RESOLVING THE STATUS OF MONILINIA spp. IN SOUTH AFRICAN STONE FRUIT ORCHARDS

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SUMMARY

Geographical distribution records of pathogens and pests are the basis for phytosanitary decision-making. Monilinia fructicola, one of the three Monilinia species responsible for brown rot of stone fruit, is listed as a regulated pest for South Africa. Many disputes about the justification of this classification have arisen during the past years as records have been published regarding the presence of this pathogen in South Africa. Results of several surveys conducted from 1985 in stone fruit orchards, revealed that M. laxa is the only causal agent of brown rot in South Africa. However, another notification of non-compliance (interception of regulated pests) was received by the National Plant Protection Organisation of South Africa in 2003 stating that M. fructicola was detected on consignments of South African Prunus fruits (P domestica) in the United Kingdom. A detection survey according to inspections and sampling relevant to the biology of Monilinia species were conducted in the identified orchards at various stages throughout the year. Molecular techniques with species-specific primers for M. fructicola, M. laxa and M. fructigena based on the EPPO Diagnostic Protocol for M. fructicola were used for the identification of presumptive positive Monilinia isolates. The absence of M. fructicola from South African stone fruit orchards was again confirmed by this survey and its status in South Africa can be reported as: absent, not known to occur, confirmed by a detection survey. The regulated status of M. fructicola in South Africa is therefore scientifically justified by the results from this survey.

Key words: Brown rot, EPPO protocols, detection survey, Monilinia fructicola, Monilinia laxa, Prunus, quarantine.

INTRODUCTION

Brown rot is one of the most important pre- and post-harvest fungal diseases known to the fruit industry (Ogawa and English, 1991; Michailides and Morgan, 1997). Three species of the fungal genus Monilinia Honey, namely Monilinia fructicola (G. Winter) Honey, M. laxa (Aderh. & Ruhland) Honey and M. fructigena Honey, are the casual organisms of brown rot (Byrde and Willets, 1977; Ogawa et al., 1995; Snyder and Jones, 1999). Amongst the three Monilinia species, M. fructicola is considered the most destructive. The three species do not occur in all areas where stone fruit are grown. M. fructicola, known as the American brown rot fungus, is common in the Americas (North and South), Australia, New Caledonia and New Zealand (CABI/EPPO, 1999; CPC, 2005), whilst the other two species, indigenous to Europe, are known as the European brown rot fungi (CABI/EPPO, 1991, 2000). All three species are known to occur together only in Central and Eastern Asia, where Prunus, Malus and Pyrus spp. originated (CABI/EPPO, 1991, 1999, 2000). During 2004, M. fructicola was reported for the first time in China after the pathogen was intercepted in the UK on Prunus fruits imported in 2003 (OEPP/EPPO, 2004; Zhu et al., 2005). In Europe, M. fructicola was moved from the A1 pest list (pests not present in the region) of the European and Mediterranean Plant Protection Organisation (OEPP/EPPO) to the A2 pest list (pests present in some EPPO countries and under official control) after detection of this pathogen in Austria, France, Spain and the Czech Republic (OEPP/EPPO, 2002a, 2002b, 2005, 2006a; Duchoslavová et al., 2007; OEPP/EPPO, 2008). In 2006, this pathogen was eradicated in Austria (OEPP/EPPO, 2006b).

All three Monilinia spp. cause similar brown rot symptoms and can be distinguished only by laboratory examination. The species are also morphologically similar in culture, and the greatest difficulty is experienced to distinguish between isolates of M. fructicola and M. laxa (OEPP/EPPO, 1988; EPPO/CABI, 1997; Lane, 2002). Several studies have reported species-specific PCR primers for the identification of M. fructicola (Fulton and Brown, 1997; Fulton et al., 1999; Snyder and
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Jones, 1999; Förster and Adaskaveg, 2000; Hughes et al., 2000; Ioos and Frey, 2000; Boehm et al., 2001; Ma et al., 2003; Côte et al., 2004a, 2004b). However, some of these methods only differentiate M. fructicola from M. laca, but have not been validated to distinguish M. fructicola from M. fructigena. According to the EPPO Council responsible for the approval of EPPO Standards, Hughes et al. (2000) and Ioos and Frey (2000) developed the most reliable PCR primers. These two primer sets were included into the standard approved by the EPPO council that describes a diagnostic protocol for M. fructicola, a regulated pest for EPPO (OEPP/EPPO, 2003a).

