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Development and validation of a fast PCR-based detection method for pathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*

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Abstract

Based on the ITS regions of the ribosomal DNA, specific primer sets were developed for the citrus pathogen *Guignardia citricarpa* and the common citrus endophyte, *G. mangiferae*, and tested for their specificity against 37 isolates of *G. citricarpa*, 29 isolates of *G. mangiferae*, 10 isolates of related species and other fungi found on citrus. The efficacy of the PCR-detection method for *G. citricarpa* was approximately 60–70% for lesions without pycnidia, and approximately 90% for lesions with pycnidia. A reliability of 99% can be reached by analysing multiple lesions per sample. An internal control was developed to monitor DNA samples for PCR inhibition; samples with PCR inhibition should be re-examined. Detection by PCR is more rapid than the current five-day incubation method prescribed by the European Union for diagnosis of black spot lesions lacking the diagnostic pycnidia. The latter method had an efficacy of 40–50%, while culturing of suspected lesions had an efficacy of 10%. Species-specific primers and ITS sequence data showed that *G. citricarpa* can occur as a symptomless endophyte in leaves. This shows that wild and cultivated plants occurring in citrus groves are potential carriers of this quarantine fungus. Application of the presently developed PCR method for the detection of *G. citricarpa* will enable citrus producing as well as importing countries to prevent further spread of this harmful organism.

Introduction

Citrus black spot, caused by *Guignardia citricarpa* Kiely, is a foliage and fruit disease of citrus, orange, mandarin, lemon, and grapefruit (Kiely, 1948; Snowdon, 1990). Affected fruits become unsightly and unsuitable for the fresh fruit market. Premature fruit drop may occur. In areas with a warm and humid climate, losses may be substantial and intensive chemical control is required (Smith et al., 1997; Baayen et al., 2002). The fungus occurs in many areas including Asia, Australia, Southern America and Southern Africa. It does not occur in the European Union (EU) or in the United States of America (USA), where it is considered a quarantine organism (Smith et al., 1997). The phytosanitary regulations of the EU restrict the import of citrus fruits, with the aim to prevent introduction (entry and establishment) of the pathogen in the Mediterranean citrus-producing regions (European Union, 2000). Fruit imports from infected countries into the EU are not entirely forbidden. According to international standards (IPPC, 1999), phytosanitary measures should not interfere unduly with international trade. Therefore, citrus fruits may be imported from infected places of production, provided that orchards are adequately treated against black spot and that all fruits harvested from the orchards involved have been found free of symptoms by the national phytosanitary authorities at harvest (European Union, 2000). During import into the EU, fresh citrus fruit consignments are re-inspected by the phytosanitary services of the importing member states, so as to

verify that the consignments meet the requirements of the EU. Citrus fruit consignments with black spot symptoms are refused.

Citrus black spot symptoms are variable in appearance and may closely resemble the symptoms caused by other citrus pathogens (Schüepp, 1961; Smith et al., 1997; Kotzé, 2000). Typical black spot symptoms are the so-called hard spot lesions: shallow lesions with a small central grey to tan crater usually with a dark brown rim, 3–10 mm in diameter. This symptom usually appears after the fruit has started to turn orange or yellow. Often, but not always, pycnidia can be seen inside the spots as tiny, slightly elevated black dots. Hard spots with pycnidia of *G. citricarpa* can be diagnosed by microscopic examination of the pycnidia for the conidia of the anamorph, *Phyllosticta citricarpa* (Sutton and Waterston, 1966; Van der Aa, 1973). Hard spots may coalesce to form virulent spots that turn brown to black, develop a leathery texture and eventually cover large parts of the fruit. In such spreading lesions, pycnidia can usually also be found. This is not the case, however, for freckle spot and so-called false melanose. This type of black spot consist of small (1–3 mm diameter), slightly depressed spots that may be tan, grey, reddish, brownish, or not discoloured at all, and generally are devoid of pycnidia. Freckle spots often occur as satellite spots around hard spot lesions, while in false melanose the fruit is covered by such tiny spots in the absence of other symptoms. In contrast to hard spot and virulent spot, freckle spot and false melanose are not very distinctive and can be easily confused with symptoms of other *Citrus* diseases such as true melanose (caused by *Diaporthe citri*), greasy spot (caused by *Mycosphaerella citri*), Alternaria spot (caused by *Alternaria citri*), or lesions caused by *Colletotrichum* spp. (Holliday, 1980; Snowdon, 1990). To a lesser extent this also holds for hard spots and virulent spots devoid of conidia. The EU currently requires that fruits with such symptoms be tested for the presence of *G. citricarpa* by removing the lesions from the peel and incubating them for five days under conditions conducive to pycnidium formation (Brodrick and Rabie, 1970). Fruits where none of the lesions produce pycnidia are considered free of the pathogen. This method is easy but time-consuming (the value of consignments may severely drop in five days), and no data are available on its effectiveness.

