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ORIGINAL ARTICLE

Two different PCR approaches for universal diagnosis of brown rot and identification of *Monilinia* spp. in stone fruit trees

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Keywords
brown rot, *Monilinia* spp., *Monilinia laxa*, *Monilinia fructigena*, *Monilinia fructicola*, peach, stone fruit.

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Abstract

Aims: To design a protocol for the universal diagnosis of brown rot by polymerase chain reaction (PCR) in plant material and subsequently *Monilinia* spp. identification.

Methods and Results: Primers for discrimination of *Monilinia* spp. from other fungal genera by PCR were designed following a ribosomal DNA analysis. Discrimination among species of *Monilinia* was subsequently achieved by developing primers using SCAR (Sequence Characterised Amplified Region) markers obtained after a random amplified polymorphic DNA study. In addition, an internal control (IC) based on the utilization of a mimic plasmid was designed to be used in the diagnostic protocol of brown rot to recognize false negatives due to the inhibition of PCR.

Conclusions: The four sets of primers designed allowed detection and discrimination of all *Monilinia* spp. causing brown rot in fruit trees. Addition of an IC in each PCR reaction performed increased the reliability of the diagnostic protocol.

Significance and Impact of the Study: The detection protocol presented here, that combined a set of universal primers and the inclusion of the plasmid pGMON as an IC for diagnosis of all *Monilinia* spp., and three sets of primers to discriminate the most important species of *Monilinia*, could be an useful and valuable tool for epidemiological studies. The method developed could be used in programmes to avoid the spread and introduction of this serious disease in new areas.

Introduction

Brown rot is one of the most important fungal diseases that affect stone and pome fruit trees such *Prunus*, *Malus* and *Pyrus* spp., causing fruit rot, death of branches and blossom blight under favourable conditions in the spring, in Europe and elsewhere. Disease severity is determined mainly by environmental factors; blossom blight can be expected in humid or rainy weather with mild day temperatures (20–25°C) and cool nights and mature fruit rot occurs at high temperatures in combination with high humidity. Leaves and shoots are also usually affected. In addition, this disease causes severe postharvest crop losses.

Three *Monilinia* species and one *Monilinia* anamorph (*Monilia* sp.) may cause brown rot. *Monilinia fructigena* and *Monilinia laxa* have been extensively reported in Europe. *Monilia polystroma*, an anamorphic species closely related to *M. fructigena*, has been only described in Japan (van Leeuwen *et al.* 2002). *Monilinia fructicola* occurs in North and South America, Japan and Australia (EPPO/CABI 1997) and has been recently introduced in France (Lichou *et al.* 2002), Austria (NPPO of Austria 2002) and Spain (Petroczy and Palkovics 2006). This species, however, is listed by EPPO as a quarantine pest within the European Union (OEPP/EPPO 2005) because a broad dissemination of this species in Europe would be devastating especially for peach, nectarine and apricot.

The first step to manage brown rot is the detection and identification of *Monilinia* spp. causing the disease. This issue is not only important for quarantine purposes, such in the case of *M. fructicola* to avoid new introductions in areas free of this fungus, but it is also essential for managing brown rot caused by all three *Monilinia* spp.

Currently, identification of *Monilinia* spp. is based on cultural and morphological characteristics and fungal isolation from the plant material involves 7–10 days incubation time (Penrose *et al.* 1976; Byrde and Willets 1977; Mordue 1979; van Leeuwen and van Kesteren 1998; De Cal and Melgarejo 1999; Lane 2002). Although morphologic characteristics may assist in the identification of typical cultures of *Monilinia* spp., some problems arise with atypical cultures. Phenotypic variability has been shown even among isolates of the same species (Byrde and Willets 1977; Harada 1977; Batra 1979; Wilcox 1989). As a result, the development of new fast and accurate identification methods has been necessary.

A monoclonal antibody-based immunoassay was developed to detect the genus *Monilinia* and to identify the species *M. fructicola* (Hughes *et al.* 1996). Another commercial monoclonal antibody-based kit was further developed by Adgen Diagnostic Systems (Auchincruive, UK). This method, however, showed some difficulties when tested using a collection of Spanish isolates (De Cal *et al.* unpublished).