South Africa is an export-driven country and exporters and the stone fruit industry (apricots, nectarines, peaches and plums) are constantly seeking access to new markets and expansion of existing markets. South Africa is currently the largest producer of stone fruit in the southern hemisphere. The total value of plum production for 2006/7 in South Africa was R 271,494,000 (DFPT, 2008). From a quarantine perspective, stone fruit is rated as “high risk crops” as a number of devastating exotic pests associated with stone fruit such as M. fructicola are currently absent from South Africa. M. fructicola is listed as a regulated pest for South Africa (Anonymous, 2008). For a pathogen to be classified as regulated pest for a country, the pest should be of economic importance to the endangered area and not yet present there, or present but not widely distributed and being officially controlled (FAO, 2002).

Many disputes have, however, arisen during the past years concerning this classification due to official records of the presence of this pathogen dating back to the 1950s. The first reference concerning the presence of M. fructicola in South Africa was by Doidge et al. (1953). Similar information was published by Heyns (1967) and Gorter (1977) about the presence of this pathogen in the South-Western and Southern Cape and also sporadic in Transvaal (= Gauteng). The situation was further complicated by reports from some European countries on detection of M. fructicola on stone fruit imported from South Africa (OEPP/EPPO, 1999; Van Leeuwen et al., 2001).

Thus, several surveys were conducted to ascertain whether this pathogen is present on stone fruit in the South-Western and Southern Cape. The results of these surveys were based solely on qualitative traits depending on colony characteristics and confirmed that M. fructicola was not present in these two stone fruit production areas, and that brown rot in South Africa is caused by M. laca only (Matthee, 1970; Schlagbauer and Holz, 1987; Den Breeyen, 1994). Based on the outcome of these surveys the status of M. fructicola in South Africa was changed to absent, formerly present (CABI/EPPO, 1999). Research done by Fourie et al. (2002) also confirmed that brown rot in South Africa is caused by M. laca. Results from this study were also based on qualitative traits depending on colony characteristics.

However, a notification of non-compliance (interception of regulated pests) was again received by the NPPO of South Africa during April 2003 from the UK, stating that M. fructicola was detected on consignments of Prunus fruits (P. domestica) traced back to orchards in South Africa (OEPP/EPPO, 2003b). The orchards where the fruit originated were traced to production units located in the Gauteng Province (previously known as the Transvaal), and to fruit of the plum cvs Flavor King, Songold and Leatitia.

The aim of this study was to ascertain whether M. fructicola is present in South African stone fruit orchards according to the terms of international standards for phytosanitary measures (ISPM) and based on diagnostics molecular protocols that contain adequate requirements for a reliable diagnosis of the specific species (OEPP/EPPO, 2003a; 2006c). The ISPMs of particular relevance are ISPM No. 6, “Guidelines for surveillance” (FAO, 1997) and ISPM no. 8, “Determination of pest status in an area” (FAO, 1998).

MATERIALS AND METHODS

Detection survey. The survey was conducted with relevance to the biology of M. fructicola in the identified orchards of the three plum cultivars located in the Gauteng Province. Three Songold orchards (average of 3.43 ha per orchard) and three Leatitia orchards (average of 3.68 ha per orchard) as well as one Flavor King orchard of 1.4 ha, with 1000 trees per ha, were inspected and sampled. The Songold and Leatitia orchards were established in 1997, 1998 and 2000 and the Flavor King orchard in 1997.

The number of trees per orchard inspected for symptoms, which included decayed or mummified fruit, blossom and twig blight and cankers, was determined according to the statistical method of Cannon and Roe (1982). To provide for a 95% confidence of sampling, 138 trees were randomly sampled per 1000 trees per ha.

The first inspection and sampling was at bud burst during August 2003, and all the orchards were monitored for mummified fruit that had fallen to the ground or remained hanging on the trees, as well as for cankers with profuse gumming. The second inspection and sampling was at full bloom stage during September 2003 when all the orchards were monitored for symptoms of blossom and twig blight. The third inspection and sampling was during October 2003 when all the orchards were monitored for twig blight symptoms and young fruit symptoms. The final inspection and sampling were carried out at later stages of harvesting during December 2003 and January 2004 with special em-
phasis on fruit expressing brown rot symptoms.