The present study was undertaken to develop a fast, sensitive and specific PCR-based detection method for the presence of *G. citricarpa* in black-spot-type lesions devoid of pycnidia. The phytosanitary regulations of the EU given in directive 2000/29/EC (European Union, 2000) are restricted to pathogenic strains of *G. citricarpa*. In terms of specificity, the method should therefore distinguish between pathogenic and nonpathogenic strains. Nonpathogenic strains of *G. citricarpa* can be isolated from symptomless *Citrus* plants and fruits (Chiu, 1955; McOnie, 1964). McOnie (1964) suggested that these strains belong to a distinct *Guignardia* species (or perhaps variety or form), but others have considered it as a saprophytic form (Sutton and Waterston, 1966) or avirulent strain of *G. citricarpa* (Kotzé, 2000). Baayen et al. (2002) have shown that such strains belong to a distinct species, *G. mangiferae* (*Phyllosticts capitalensis*), a common endophyte in many plant families and coincidentally also occurring on *Citrus* species. *G. mangiferae* can be distinguished from *G. citricarpa* with difficulty after 10–14 days by cultural and morphological characters. ITS sequence and AFLP polymorphism analyses have shown that *G. citricarpa* and *G. mangiferae* are only distantly related in terms of phylogeny (Baayen et al., 2002). Differences in ITS sequence between *G. citricarpa* and *G. mangiferae* were therefore considered adequate for the development of PCR primers.

Many other PCR-detection methods for fungi have similarly been based on ITS sequences (Böhm et al., 1999; Bonants et al., 1997; Lacourt et al., 1997; Niepold and Schober-Butin, 1997; Ristaino et al., 1998; Schubert et al., 1999). In some cases, a nested PCR-approach was followed so as to increase sensitivity (Bonants et al., 1997; Lacourt et al., 1997). In order to eliminate false negatives due to PCR-inhibition by polyphenolics, humic acids and other ingredients present in the isolated DNA sample (De Boer et al., 1995; Jobes et al., 1995), an internal control was developed containing primer sequences specific for *G. citricarpa*. Development of an internal control has been proven to work efficiently to monitor for PCR inhibition (Courtney et al., 1999). The PCR method for detecting *G. citricarpa* in citrus fruit peel was validated on fruit samples from import inspection, and compared to the incubation method currently used in the EU, and classical isolation of the fungus by culturing on appropriate media.

Materials and methods

Fungal and plant material

Isolates of *G. citricarpa*, *G. mangiferae*, other *Guignardia* and *Phyllosticta* species, and reference

cultures of *A. alternata*, *D. citri*, *Colletotrichum* sp., and *Penicillium* sp. used in this study are listed in Table 1. Apart from cultures from the collections of the authors, cultures were used that were generously supplied by Tian Schutte, Outspan Citrus Centre, Nelspruit, South Africa, and Huub van der Aa and Gerard Verkleij, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Orange (*Citrus sinensis*) and lemon (*C. limon*) fruits with black spot symptoms were collected during import inspection in The Netherlands from 1999 to 2001 and dissected for collecting supposed black spot lesions. Dissected lesions were stored at −20 °C, or used fresh. Additional oranges with black spot symptoms intercepted at Antwerpen, Belgium, were generously supplied by Caroline Crepel and Sven Inghelbrecht, Centrum voor Landbouwkundig Onderzoek, Departement Gewasbescherming, Merelbeke, Belgium. Prior to use, all supposed black spot lesions were examined with a stereo microscope for the presence or absence of pycnidia. No further checks were carried out to avoid confusion with other fungi (e.g. acervuli of *Colletotrichum* spp.), because this can only be done in a destructive manner, which would have affected the validation tests.

Culturing method

Supposed black spot lesions were cleaned and disinfected with a tissue with 70% ethanol. Lesions were aseptically cut out from the peel, removing as much of the underlying pith as possible, divided into four pieces, placed on cherry decoction agar (Gams et al., 1988), and incubated in the dark at 22 °C for 14 days, after which the plates were checked for outgrowth of *G. citricarpa* cultures.

Incubation method (current EU protocol)

Supposed black spot lesions devoid of pycnidia were cleaned and disinfected with a tissue with 70% ethanol. Lesions were placed in a Petri dish with a sterile filter paper moistened with water, kept in constant light at 27 °C for five days, and checked for the presence of newly formed pycnidia. In some experiments, lesions were cut into two halves, of which one was used for PCR and the other for incubation. In other experiments, whole lesions were incubated.

