA (rDNA) van een grote groep isolaten, bevestigde grotendeels de soortsindeling op basis van morfologie (Hoofdstuk 3). Echter, er kwamen vier verschillende nucleotidesequenties voor, waarvan die van Japanse *M. fructigena* isolaten niet eerder gevonden was. Japanse *M. fructigena* isolaten verschilden van Europese *M. fructigena* isolaten op vier (base)plaatsen in het ITS 1 gebied, en op één in het ITS 2 gebied. Een fylogenetische analyse van de ITS-nucleotidesequenties maakte duidelijk dat de Japanse *M. fructigena* isolaten nauwer verwant waren aan de Europese *M. fructigena* isolaten dan aan de andere twee soorten. De bandenpatronen verkregen door toepassing van RAPD-PCR met zes random 10-base primers, lieten geen duidelijke groepering zien naar regio noch in *M. laxa* noch in *M. fructigena*. Echter, Japanse *M. fructicola* isolaten kwamen samen in één cluster voor, en deze waren nauw gerelateerd aan enkele van de *M. fructicola* isolaten uit Nieuw Zeeland. Een 'group-I'-intron dat eerder ontdekt was in de kleine subunit van rDNA (SSU-rDNA) in *M. fructicola* isolaten van buiten Japan, bleek slechts aanwezig te zijn in één van de vijf Japanse isolaten.

De gevonden genetische variatie binnen *M. fructigena* was een stimulans tot verder onderzoek naar eventuele morfologische en/of biologische verschillen tussen Japanse en

Europese *M. fructigena* isolaten. Een groep van zes Japanse *M. fructigena* isolaten werd vergeleken met een groep van zes Europese (Hoofdstuk 4). De groeisnelheid op PDA, stromavorming op kersenagar, en de grootte van conidia *in vitro* en *in vivo* werden bepaald. Een opmerkelijk verschil in stromavorming werd waargenomen, de oppervlakte aan stroma in de Japanse isolaten varieerde van 4.11 tot 5.19 cm^2 per Petri schaal, in de Europese isolaten werd slechts 0 tot 0.85 cm^2 stroma gevormd. De conidia van Japanse isolaten waren significant kleiner dan die van Europese isolaten (*t*-test, $P = 0.01$). De gemiddelde groeisnelheid, genomen over alle isolaten in een groep, was significant hoger in de groep van Japanse isolaten (*t*-test, $P = 0.01$), maar individuele Europese isolaten lieten een vergelijkbare groeisnelheid zien als Japanse isolaten. Er werden geen verschillen gevonden tussen beide groepen in latentieperiode, groeisnelheid van lesies, en sporulerend vermogen op vruchten van appel en peer. De duidelijke genetische en morfologische verschillen tussen beide groepen leidde tot de beschrijving van een nieuw *Monilia* anamorf, *Monilia polystroma* Van Leeuwen, waarbinnen de voormalige Japanse *M. fructigena* isolaten vallen.

In twee opeenvolgende jaren werd de ontwikkeling van *M. fructigena* vruchtrot in een appelboomgaard bestudeerd in tijd en ruimte (Hoofdstuk 5). Het proefveld bestond uit een enkele-rij systeem waarin het vroegrijpende ras James Grieve en de later rijpende Cox's Orange Pippin werden afwisselend. De ziekteincidentie in James Grieve in het veld nam toe met een vrijwel constante snelheid, uiteindelijk ging in beide jaren 4.2 tot 4.3 % van de appels verloren door vruchtrot veroorzaakt door *M. fructigena*; voor Cox's Orange Pippin was dit 4.4 % in 1997, en 2.7 % in 1998. Aanvankelijk was de snelheid waarmee ziekteincidentie toenam laag in Cox's Orange Pippin, maar deze nam opmerkelijk toe na de oogst van James Grieve. Naoogst verliezen bedroegen gemiddeld 1.5-2.0 % voor beide rassen, en er werden geen significante verschillen gevonden tussen de rassen (*t*-test, $P = 0.05$). De mate van clustering van door *Monilinia* aangetaste vruchten binnen bomen én die van bomen met *Monilinia*-vruchten, werd bepaald met respectievelijk 'Lloyd's index of patchiness' (LIP) en ruimtelijke autocorrelatie. Clustering van *Monilinia*-vruchten binnen bomen was significant in beide rassen in het eerste jaar ($P = 0.05$), en was nadrukkelijker aanwezig in het tweede jaar ($P = 0.01$). Clustering van bomen met *Monilinia*-vruchten binnen een rij was minder duidelijk aanwezig. In 1998 werden significante ($P = 0.05$), positieve correlatiecoëfficiënten gevonden tussen bomen die twee, drie en vier bomen van elkaar verwijderd stonden in James Grieve, maar opmerkelijk genoeg werd geen significante, positieve correlatie gevonden tussen (directe) buurbomen. In Cox's Orange Pippin werd een significante ($P = 0.05$), positieve correlatie gevonden tussen buurbomen in het tweede jaar.