**Fungal isolates and colony characteristics.** Isolations were made from the 420 samples. Small pieces of tissue (mummified fruit, twigs and young fruit) were plated directly onto malt agar (MA; Biolab, South Africa), potato carrot agar (PCA; 20 g Biolab agar, 20 g carrot, 20 g potato, 1000 ml water) and potato-dextrose agar (PDA; Biolab, South Africa) amended with 2% streptomycin. The plates were incubated at 25°C. Plates were monitored daily for presumptive *Monilinia* isolates based on general colony characteristics. Hyphae growing out from the tissue pieces were subcultured onto fresh PDA and water agar (WA; Biolab) to induce sporulation. Isolates were incubated at 25°C under near-ultraviolet light (Dhingra and Sinclair, 1995). Presumptive positive isolates were hyphal-tipped to obtain pure cultures and maintained on PDA. Disks of all the cultures (6-day-old) were transferred to oatmeal agar (OMA; Biolab) in Petri dishes, and placed in a pattern as described by Sonoda et al. (1982), to examine the interaction between the different cultures.

**DNA isolation and amplification using species-specific primers for the identification of *M. fructicola.*** All the presumptive positive *Monilinia* isolates were grown on PDA. Twenty isolates collected from stone fruit during the 1996/97, 1997/8 and 1998/99 seasons (Fourie et al., 2002) were also included. Total genomic DNA was extracted using the Qiagen DNeasy Plant Maxi Kit (Hilden, Germany). The species-specific primer pair 5'-TATGCTCGCCAGAGGATAATTA-3' (Mfc-F1) and 5'-GATTTTAGAGCCTGCCATTA-3' (Mfc-R1) developed by Hughes et al. (2000) was used. These primers are based on subtle DNA sequence differences in the ITS 1 and 2 regions of the nuclear rRNA gene repeat. The species-specific primer pairs were custom synthesised by Integrated DNA Technologies (Coralville, USA). Amplification with the primers specific for *M. fructicola* was performed using the PCR conditions recommended by Hughes et al. (2000) as published in the EPPO-approved diagnostic protocol for *M. fructicola* (OEPP/EPPO, 2003a), except for the annealing temperature, which was increased from 59°C for 1 min to 62.5°C for 30 sec. Positive controls (DNA

![Fig. 1. Gel documentation of PCR products following PCR amplification of DNA extracted from *Monilinia* cultures ex 'Leatitia' plum with species-specific primers for *M. fructicola.* Samples on the gel are as follows: lane 1 denotes a 100-bp DNA ladder; lanes 2-16, isolates from 'Leatitia' plum. Isolates in lanes 17-19, lane 20 and lane 21 are *M. fructicola*, *M. laxa* and *M. fructigena* positive controls, respectively; lane 22 denotes a negative water control; lane 25 denotes a 100-bp DNA ladder; lanes 26-40, isolates from 'Leatitia' plum. Isolates in lanes 41-43, lane 44 and lane 45 are *M. fructicola*, *M. laxa* and *M. fructigena* positive controls, respectively; lane 46 denotes a negative water control.](image1)

![Fig. 2. Gel documentation of PCR products following PCR amplification of DNA extracted from *Monilinia* cultures ex 'Songold' plum with species-specific primers for *M. fructicola.* Samples on the gel are as follows: lane 1 denotes a 100-bp DNA ladder; lanes 2-16, isolates from 'Songold' plum. Isolates in lanes 17-19, lane 20 and lane 21 are *M. fructicola*, *M. laxa* and *M. fructigena* positive controls, respectively; lane 22 denotes a negative water control; lane 23 denotes a 100-bp DNA ladder; lanes 24-38, isolates from 'Songold' plum. Isolates in lanes 39-41, lane 42 and lane 43 are *M. fructicola*, *M. laxa* and *M. fructigena* positive controls, respectively; lane 44 denotes a negative water control.](image2)
of *M. fructicola*, which was obtained from The Central Science Laboratory, Sand Hutton, York, UK) and negative controls (using water instead of template DNA) were included. DNA of *M. fructigena* and *M. laxa* (obtained from The Central Science Laboratory, Sand Hutton, York, UK) were also included. PCR products were analysed by electrophoresis at 100 V for 1 h in a 1% (w/v) agarose gel and visualized under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, UK) after ethidium bromide staining.