Development of specific PCR primers

ITS sequences obtained in a previous study (Baayen et al., 2002) were aligned with MegAlign Software (DNA Star Inc., Madison, WI). A series of species-specific primers was developed, based on sequence differences between *G. citricarpa* and *G. mangiferae*. The robustness and specificity of various primer combinations were evaluated using DNA from 37 isolates of *G. citricarpa*, 29 isolates of *G. mangiferae*, and 10 isolates of reference species belonging to the genera *Guignardia* and *Phyllosticta* (Table 1). Specificity was also tested against cultures of the common citrus pathogens *A. alternata*, *Colletotrichum acutatum*, *C. gloeosporioides*, *D. citri* and *Penicillium* sp., and against the peel of oranges and lemons. DNA extraction, amplification and sequencing of these isolates were performed as described by Baayen et al. (2002). The primer pair selected for *G. citricarpa* was GCF3: 5′-AAAAAGCCGCCCGACCTACCT-3′ and GCR7: 5′-TGTCCGGCGGCCAG-3′, resulting in a 490 bp amplicon. The primer pair selected for *G. mangiferae* was GCF2: 5′-TAACTTCTATTGAAA GGTTCCAGAGT-3′ and GCR4: 5′-TCAGGACTT-CACAAAATGAATTCTT-3′, resulting in a 210 bp amplicon. The citrus black spot pathogen primer(s) and their use in diagnostic assays are the subject of an international patent application by the University of Oregon.

DNA extraction and sequencing

DNA from fungal cultures was extracted and sequences were obtained using protocols described in Baayen et al. (2002). To isolate DNA from lesions, lesions were dissected from the peel, removing as much of the surrounding pith and peel tissue as possible. Dissected lesions were grinded in an 1.5 ml Eppendorf vial with sand in 300 µl cell lysis solution (Puregene kit, Gentra/Biozym, Landgraaf, the Netherlands), after which 1.5 µl proteinase K (20 mg/ml) was added, the mixture incubated for 1.5 h at 55 °C, and then cooled down to room temperature. After adding 100 µl protein precipitation solution (Puregene kit, Gentra/Biozym), the mixture was vortexed for 20 s, kept on ice for 5 min, centrifuged at 14,000 rpm for 3 min, and the supernatant transferred to a clean Eppendorf vial, mixed with 300 µl isopropanol, and centrifuged at 14,000 rpm for 5 min to precipitate nucleic acids. The pellet was washed with 70% ethanol, dried, taken up in 20 µl TE buffer (10 mM Tris–HCl, 1 mM EDTA,

Table 1. Isolates of the citrus pathogen *G. citricarpa*, the citrus endophyte *G. mangiferae*, *Guignardia* and *Phyllosticta* species from other host species, and other common citrus pathogens used in the specificity tests for PCR-primer set GCF3/GCR7. Isolates were classified as *G. citricarpa* or *G. mangiferae* according to a previous morphological and molecular study (Baayen et al., 2002)

Taxon	Number of isolates tested	Host	Origin	Size of amplicon generated with primer set GCF3/GCR7	Size of amplicon generated with primer set GCF2/GCR4
Alternaria alternata	1	*Citrus paradisi*	Florida	None	None
Alternaria alternata	2	*Citrus reticulata*	Unknown	None	None
Colletotrichum acutatum	1	*Capsicum* sp.	Indonesia	None	None
Colletotrichum acutatum	1	*Fragaria ananassa*	The Netherlands	None	None
Colletotrichum gloeosporioides	4	*Citrus paradisi*	Florida	None	None
Colletotrichum gloeosporioides	1	*Citrus reticulata*	Unknown	None	None
Colletotrichum gloeosporioides	1	*Malus sylvestris*	The Netherlands	None	None
Diaporthe citri	1	*Citrus sinensis*	Surinam	None	None
Diaporthe citri	1	*Citrus* sp.	Surinam	None	None
Guignardia aesculi	1	*Aesculus hippocastanum*	Germany	±400	None
Guignardia bidwellii	1	*Parthenocissus tricuspidatus*	Unknown	±1000	None
Guignardia citricarpa	2	*Citrus aurantium*	Brazil/India	490	None
	1	*Citrus limettioides*	South Africa	490	None
	1	*Citrus limon*	Argentina	490	None
	2	*Citrus limon*	South Africa	490	None
	1	*Citrus limon*	Brazil	490	None
	2	*Citrus reticulata*	South Africa	490	None
	5	*Citrus sinensis*	Brazil	490	None
	19	*Citrus sinensis*	South Africa	490	None
	2	*Citrus* sp.	Brazil	490	None
	1	*Citrus* sp.	Australia (ex China)	490	None
	1	*Sapotaceae*	Puerto Rico	[2]	None
Guignardia mangiferae	1	*Allophylus africanus*	South Africa	None	210
	1	*Artocarpus* sp.	Thailand	None	210
	1[1]	*Camellia japonica*	USA	None	210
	1	*Citrus aurantiifolia*	Brunei	None	210
	1	*Citrus jambhiri*	Australia	None	210
	2	*Citrus limon*	South Africa	None	210
	2	*Citrus limon*	Argentina	None	210
	1	*Citrus limon*	Taiwan	None	210
	9	*Citrus paradisi*	Florida	None	210
	2	*Citrus reticulata*	Hong Kong	None	210
	1	*Citrus sinensis*	Brazil	None	210
	1	*Citrus* sp.	Japan	None	210
	1	*Encephalartos ferox*	South Africa	None	210
	1	*Mangifera indica*	Ghana	None	210
	1	*Musa* sp.	Australia	None	210
	1	*Paphiopedilum callosum*	Germany	None	210
	1	*Viscum obscurum*	South Africa	None	210
	1	*Zamia integrifolia*	Florida	None	210
Guignardia philoprina	1	*Ilex aquifolium*	Germany	None	None
Guignardia vaccinii	1	*Oxycoccus macrocarpos*	Unknown	None	None
Penicillium sp.	1	*Citrus reticulata*	Unknown	None	None
Penicillium sp.	3	*Citrus sinensis*	South Africa	None	None
Phyllosticta beaumarisii	1	*Muehlenbeckia adpressa*	Australia	None	None
Phyllosticta eugeniae	1	*Eugenia aromatica*	Indonesia	None	220
Phyllosticta hypoglossi	1	*Ruscus aculeatus*	Italy	None	None
Phyllosticta spinarum	1	*Hedera helix*	Italy	None	None
	1	Unknown	Japan	None	None
Phyllosticta telopeae	1	*Telopea speciosissima*	Australia	None	None