Many efforts have been made to develop molecular DNA-based methods for detection and differentiation of *Monilinia* species. Polymerase chain reaction (PCR) primers for the *M. fructicola* small sububit (SSU) rDNA intron and some of the SSU sequence were developed for species identification (Fulton and Brown 1997). Nevertheless, the method was not reliable as the intron-containing PCR product was not amplified from some isolates of *M. fructicola*, suggesting the lack of the intron in these isolates (Fulton *et al.* 1999; Förster and Adaskaveg 2000; Hughes *et al.* 2000; Côté *et al.* 2004). The sequences of the ribosomal internal transcribed spacer (ITS) regions of the three *Monilinia* species have been reported (Holst-Jensen *et al.* 1997; Fulton *et al.* 1999), and several molecular methods based on ITS sequence variations or combined with other genetic markers were developed (Snyder and Jones 1999; Förster and Adaskaveg 2000; Ioos and Frey 2000). Snyder and Jones (1999) proposed a diagnostic method based on restriction fragment length polymorphism (RFLP) of PCR-amplified ITS1, digested with the endonuclease *Mse*I, which allowed differentiation between *M. laxa* and *M. fructicola*. This method should also differentiate *M. fructigena* from the other two species, according to the published ITS1 sequences. Snyder and Jones (1999) also assessed a microsatellite-primed PCR assay to differentiate *M. laxa* from *M. fructicola*. Microsatellite sequences, however, could not be used for direct species diagnosis from infected fruits, because plant DNA may be simultaneously amplified. Other molecular identification methods based on unique species-specific repetitive sequences and microsatellite regions were also developed (Boehm *et al.* 2001; Ma *et al.* 2003). Côté *et al.* (2004) developed an identification method using multiplex PCR that differentiated all three *Monilinia* species and *Monilia polystroma*.

Although PCR is an important tool for detection of plant pathogens, its routine application in diagnosis of diseased plant tissue is usually restricted by the presence of plant compounds that inhibit the amplification of nucleic acids. Inhibition of amplification of the target sequence results in false negatives because of the failure of the PCR (Wilson 1997). Reaction inhibition can be partially overcome by the use of appropriate DNA extraction protocols (Cubero *et al.* 1999; Llop *et al.* 1999; Förster and Adaskaveg 2000; Ma *et al.* 2003; Hoorfar *et al.* 2004) but these sometimes are not efficient enough in the total removal of inhibitors. To recognize false negatives because of inhibition of PCR, different approaches of production and application of internal controls (IC) have been used in the diagnostic protocols of plant diseases (Sachadyn and Kur 1998; Louws *et al.* 1999; Cubero *et al.* 2001, 2002).

The purpose of this study was to design a protocol for the universal diagnosis of brown rot by PCR in plant material and subsequent identification of *Monilinia* spp. A new set of primers has been designed to amplify a DNA fragment corresponding to a sequence within the rDNA of all *Monilinia* spp. causing brown rot on stone fruit. Furthermore, three new set of primers were designed based on SCAR markers obtained by random amplified polymorphic DNA (RAPD) to detect and discriminate the three *Monilinia* spp. causing brown rot on stone fruit. In addition, an internal control, based on the utilization of a mimic plasmid, was designed to be used with universal primers in the diagnostic protocol for brown rot in order to recognize false negatives because of the inhibition of PCR.

Materials and methods

Fungal isolates

Fungal isolates used in this study are listed in Table 1. Isolates of *Monilinia*, *Epicoccum*, *Penicillium*, *Alternaria* and *Aspergillus* spp. were obtained from stone and apple fruit from orchards worldwide, especially from different production areas of Spain. *Monilinia* isolates from other countries different to Spain were provided as nonliving fungi to comply with quarantine regulations.

Table 1 Fungal isolates used to evaluate four sets of primers based on ribosomal sequences (*Mon3·1/Mon3·2*), and polymorphic bands after RAPD analysis (*LaxaS/LaxaAS*, *ColaS/ColaAS*, *GenaS/GenaAS* specific for *Monilinia laxa*, *Monilinia fructicola* and *Monilinia fructigena*, respectively)