**DNA isolation and amplification using species-specific primers for the identification of *M. laxa***. Thirty fungal isolates were randomly selected. The species-specific primer pair 5’-TATGCTGCGCACAGAATATC-3’ (IST1Mlx) and 5’-TGGGTGTTGGCAGAAACGACACC-3’ (IST4Mlx) developed by Ioos and Frey (2000) was used in the amplification reaction. These primers are based on base substitutions between the three *Monilinia* species clustered in two polymorphic regions, one located in the ITS1 and the other in the ITS2. The species-specific primer pairs were custom synthesised by Integrated DNA Technologies (Coralville, USA). Amplification with the species-specific primers for *M. laxa* was performed using the PCR conditions recommended by Ioos and Frey (2000) as published in the diagnostic protocol for *M. fructicola*, provided by EPPO (OEPP/EPPO, 2002). Positive controls (DNA of *M. laxa*, obtained from The Central Science Laboratory, Sand Hutton, York, UK) and negative controls (using water instead of template DNA) were included. DNA of *M. fructicola* and *M. fructigena* (obtained from The Central Science Laboratory, Sand Hutton, York, UK) were also included. PCR products were analysed as described above.

**RESULTS**

**Detection survey**. A total of 414 trees for each of the Songold and Leatitia orchards and 138 trees for Flavor King were monitored at each of the four inspection intervals. During the surveillance period 98 samples were collected from the Flavor King orchard, 154 from the Leatitia orchards and 168 from the Songold orchards. Mummified fruit and cankers on twigs were the most prevalent type of symptoms found in all the identified orchards.

**Fungal isolates and morphology**. Of the 420 samples taken during the surveillance period, 172 presumptive *Monilinia* isolates were obtained through visual examination based on morphological features such as colony margins, lobing and colour of the sporulating areas. On the oatmeal agar plates, no distinct black lines, which are a positive identification for *M. fructicola*, were observed between any of these cultures after 10 days incubation and the 172 isolates were regarded as *M. laxa*. A light line was, however, observed between some of the cultures. Therefore, for conclusive identification, 90 isolates (including the aforementioned), 30 each of the different cultivars, were selected for molecular analysis.

DNA isolation and amplification using species-specific primers for the identification of *M. fructicola*. None of the 90 DNA samples of the presumptive positive isolates or DNA from the 20 isolates from Fourie et al. (2002) gave positive results following PCR amplification with the species-specific primers for *M. fructicola*. Only DNA from the three *M. fructicola* positive controls yielded an amplicon of 280 bp (Fig. 1-4).

DNA isolation and amplification using species-specific primers for the identification of *M. laxa*. All the samples gave positive results following PCR amplification with the species-specific primers for *M. laxa*. All the isolates, including the positive control (DNA of *M. laxa*) yielded an amplicon of 350 bp. No amplification was observed in the lanes containing the reference DNA of *M. fructicola* or *M. fructigena*.

![Fig. 3. Gel documentation of PCR products following PCR amplification of DNA extracted from Monilinia cultures ex ‘Flavor King’ plum with species-specific primers for M. fructicola.](image-url)
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In this study, a molecular approach was followed to determine the presence of *M. fructicola* in South African stone fruit orchards after a thorough detection survey was conducted. None of the isolates tested with the species-specific PCR primers gave positive results for *M. fructicola*. The PCR test was performed using the PCR conditions of the EPPO-approved and recommended diagnostic protocol for *M. fructicola* (OEPP/EPPO, 2003a), with one exception: annealing temperature was adjusted to 62.5°C for 30 sec to eliminate non-specific amplification, which was observed at the recommended annealing temperature of 59°C for 1 min (results not shown). The absence of *M. fructicola* was validated by the positive results following PCR of 30 randomly selected isolates with species-specific primers for *M. laxa*.

Since 1985, various surveys have been conducted in stone fruit orchards in all the production areas (Western Cape and Gauteng), and *M. fructicola* has never been detected (Matthee, 1970; Schlagbauer and Holz, 1987; Den Breeyen, 1994; Fourie et al., 2002). This study therefore confirms these reports, which were based on morphological identification and provides the scientific justification to the NPPO of South Africa to support the status of *M. fructicola* as a regulated pest. The status of *M. fructicola* in South Africa can therefore be described as: “Absent, not known to occur, confirmed by a detection survey”.

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