[1]Received as *G. camelliae*.
[2]Detection based on species-specific primers derived from other genomic regions than the ITS region of the ribosomal DNA gene.

pH 8.0), and 1 µl RNAse (4 mg/ml) was added, after which the mixture was incubated for 30 min at 37 °C. The volume was made up to 50 µl with TE buffer, and purified over a spin column (BioRad, Veenendaal, the Netherlands) filled with PVPP (Sigma, Zwijndrecht, the Netherlands).

PCR amplification

The reaction mixture contained 5 µl DNA suspension; 2.5 µl of 10×-concentrated reaction buffer containing 1.5 mM $MgCl_2$; 2.5 µl 600 µM dNTPs; 0.25 µl of each primer at 60 µM; 0.2 µl Taq DNA polymerase (5 U/µl; Roche, Mannheim, Germany); 0.25 µl internal control, and was filled up with MilliQ water to a final volume of 25 µl. Amplification was performed in thin-walled PCR tubes in a DNA thermal cycler (PTC200; MJ Research, Watertown, MA, USA) with heated lid programmed as follows: 1 cycle of 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 65 °C, and 60 s at 72 °C. One cycle of 10 min at 72 °C was conducted after the 30 cycles. After amplification, 5 µl of the reaction mixture was loaded onto a 1.0% agarose gel in 0.5× TBE buffer, separated by electrophoresis, stained with ethidium bromide, and viewed and photographed under UV light. A negative control (no DNA target) was included in every experiment to test for contamination, as well as a positive control (DNA from a reference strain of the pathogen). The positive control, but not the negative control, was to yield an amplicon of 490 bp with primer pair GCF3-GCR7. Isolates yielding an amplicon of this size were identified as *G. citricarpa*. Isolates yielding an amplicon of 210 bp with primer pair GCF2-GCR4 were identified as *G. mangiferae*.

Development of internal control

An internal control was developed to monitor DNA samples for PCR-inhibition during tests for *G. citricarpa*. Heterologous DNA from ryegrass was amplified with the primers specific for *G. citricarpa* (GCF3 and GCR7) at a lower annealing of temperature 55 °C. PCR products were cloned into a pGEM-T vector (Promega, Madison, WI, USA), and white colonies were selected with a suitable insert. A clone with an insert of 230 bp was selected to serve as internal control.

Validation

The value of the PCR diagnosis was assessed in a series of trials with lesions from oranges and lemons from intercepted consignments known to be infected with *G. citricarpa*.

In a first experiment, 40 lesions with tiny black structures, supposed to represent pycnidia, were each divided in two equal parts, when possible with equal numbers of pycnidia for each half. One of the halves was used for culturing the fungus on cherry decoction agar; the other half was subjected to the PCR method described above. *Guignardia* cultures obtained on cherry decoction agar were identified as *G. citricarpa* by PCR.

In a second experiment, 40 black-spot-like lesions without pycnidium-like black structures were similarly cut into two halves, but the first halves of the lesions were subjected to the EU incubation protocol instead of culturing them on cherry decoction agar. The other halves were again subjected to the PCR method.

In a third experiment, with smaller lesions that could not safely be cut into two halves and that were devoid of pycnidium-like structures, 20 lesions were cultured on cherry decoction agar, 20 lesions were incubated according to the EU protocol, and 20 lesions were subjected to the PCR method. *Guignardia* cultures obtained on cherry decoction agar were identified as *G. citricarpa* by PCR.

In a fourth experiment, 25 lesions without pycnidia were each divided in two halves, of which the first half was incubated according to the EU protocol, and the other tested with the PCR method. After five days incubation, the first halves were tested for the presence of *G. citricarpa* and *G. mangiferae* by PCR.