					PCR results			
Taxon	Isolate	Host	Year of isolation	Origin	*IMon3·1/ IMon3·2*	*ILaxaS/ ILaxaAS*	*IGenaS/ IGenaAS*	*IColaS/ IColaAS*
M. laxa	T3, T5, T6, T7	Peach	1998	Tudela, Navarra*	+	+	–	–
	L2, L4, L8, L12, L22	Peach	1998	Lérida*	+	+	–	–
	ES12, ES13	Apricot	1998	Zaragoza*	+	+	–	–
	ES14, ES15, ES23, E32	Apricot	1996	Cehegin, Murcia*	+	+	–	–
	ES16, ES21	Apricot	1996	Moratalla, Murcia*	+	+	–	–
	ES19, ES31	Apricot	1996	Bullas, Murcia*	+	+	–	–
	ES20, ES27	Almond	1996	Moratalla, Murcia*	+	+	–	–
	ES25, ES37	Apricot	1996	Caravaca, Murcia*	+	+	–	–
	ES50	Almond	1996	Zaragoza*	+	+	–	–
	ES52	Almond	1997	Zaragoza*	+	+	–	–
M. fructigena	ES41	Apple	1996	Jubia, La Coruña*	+	–	+	–
	ES45	Peach	1996	Jubia, La Coruña*	+	–	+	–
	ES49	Plum	1996	Jubia, La Coruña*	+	–	+	–
	ES 51	Apple	1997	Zaragoza*	+	–	+	–
	W13	Quince	1996	Portugal	+	–	+	–
	W14	Apple	1995	Japan	+	–	+	–
	W17	Plum	1993	Poland	+	–	+	–
	W18	Peach	1996	Holland	+	–	+	–
M. fructicola	W1	Apple	1989	Japan	+	–	–	+
	W2	Apricot	1994	New Zealand	+	–	–	+
	W7	Plum	1994	USA	+	–	–	+
	W11	Plum	1996	USA	+	–	–	+
	W16	Peach	1992	Japan	+	–	–	+
Epicoccum nigrum	Epi. A	Peach	2003	Gimenells, Lérida*	–	–	–	–
	Epi. B	Peach	2003	Sudanell, Lérida*	–	–	–	–
	Epi. C	Peach	2003	Torres Form, Lérida*	–	–	–	–
Penicillium spp.	Penic. A	Peach	2003	Gimenells, Lérida*	–	–	–	–
	Penic. B	Peach	2003	Sudanell, Lérida*	–	–	–	–
	Penic. C	Peach	2003	Torres Form, Lérida*	–	–	–	–
Alternaria spp.	Alt. A	Peach	2003	Gimenells, Lérida*	–	–	–	–
	Alt. B	Peach	2003	Sudanell, Lérida*	–	–	–	–
	Alt. C	Peach	2003	Torres Form, Lérida*	–	–	–	–
Aspergillus niger	Asp.	Peach	2003	Sudanell, Lérida*	–	–	–	–
Botrytis cinerea	Botry 1283	Unknown	1993	Almería*	–	–	–	–
	Botry 1284	Unknown	1993	Almería*	–	–	–	–
	Botry 1285	Unknown	1993	Almería*	–	–	–	–

*Located in Spain.
RAPD, random amplified polymorphic DNA; PCR, polymerase chain reaction.

Fungal isolations from fruit were made on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) amended with 0·5 g l^{-1} of streptomycin sulfate. Species identification was performed according to a protocol previously described (De Cal and Melgarejo 1999). All isolates were stored as conidial suspensions in 20% glycerol at −80°C, or as cultures on PDA at 4°C for long- and short-term storage, respectively. For conidial and mycelial production, all isolates were cultured on PDA plates at 22°C in the dark.

DNA extraction from pure cultures

DNA was extracted following the method described by Keijer *et al.* (1996) with minor modifications. Briefly, three mycelial plugs of about 5 mm^3 of each isolate were taken from the edges of 7-day-old actively growing colonies of the fungus on PDA, transferred to 50 ml of liquid complete media (LCM: yeast extract, malt extract and glucose, 5·0 g l^{-1} each) and incubated with shaking for 21 days at 25°C. Mycelial mats were harvested by

filtration, washed with sterile distilled water and lyophilized. About 75 mg of the lyophilized fungus was ground in a mortar, resuspended in 500 μl of DNA-extraction buffer [20 mmol l^{-1} Tris HCl pH 8·0, 50 mmol l^{-1} EDTA pH 8·0 and 0·4% (w/v) sodium dodecyl sulfate (SDS)], incubated for 5 min at room temperature and centrifuged at 17 000 ***g***. The supernatant was extracted twice with an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and cleaned with chloroform : isoamyl alcohol (24 : 1). After RNase treatment, DNA was precipitated in isopropanol, washed with 70% ethanol and re-suspended in 50 μl of ultra pure water.