Om de concentratie van *M. fructigena* conidia in de lucht te bepalen in een appelboomgaard, werd gedurende twee opeenvolgende seizoenen een 'Burkard' sporevanger geplaatst. Relaties tussen het aantal conidia dat gevangen werd en gemeten omgevingsfactoren werden bestudeerd (Hoofdstuk 6). De hoogste sporenconcentratie die gemeten werd per uur was 233 conidia per m^3 in 1997, in 1998 waren de concentraties lager. Voor regressieanalyse werd een algemeen lineair model ('generalised linear model', GLM) gebruikt, waarin het aantal conidia dat gevangen werd per uur gerelateerd werd aan de relatieve vochtigheid, temperatuur, mm neerslag, windsnelheid en windrichting in het veld met een logaritmische linkfunctie. Uit enkelvoudige regressie bleek dat relatieve vochtigheid en temperatuur de variatie in sporevangsten het best verklaarden. De methode van voorwaartse selectie ('forward selection') werd gebruikt om een meervoudig regressiemodel te ontwikkelen. In 1997 bevatte het best verklarende model alle weersfactoren die gemeten waren behalve neerslag, in het beste model voor 1998 kwamen alleen relatieve vochtigheid en temperatuur voor.

Vruchten die geïnfecteerd zijn door *M. fructigena* drogen langzaam uit en mummificeren uiteindelijk ('mummies') in de meeste gevallen. In het volgende seizoen worden nieuwe conidia geproduceerd op deze mummies (primair inoculum). Mummificatie en sporulatie van pitvruchten geïnfecteerd door *M. fructigena* werd bestudeerd in een veldexperiment en tevens onder gecontroleerde omstandigheden (Hoofdstuk 7). Golden Delicious vruchten die geïnfecteerd werden in september en oktober mummificeerden niet, maar sporuleerden rijkelijk na overwintering. Geconcludeerd werd dat zowel vroeg als laat in het seizoen geïnfecteerde vruchten bijdragen aan de hoeveelheid primair inoculum in het volgende seizoen. Voor reductie van primair inoculum in de boomgaard zouden alle geïnfecteerde vruchten verwijderd moeten worden tijdens de winter, niet alleen de gemummificeerde vruchten. Uit een studie naar het effect van verschillende klimaatregimes na infectie op uiteindelijke mummificatie en (her)sporulatie na een koudeperiode, bleek dat het percentage sporulerende vruchten sterk afnam in de klimaten van 20 °C en RV 75-85 % en 25 °C en RV 65-75 %. Na een incubatieperiode van 12 weken bij 25 °C sporuleerde geen enkele vrucht meer. Na 8 weken incubatie was het sporulerend vermogen ('sporulation intensity') per sporulerende vrucht significant hoger in een klimaat van 10 °C en RV 85-95 % vergeleken met dat in de klimaten van 20 en 25 °C (Kruskal-Wallis test, $P = 0.05$).

Een risicoanalyse werd uitgevoerd voor het quarantaine pathogeen *M. fructicola* voor de landen van de Europese Unie (EU) (Hoofdstuk 8). Via de import van fruit, vooral steenfruit, en de import van plantmateriaal behorende tot de familie der Rosaceae kan dit pathogeen de EU binnenkomen. Hoewel de import van fruit veruit de grootste bulk aan materiaal vertegenwoordigt, is de waarschijnlijkheid dat het pathogeen zich vestigt veel groter als het aanwezig is op plantmateriaal. Vestiging van *M. fructicola* in Europa zou vooral de steenfruit industrie in Zuid-Europa treffen, en zou waarschijnlijk resulteren in grotere verliezen in het veld en grotere naoogst verliezen. Het vóórkomen van fungicideresistentie in *M. fructicola* zou de problemen nog kunnen vergroten. Recent ontwikkelde moleculaire 'tools' voor snelle detectie van *M. fructicola* in geïmporteerde producten zouden een belangrijk instrument kunnen zijn in de pogingen om dit quarantaine organisme buiten Europa te houden.

Tenslotte worden in Hoofdstuk 9 de resultaten van het onderzoek gepresenteerd in dit proefschrift bediscussieerd, en worden aanbevelingen gegeven voor verder onderzoek. De fytosanitaire status van het nieuw beschreven anamorf *M. polystroma* en het verspreidingsgebied van deze nieuwe soort vragen om meer onderzoek. In de epidemiologie zou meer aandacht besteed moeten worden aan het proces van regeneratie van conidia op geïnfecteerde vruchten. Als dit proces geblokkeerd zou kunnen worden, zal de hoeveelheid primair inoculum in het voorjaar sterk teruggebracht kunnen worden.

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Curriculum vitae

Gerard van Leeuwen werd geboren op 5 november 1966 te IJsselstein, Utrecht. Na het behalen van het VWO-diploma aan het St. Bonifatiuscollege te Utrecht in 1985 werkte hij een jaartje op het ouderlijk bedrijf alvorens een studie te beginnen aan de Hogere Tuinbouwschool te Utrecht. Na afronding van deze studie werkte hij één jaar als voorlichter in de fruitteelt bij DLV Geldermalsen. Vanaf oktober 1991 was hij werkzaam bij kleinfruitbedrijf H. van Hemert in Ammerzoden als verkoper/voorlichter. In september 1992 begon hij aan de Landbouwuniversiteit te Wageningen aan een doorstroomstudie Plantenziektekunde, die hij in 1996 afrondde. Maart 1996 trad hij in dienst bij de vakgroep Fytopathologie van de Landbouwuniversiteit als assistent in opleiding en verrichtte het onderzoek dat beschreven is in dit proefschrift.