Results

Development of specific PCR primers

Based upon the ITS sequences of *G. citricarpa*, *G. mangiferae*, and related *Guignardia* and *Phyllosticta* species (Baayen et al., 2002), 4 forward and 8 reverse primers were developed for *G. citricarpa* and *G. mangiferae*, and 24 combinations of these were tested for their specificity against 37 isolates of *G. citricarpa*, 29 isolates of *G. mangiferae*, 10 isolates of related *Guignardia* and *Phyllosticta* species, and 17 isolates of other fungi present on citrus (*Alternaria alternata*, *Diaporthe citri*, *Colletotrichum* sp., *Penicillium* sp.). Primer set GCF3 + GCR7 was specific for *G. citricarpa* (amplicon size: 490 bp) and differentiated this species reliably from the common citrus endophyte, *G. mangiferae*, and other fungi

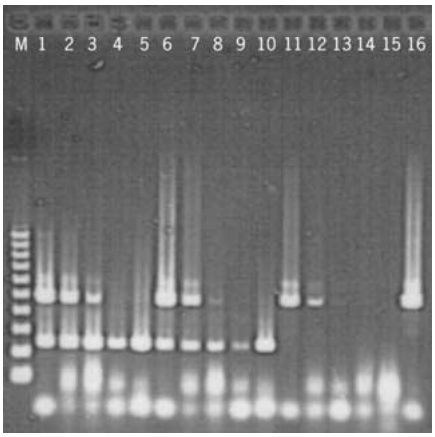

Figure 1. Amplification of a 490 bp product (upper band) from DNA of *G. citricarpa* and a 230 bp internal control amplicon (lower band) in PCR reactions with primers GCF3/GCR7. A dilution series of DNA of *G. citricarpa* (2 ng, 0.2 ng, 20 pg, 2 pg and 0 pg) was mixed with DNA of the internal control (lane 1–5: 5 pg; lane 6–10: 2 pg; lane 11–15: 0 pg). Lane 16: positive control; M: 100 bp markers.

occurring on citrus fruits, none of which gave a signal (Table 1). Primer set GCF3 + GCR7 also differentiated *G. citricarpa* from *G. philoprina*, *G. vaccinii*, *Phyllosticta beaumarisii*, *P. eugeniae*, *P. hypoglossi*, *P. spinarum*, and *P. telopeae*, none of which gave a signal either. However, amplicons were generated from *G. bidwellii* and *G. aesculi*, neither of which occurs on citrus. The amplicons generated with the latter two species differed in size from those found with *G. citricarpa* (fragments were sized approximately 1000 and approximately 400 bp, respectively). A dilution series of DNA showed that in a single PCR 20 pg DNA of *G. citricarpa* could be detected (Figure 1). DNA of *G. citricarpa* which was diluted 20-fold with DNA of *G. mangiferae* still resulted in a clear PCR signal. Primer set GCF2 + GCR4 was specific for *G. mangiferae* (amplicon size: 210 bp), except that an amplicon of 220 bp was generated with *P. eugeniae* (Table 1).

Internal control

An internal control was developed to monitor DNA samples for PCR inhibition. Under specific reaction conditions, amplification of this plasmid DNA resulted in a single band of 230 bp. Optimalisation studies showed that addition of 10 pg of this plasmid DNA to the PCR mix suffices well. PCR reactions were performed on a dilution series of DNA in presence and absence of the internal control (Figure 1). The sensitivity of detection of *G. citricarpa* was not influenced negatively by addition of 10 pg internal control plasmid DNA.

Testing on lesions

Primer set GCF3 + GCR7 was tested on DNA samples from individual lesions with and without pycnidia. Good results were obtained with DNA extracted from single lesions (Figure 2). Non-specific reaction products were observed in lane 7 and 8 besides the specific 490 bp band. Pooling of lesions, however, often resulted in inhibition of the amplification reaction (e.g. lane 11, 13, 14, 16 in Figure 3). DNA from *G. citricarpa*-positive lesions no longer produced a signal when pooled with DNA from other lesions. Inhibition was not observed when no more than 2 lesions were combined during DNA extraction (not shown). The internal control was also found to be reliable in PCR reactions using DNA samples from lesions (Figures 2 and 3).

Efficacy of PCR detection compared with culturing on media

PCR with primer set GCF3 + GCR7 generated a 490 bp amplicon for 34 out of 40 halves of lesions with pycnidia-like black structures (Table 2). Culturing of the corresponding halves of the 40 lesions produced 21 *G. citricarpa* cultures, of which the identity was verified by PCR with primer set GCF3 + GCR7. Often *Colletotrichum* sp. and incidentally *Alternaria* sp. and *Penicillium* sp. were isolated from the lesions, alone or in combination with *G. citricarpa*. The total number of lesions in which *G. citricarpa* was detected was 37; the remaining 3 lesions produced only *Colletotrichum* sp. cultures and the black pycnidia-like structures in these may have been acervuli of *Colletotrichum* sp. rather than pycnidia of *G. citricarpa*. Assuming that this was the case, the efficacy of the PCR-detection method was 92% and that of culturing 57%. Two out of the three cases where *G. citricarpa* had been cultured from the lesions but not detected by PCR were due to PCR inhibition, as shown by the absence of the internal control amplicon. Inhibition often occurred when large numbers of pycnidia were present in the lesions (not shown), suggesting that elements of the black