DNA extraction from plant material

DNA extractions from plant material were performed using the method described by Keb-Llanes *et al.* (2002) with some modifications: 0·5 g of plant tissue was homogenized in EB-2 buffer [100 mmol l^{-1} Tris-HCl (pH 8·0), 50 mmol l^{-1} EDTA, 100 mmol l^{-1} NaCl, 10 mmol l^{-1} β-mercaptoethanol] and transferred to a microcentrifuge tube containing 600 μl of EB-2 and 200 μl of EB-1 [2% (w/v) hexadecyltrimethylammonium bromide (CTAB), 100 mmol l^{-1} Tris-HCl (pH 8·0), 20 mmol l^{-1} EDTA, 1·4 mol l^{-1} NaCl, 4% (w/v) polyvinylpyrrolidone (PVP), 0·1% (w/v), ascorbic acid, 10 mmol l^{-1} of β-mercaptoethanol and 20% SDS]. After vortexing and incubation at 65°C for 30 min, with occasional shaking, samples were ice cooled, mixed with 300 μl of 5 mol l^{-1} cold potassium acetate and incubated for 5 min on ice. After centrifugation at 4°C and at 20 000 ***g*** for 15 min, the supernatant was mixed with an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and centrifuged at 4°C and at 16 000 ***g*** for 10 min. The aqueous phase was mixed with an equal volume of chloroform–isoamylic alcohol (24 : 1) and centrifuged at 4°C and at 16 000 ***g*** for 10 min. The supernatant was mixed with 0·6 volume of isopropanol, incubated on ice for 60 min and centrifuged at 4°C and at 16 000 ***g*** for 15 min. Precipitate was finally washed with 70% ice-cold ethanol, re-suspended in 200 μl of ultra pure water and stored at −20°C until further use.

PCR amplification of ribosomal DNA regions

DNA from isolates shown in Table 1 was used as the target for PCR. Ribosomal DNA was amplified using primers ITS 5 and ITS 4A (Larena *et al.* 1999). PCR was performed in 25 μl total volume containing, 10 mmol l^{-1} Tris-HCl (pH 8·3), 50 mmol l^{-1} KCl, 100 mmol l^{-1} each of dNTP, 2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} of each primer, 1 U of Taq DNA Polymerase (Biotools B&M Labs, SA, Madrid, Spain) and 10 ng of template genomic DNA. Amplification reaction conditions consisted of 94°C for 7 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and a final elongation step of 72°C for 10 min. PCR products were visualized under ultraviolet (UV) light in 1% agarose gels stained with ethidium bromide.

PCR products were extracted from agarose gels using the QIAEX II extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions and sequenced using universal primers ITS 1 and ITS 4 (White *et al.* 1990).

Sequence analysis

Sequences were aligned and compared with those available in the GenBank database using BioEdit Sequence Alignment Editor 5·0·6 (Hall 1999) and CLUSTAL W 1·82 (Thompson *et al.* 1994). Sequences from *Monilinia, Botrytis, Sclerotinia, Myriosclerotinia, Dumontinia, Grovesinia, Epicoccum, Penicillium, Alternaria* and *Aspergillus spp.* were compared. The analytical program MEGA version 2.1 (Kumar *et al.* 2001) was used to construct a dendrogram using the neighbour-joining method (Saitou and Nei 1987). The Jukes-Cantor model (Jukes and Cantor 1969) and '*p*' distance were used to calculate genetic pair-wise distances among sequences. Reliability of clusters was evaluated by bootstrapping with 1000 replicates.

Primer design and PCR conditions for *Monilinia* spp.

Oligonucleotide primers *IMon3·1* (GCTCGCCAGAGAATAAYY) and *IMon3·2* (AGACTCAATACCAAGCTGT) were designed for detection of *Monilinia* species. DNA from isolates shown in Table 1 were used as targets in PCR performed as before. Amplification reaction conditions consisted of 94°C for 7 min followed by 30 cycles of 94°C for 1 min, 61°C for 1 min and 72°C for 1 min and a final elongation step of 72°C for 10 min. PCR products were visualized as before.