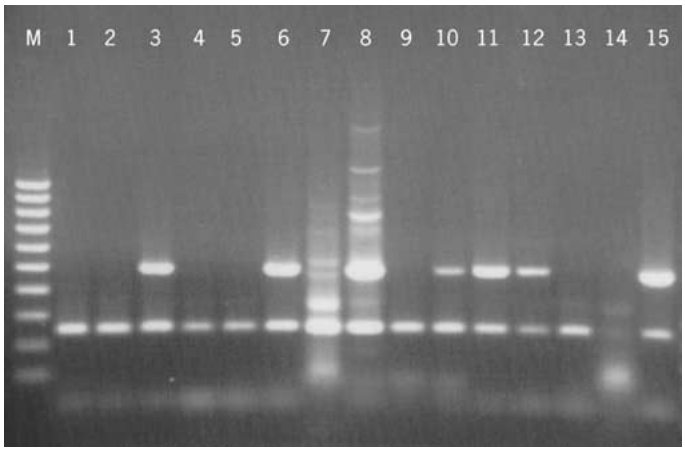

Figure 2. Amplification of a 490 bp product (upper band) and 230 bp internal control (lower band) with *G. citricarpa*-specific primer set GCF3/GCR7 in DNA from single lesions (lane 1–12). Lane 13: internal control; lane 14: negative control; lane 15: positive control; M: 100 bp markers.

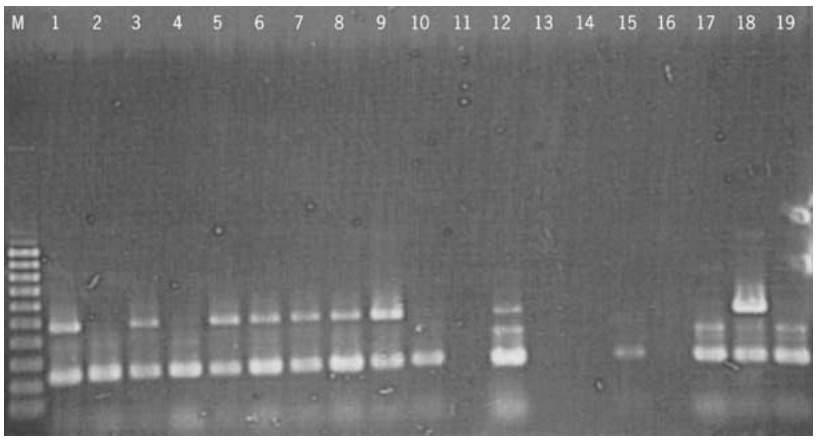

Figure 3. Amplification of a 490 bp product (upper band) and 230 bp internal control (lower band) with *G. citricarpa*-specific primer set GCF3/GCR7 in DNA from single lesions with pycnidia (lane 1–10) and similar lesions but pooled in groups of four (lane 11–17). Lane 18: positive control; lane 19: negative control; M: 100 bp marker.

pycnidial wall and surrounding host tissue (melanins, phenolics) are responsible for the inhibition. Extracts from healthy citrus peel did not adversely affect amplification (not shown).

Efficacy of PCR detection compared with incubation of lesions under continuous light

PCR with primer set GCF3 + GCR7 generated a 490 bp amplicon for 23 out of 40 halves of lesions without pycnidia (Table 3). Incubation of the corresponding halves of the 40 lesions at 27 °C for five days in continuous light according to the EU diagnosis protocol produced pycnidia from *G. citricarpa* cultures for 14 out of 40 lesions. Seven of those lesions were PCR positive and 7 PCR negative.

The total number of lesions in which *G. citricarpa* was detected was 30. Although the lesions resembled those of *G. citricarpa*, the remaining 10 negative lesions may have not been caused by *G. citricarpa* because other citrus fruit pathogens such as *Colletotrichum* sp. cause similar tiny spots. Assuming that only 30 lesions had carried *G. citricarpa*, the efficacy of PCR was 77% and that of incubation 47%.

Table 2. Distribution of PCR signals with primer set GCF3/GCR7 (amplicon: 490 bp) over lesion classes grouped according to the fungi that were isolated from them. All 40 lesions contained pycnidium-like black structures

Fungi isolated from lesion	Number of PCR-positive lesions	Number of PCR-negative lesions
G. citricarpa	13	2*
G. citricarpa, *Colletotrichum* sp.	4	0
G. citricarpa, *Penicillium* sp.	1	1*
Colletotrichum sp.	10	3**
Colletotrichum sp., *Alternaria* sp.	2	0
Colletotrichum sp., *Penicillium* sp.	1	0
Alternaria sp.	2	0
None	1	0
Total	34	6****

*Each asterisk designates one lesion with PCR inhibition.

Table 3. Distribution of PCR signals with primer set GCF3/GCR7 (amplicon: 490 bp) over lesion classes grouped according to the outcome of the incubation test for 40 lesions without pycnidia

Outcome of incubation test for lesions without pycnidia	Number of PCR-positive lesions	Number of PCR-negative lesions
Positive	7	7
Negative	16	10
Total	23	17

Comparison of PCR detection, incubation, and culturing for lesions without pycnidia

PCR with primer set GCF3 + GCR7 generated a 490 bp amplicon for 14 out of 20 tiny lesions without pycnidia (Table 4). Incubation of 20 of such lesions at 27 °C for five days in continuous light according to the EU diagnosis protocol resulted in 8 lesions producing pycnidia of *G. citricarpa*. Only 2 out of 20 of such lesions produced *G. citricarpa* cultures on cherry decoction agar. Because the lesions were tiny and could be analysed by only one of the three methods, it is not possible to estimate the total number of infected lesions. Assuming that all lesions had been produced by *G. citricarpa*, the efficacy of detection by PCR was 70%, that of the incubation method 40%, and that of culturing 10%. The real efficacy may have been slightly higher, depending on the actual infection percentage, which must have been in the range of 70–100%.

Table 4. Number of *G. citricarpa*-positive lesions out of 20 tested with PCR with primer set GCF3/GCR7, the EU incubation test for lesions without pycnidia, and culturing on cherry decoction agar, and the relative efficacy of these methods

Detection method	Number of positive lesions out of 20	Efficacy range
PCR test	14	70–100%
Incubation test	8	40–57%
Culturing	2	10–14%

PCR test for the presence of G. citricarpa *and* G. mangiferae *in lesions without pycnidia before and after incubation*

The possible interaction between endophytic *G. mangiferae* and pathogenic *G. citricarpa* in lesions without pycnidia was evaluated for oranges from a consignment that had been refused in Antwerpen harbour because of the presence of black spot lesions and pycnidia of *G. citricarpa*. Tiny black-spot-like lesions were collected for incubation and PCR. Five lesion halves produced pycnidia in the incubation test, and 20 lesions were negative of which four possibly contained blackish structures resembling fructification initials (pycnidia of *G. citricarpa*, acervuli of *Colletotrichum* sp., or others) (Table 5). The complementary halves of the five incubation-positive lesions also gave a positive PCR signal for *G. citricarpa*. After incubation, all five *G. citricarpa*-positive lesions and additionally one of the questionable ones gave a positive signal for *G. citricarpa*, bringing the total number of *G. citricarpa*-positive lesions in this particular lot at 6 out of 25. Three of the lesions producing pycnidia gave a signal for *G. mangiferae* additional to *G. citricarpa*. None of the other lesions gave a signal for *G. mangiferae*.

Application of PCR for the detection of G. citricarpa *in wild plant species*

In a survey of ITS sequences (ITS1-5.8S-ITS2) from about 150 endophytic *Guignardia* isolates from diverse

Table 5. Number of black-spot-like lesions (out of 25) that produced pycnidia in the EU incubation test for lesions without pycnidia, compared to the PCR test for *G. citricarpa* (primer set: GCF3/GCR7; before and after incubation) and *G. mangifera* (primer set: GCF2/GCR4)

Outcome of incubation test for lesions without pycnidia	PCR-positive lesions for *G. citricarpa*		PCR-positive lesions for *G. mangiferae*
	Before incubation	After incubation	
Positive ($n = 5$)	5	5	3
Questionable ($n = 4$)	0	1	0
Negative ($n = 16$)	0	0	0

hosts and localities, one isolate on non-Citrus hosts (Table 1) was discovered with ITS sequences identical to that of *G. citricarpa* (Baayen et al., 2002). This isolate came from leaves of an unidentified sapotaceous plant collected in Puerto Rico. Although DNA from this isolate was not tested with ITS species-specific primers, other species-specific primer pairs based on variable sequences of the external transcribed spacer (B. Wenleder and G.C. Carroll, unpublished) did give strong positive signals for *G. citricarpa*. Apparently, both *G. mangiferae* and *G. citricarpa* can colonize leaves of other host plants and persist there as endophytes. This is the first proven report of *G. citricarpa sensu stricto* in a host outside the Rutaceae.

Discussion

As long as black spot lesions carry the pycnidia of *G. citricarpa*, diagnosis is fast and easy. Unfortunately, in many instances black spot lesions are observed without any pycnidia. Especially when they are small, such lesions are very similar to those caused by *Alternaria* sp., *Colletotrichum* sp., and other fungi (Baayen et al., 2002) occurring on citrus. As a consequence, consignments with such symptoms must be held to check whether or not the symptoms in the consignment are those of *G. citricarpa*.