PCR amplification using RAPD techniques

Two *M. laxa* isolates (ES-37 and T-5) were used to set up appropriate RAPD conditions. Eighty primers from the OPB, OPD, OPN and OPR series (Operon Technologies, Alameda, CA, USA) were selected and tested to be used for isolate discrimination. After PCR optimization all fungal isolates shown in Table 1 were analysed.

The amplification reactions were performed in a volume of 25 μl containing 1x buffer, 100 mmol l^{-1} of each dNTP, 1·5 μmol l^{-1} of primer, 2 mmol l^{-1} of $MgCl_2$, 1·25 U of Ultratools DNA Taq Polymerase and 10 ng of DNA template. Amplification conditions consisted of an

initial denaturation of 94°C for 6 min, followed by 40 cycles of 1 min at 94°C, 2 min at 38°C, 2 min at 72°C and the reaction was completed with a final extension of 72°C for 10 min. Amplified products were analysed as before in 1·5% agarose gels.

Design of species-specific primers for *Monilinia* spp. from RAPD markers

After RAPD analysis using primers OPR-09, OPN-06 and OPB-06, polymorphic bands specific for *M. laxa*, *M. fructigena* and *M. fructicola* were obtained. RAPD fragments were purified from agarose gels, cloned into pGEM-T vector (Promega, Madison, WI, USA) and sequenced at the sequencing service of Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Madrid, Spain).

The sequences obtained were used to design primers specific for each of the *Monilia* spp. Primers *ILaxaS* (TGAGCACGAGTGAATGTATAG) and *ILaxaAS* (TGAGCACGAGGGCATATC) were designed to detect only *M. laxa*, primers *IGenaS* (TGCTCTGCCCGTACCCAG) and *IGenaAS* (GGATTTATTGTGATGTAGTTTCG) for *M. fructigena* and primers *IColaS* (GAGACGCACACAGAGTCAG) and *IColaAS* (GAGACGCACATAGCATTGG) for *M. fructicola*.

The ability of these primers to detect *Monilinia* spp. was assessed by performing PCR using DNA obtained from the fungal collection listed in Table 1. Amplification reactions were carried out in a volume of 25 μl containing 1x buffer, 100 mmol l^{-1} of each dNTP, 1 μmol l^{-1} of each primer, 2 mmol l^{-1} of $MgCl_2$, 1 U of *Tth* DNA polymerase and 10 ng of DNA template. PCR reactions were performed with a cycle profile of an initial step of 94°C for 7 min, followed by 30 cycles of 1 min at 94°C, 1 min at 65°C and 1 min at 72°C and a final extension step of 72°C for 10 min. Amplification products were analysed as before.

IC construction for PCR using primer set *IMon3·1/IMon3·2*

An IC plasmid was constructed by following an approach similar to that described previously for *Xanthomonas axonopodis* pv. citri (Cubero *et al.* 2001). Primers *ICimon3·1* (GCTCGCCAGAGAATAAYYTATATACTGCAA) and *ICimon3·2* (AGACTCAATACCAAGCTGTAGTACAGGAAGG) containing 5′ termini identical to primers designed to amplify the ribosomal region in *Monilinia* spp. and 3′ termini complementary to a sequence inside the plasmid pGXIS (Cubero *et al.* 2001) were used to amplify pGXIS. The PCR product of 400 bp was cloned into pGEM-T vector (Promega) to create pGMON used to transform *Escherichia coli* cells. Adjusted concentrations of purified pGMON were added and used as IC for PCR for *Monilinia* spp.

IC titration

The concentration of pGMON that could be added to PCR reactions, without a decrease in the sensitivity in detection of *Monilinia* spp. was evaluated as described before (Cubero *et al.* 2001, 2002). Competitive PCR were performed with DNA ranging from 0·1 to 10 ng, obtained from serial dilutions of *Monilinia* spp. DNA in the presence of 0·015, 0·15 and 1·5 pg of pGMON per reaction. Amplification conditions were as described before but with the addition of IC.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers EF153013 to EF153017 and EF207413 to EF207429.

Results

Analysis of rDNA sequences

Sequence analysis of rDNA revealed high levels of similarity among the fungal isolates studied. The mean similarity level (±SD) was 98·2 ± 0·4, with 30 variable nucleotide positions and a maximum of 20 pair-wise nucleotide differences over a sequence of 459 nucleotides when ITS1, 5·8S rDNA and ITS2 were analysed together. Sequences of the 5·8S rDNA showed a 100% similarity among all the isolates. However, some diversity was shown at the ITS1 and ITS2 levels. Analyses of the combined ITS1 and ITS2 sequences showed a mean similarity level of 97·1 ± 0·6 among all fungal isolates and included the maximum 20 nucleotide pair-wise nucleotide differences and the 30 variable positions over a sequence of 290 nucleotides. The variability in the ITS rDNA among the different fungal isolates used in this study was sufficient to discriminate *Monilinia* spp. from other related fungal genera as shown in the dendrogram in Fig. 1. The dendrogram based on pair-wise comparison of the ITS1 and ITS2 analysed showed that *Monilinia* spp. grouped together, within this *Monilinia* cluster, three subgroups corresponding to the three *Monilinia* species could be elucidated (Fig. 1).

Discrimination of *Monilinia* spp. from other fungal genera by PCR

Variable regions in ITS1 and ITS2 were used to design primers to specifically amplify DNA from *Monilinia* spp.

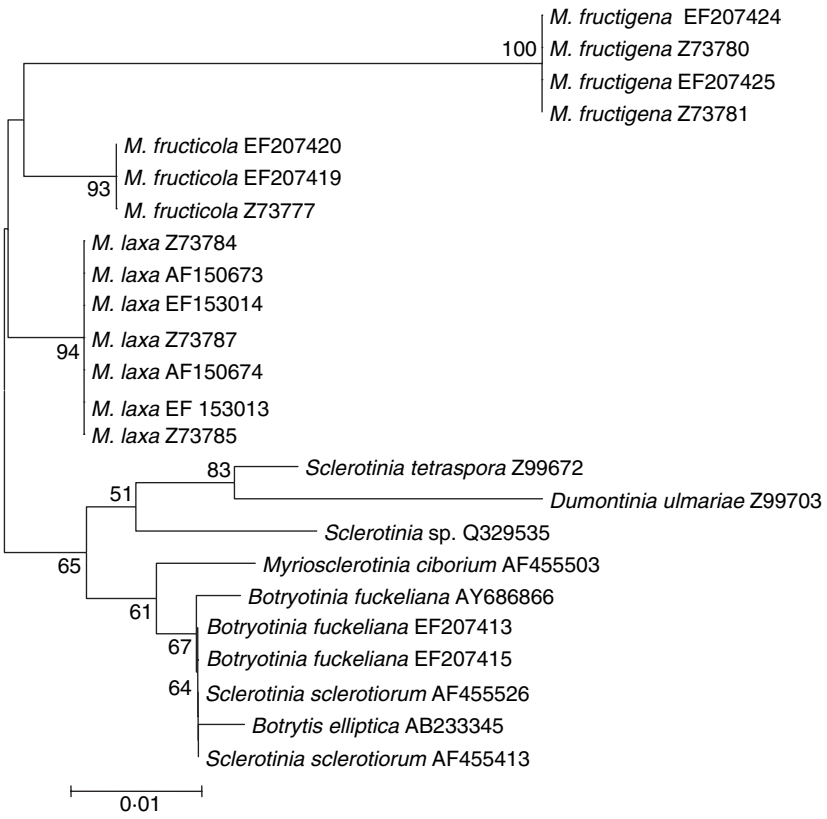

Figure 1 Neighbour-joining dendrogram showing phylogenetic relationships based on pair-wise comparison using Jukes-Cantor parameter. The distance between two isolates is obtained by adding the lengths of the connecting horizontal branches using the scale at the bottom. Bootstrap values (based on 1000 replicates) are indicated at the nodes.

```
                                        Imon3·1                 Imon3·2
                                   5′GCTCGCCAGAGAATAAYY 3′   3′ TGTCGAACCATAACTCAGA 5′
Monilinia fructigena ES49          GCACGCTCGCCAGAGAATAACCAAAC  AAGCACAGCTTGGTATTGAGTCTATGC
Monilinia fructigena Z73781        ...........................  ...........................
Monilinia fructicola W1            .T.T..........G....TT....  ...........................T
Monilinia fructicola Z73777        .T.T..........G....TT....  ...........................T
Monilinia laxa T3                  .T.T.................T.....  ...........................T
Monilinia laxa AF150673            .T.T.................T.....  ...........................T
Botrytis spp. 1284                 .T.T................C.A....  ....TT.....................T
Botrytis elliptica AB233345        .T.T.............A.C.A....  ....TT.....................T
Botrytinia fuckeliana AY686866     .T.T................C.A....  ....TT.....................T
Myyriosclerotinia ciborium AF455503.T.T..........T...C--....  ....TT.....................T
Sclerotinia sclerotium AF 455526   .T.T................C.A....  ....TT.....................T
Dumontinia ulmariae Z99703         .T.T...............T.A....  ....T.................C...T
                                                  ITS1                    ITS2
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Figure 2 Internal transcribed spacer ITS1 and ITS2 sequences used to design primers *Imon3·1* and *Imon3·2* for polymerase chain reaction (PCR) amplification of *Monilinia* spp. Primers *Imon3·1* and *Imon3·2* are shown by italic and boldface types at the top of the corresponding sites in rDNA sequences.

(Fig. 2). Because of a slight variation of these variable regions among *Monilinia* spp., two degenerated positions were included at 3′ terminus of the upstream primer (*Imon3·1*) complementary to ITS (Fig. 2).

Results of the amplifications performed with the primers *Imon3·1* and *Imon3·2* are shown in Table 1. This set of primers was used to amplify target sequence in DNA extracted from 38 isolates of *Monilinia* spp. from different geographic origins and different host plants. The expected 250-bp product was produced with the DNA from *Monilinia* and was not obtained with DNA extracted from isolates of other fungal genera associated with fruits such as *Epicoccum*, *Penicillium*, *Alternaria* and *Botrytis* (Fig. 3, Table 1). Therefore, primers *Imon3·1* and *Imon3·2* allowed

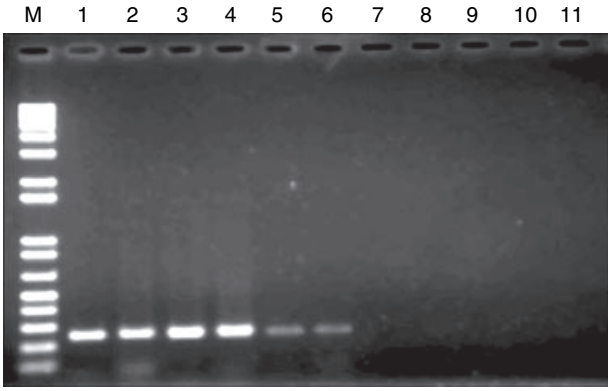

Figure 3 Polymerase chain reaction (PCR) products obtained after amplification of DNA extracted from isolates ES19 and T7 of *Monilinia laxa* (lanes 1 and 2), ES49 and W17 of *Monilinia fructigena* (lanes 3 and 4), W1 and W7 of *Monilinia fructicola* (lanes 5 and 6), 1283 of *Botrytis cinerea* (lane 7), EpiA of *Epicoccum nigrum* (lane 8), Penic. A of *Penicillium* sp. (lane 9) and Alt-A of *Alternaria* sp. (lane 10). Negative control (lane 11) and 1-kb plus ladder (Invitrogen, Life Technologies, Carlsbad, CA, USA) as molecular marker (M) are also shown.

simultaneous identification of the three species of *Monilinia* that cause brown rot of fruit trees.

Discrimination among species of *Monilinia* by PCR

RAPD PCR analysis showed different fingerprints among species of *Monilinia* spp. Under our conditions primers OPR-09, OPN-16 and OPB-06 yielded polymorphic bands of 314, 386 and 500 bp specific for *M. laxa*, *M. fructicola* and *M. fructigena*, respectively.

Based on the polymorphic fragment obtained using primer OPR-09, primers *IlaxaS* and *IlaxaAS* were designed to specifically amplify DNA from *M. laxa*, based on the fragment obtained using primer OPN-16, primers *IColaS* and *IColaA* were designed to specifically amplify DNA from *M. fructicola* and based on the fragment obtained using primer OBP-16, primers *IGenaS* and *IGenaAS* were designed to specifically amplify DNA from *M. fructigena.* These primers were evaluated in a fungal isolate collection and the expected PCR products of 314, 386 or 500 bp were produced only in *M. laxa*, *M. fructicola* and *M. fructigena*, respectively (Table 1). The specific PCR products were not obtained with DNA extracted from other fungi (Table 1).

Development of an IC to be used with primers *Imon3·1* and *Imon3·2*

Amplification of the plasmid created as an IC, pGMON, resulted in a 400-bp DNA fragment, whereas amplification from the target in *Monilinia* spp. yielded a 250-bp fragment. The two fragments were easily differentiated in agarose gels. pGMON at a concentration of 0·015 pg μl^{-1} provided sensitivity for detection of *Monilinia* spp. comparable with that obtained without the addition of this plasmid in the PCR.

In plant material, PCR products from the IC and the target sequence were obtained simultaneously from most of the samples analysed. However, high concentrations of the target sequence present in the initial extract prevented the amplification of the IC in the competitive reaction. No bands, or faint bands, indicated PCR inhibition because of a poor quality of the DNA extraction as a consequence of the probable presence of PCR inhibitors. This type of sample would be considered wrongly negative without the inclusion of the IC in the reactions.

Discussion

With increasing movement of fresh fruits worldwide, border monitoring and surveillance become more challenging. Especially with perishable commodities, timely methods for the identification of potential quarantine pests are an important tool for inspection agencies. Usage of DNA-based methods is progressively increasing for timely identification of quarantine pests (Martin *et al.* 2000). Molecular methods based on the PCR technique give results more rapidly than identification following isolation of the potential quarantine pests, and can be designed to directly and specifically identify the species that is infecting a commodity.

We developed primers for discrimination of *Monilinia* spp. from other fungal genera in pure culture and in diseased plant material. Genus-specific primers were developed from rDNA region, whereas species-specific primers were designed from unique RAPD fragments that were cloned and sequenced. This latter method has been used successfully in developing *M. fructicola*-specific PCR primers by other authors (Förster and Adaskaveg 2000).

Analysis of the rDNA sequences revealed a limited diversity among fungal species that comprise the microflora of the plant species affected by *Monilinia* spp. This high level of similarity was especially remarkable within the *Monilinia* genus. Still, combined analysis of ITS1 and ITS2 was sufficient to discriminate *Monilinia* from other fungi and moreover, to identify the three species of *Monilinia* as reported previously by other authors using different isolates (Holst-Jensen *et al.* 1997; Fulton *et al.* 1999). In this work, ITS has not been used to design specific primer to identify each species of *Monilinia* but for universal diagnosis of brown rot (i.e. identification of the genus). Primers *Imon3·1* and *Imon3·2* were addressed to detect all *Monilinia* spp. causing this disease. These primers were based on the limited sequence variability found

in the ITS ribosomal region among the fungus species usually isolated in rosaceous fruit trees and the high similarity within the *Monilinia* genus. In contrast, primers *IlaxaS/llaxaAS, IColaSI/IColaA* and *IGenaSI/IGenaAS* used to discriminate species within the genus *Monilinia*, were obtained following a different approach based on sequence information of polymorphic RAPD bands. These polymorphic bands allowed the design of primers based on fungal genome regions different from rDNA and variable enough to discriminate the three *Monilinia* spp.

To address difficulties because of PCR inhibition in the detection on the *Monilinia* species from plant material, we report the use of an IC designed to be used with primers *Imon3·1* and *Imon3·2* in order to improve the accuracy of the diagnostic protocol for brown rot. Addition of this IC to each PCR reaction allows us to distinguish between false and true negatives due to PCR inhibitors in reactions performed for the detection of *Monilinia* spp. in plant material. To optimize the concentration of plasmid pGMON in the reactions, without creating an appreciable decrease in the sensitivity of the PCR assay for the detection of the fungus, a titration of the IC was perfomed. In our conditions and according to previous results in other plant pathogen models (Cubero *et al.* 2001, 2002), the addition of 0·015 pg μl^{-1} of pGMON ensures the attainment of at least one PCR product after the amplification. If DNA from the fungus in the sample is high, only a PCR product from the target sequence is obtained. If there is no target DNA in the sample, then only product from the IC is present. Co-amplification from the target and IC occur if the target DNA is present at appropriate concentrations and finally, if no amplification products are obtained, DNA extracted was not of high enough quality to be amplified and DNA extraction and PCR should be repeated. Without the addition of the IC this last sample would be considered wrongly brown rot negative.

The detection protocol presented here, which combined a set of universal primers and the inclusion of an IC for diagnosis of brown rot and three set of primers to discriminate the most important species of *Monilinia,* could be a useful and valuable tool for epidemiological studies and can be used in programmes to avoid the spread and introduction of this serious disease in new areas.

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