The classical method for diagnosis of lesions without pycnidia is culturing, which takes two weeks. The present study shows that the efficacy of culturing is extremely low, approximately 10%. Even in the case of lesions containing pycnidia of *G. citricarpa*, the recovery was no higher than approximately 50%.

Since July 2000, the EU has decided that fruits from consigments with symptoms of black spot but lacking the pycnidia should be subjected to a five-day incubation protocol. Our results show that the incubation method is moderately effective. In the second and third validation experiment, its efficacy was approximately 35–40%. In a fourth experiment, only 5 out of 25 lesions were positive for *G. citricarpa*; however, in this experiment only one more positive was found with PCR, indicating that the efficacy of the incubation method in this particular case may have been higher. This protocol is technically simple, the main problem with this method remaining the duration of the test.

The PCR-detection method described in this paper provides a fast (about two days) and reliable alternative to the culturing and incubation methods. The primer set GCF3/GCR7 is selective for *G. citricarpa*, and will not amplify endophytic *G. mangiferae* in the peel of citrus fruits, or other fungi (*A. alternata*, *D. citri*, *Colletotrichum* sp., *Penicillium* sp.) present in or on the fruit peel. Based on sequence comparison from two other fungi occurring on citrus (*M. citri* and *A. citri*), it was expected that they will not give positive PCR amplicons with mentioned primer set. Two distantly related *Guignardia* species (*G. aesculi*, *G. bidwellii*) give a signal, but neither species occurs on citrus and the amplicons produced with these species differ in size from that produced with *G. citricarpa*. Introduction of a molecular probe to verify the amplicon produced, would increase the value of the method even more. The efficacy of the PCR method was superior to the culturing and incubation methods, with recovery rates of approximately 60–70% for lesions without pycnidia, and approximately 90% for lesions with pycnidia.

PCR-negative samples, which were incubation positive, can be explained by uneven distribution of the fungus in suspected lesions. For a quarantine fungus, one would expect the pathogenic agent to be present homogeneously throughout all lesions. Unfortunately, repeated culturing studies in our laboratory have consistently shown that the fungus is not present inside lesions in such a manner. It seems that the fungus acts as primary source of damage but is rapidly followed by others, in particular the ubiquitous endophyte *C. gloeosporioides*, which then switches to pathogenic behaviour and becomes responsible for much of the actual 'black spot' damage on fruits. Pooling of more than two lesions for DNA extraction would then be an improvement, but often resulted in inhibition of the amplification response. Tenfold dilution of the material

prior to amplification resolved this in a number of cases. Inhibition only seemed to occur with pooled lesions with pycnidia, or single lesions with many (5–10) pycnidia. Our results therefore suggest that melanins, phenolics and other pycnidium-related compounds may be responsible for the inhibition phenomenon. Additional DNA clean up kits should be included to increase purity of the DNA sample. Introduction of an internal control in the PCR reaction enables us to monitor for PCR inhibition. For quarantine diagnosis, care should nevertheless be taken not to combine more than two lesions for extraction and processing, even though pycnidia will be absent from the tested material.

Application of a PCR-test method allowed *G. citricarpa* to be detected in leaves of a non-host plant, an unidentified member of the Sapotaceae. This unexpected findings shows that *G. citricarpa* can invade wild plant species in addition to commercial *Citrus* cultivars, as suggested by Kiely (1948). Kiely's original claim was refuted by McOnie (1964), who demonstrated that the fungus growing endophytically in native species (*G. mangiferae,* Baayen et al., 2002) was different from the pathogenic *G. citricarpa*. Our findings show that *G. citricarpa* can occasionally invade other host species and perhaps form a reservoir there. Whether the fungus can grow and sporulate on leaves of non-host plants is unknown, but the question may prove of considerable importance in enforcing quarantine regulations. The possibility that *G. citricarpa* may colonize leaves of non-host plants on a wide scale calls for a thorough survey of the endophytic mycoflora of non-host plants growing in and near Citrus orchards. Such a survey will be greatly facilitated through the use of PCR testing with species-specific primers.

Our results show that *Colletotrichum* sp. is associated with symptoms that are similar to those of *G. citricarpa*. *C. gloeosporioides* is a common endophyte of citrus fruit that may cause postharvest anthracnose; *C. acutatum* causes flower petal infection, followed by postbloom fruit drop, and so-called key lime anthracnose (Brown et al., 1996; Timmer et al., 1998). Specific PCR-primers for *Colletotrichum* sp. could help in correct diagnosis. *C. gloeosporioides* cultures are commonly found when the peel of healthy oranges is cultured on agar media. The apparent impossibility to distinguish small *G. citricarpa* lesions on citrus fruits from those produced by a so common fungus as *C. gloeosporioides* is troublesome. It necessitates the systematic inspection and diagnosis of citrus fruit imports from countries where *G. citricarpa* has become established. The same does apparently not hold for *G. mangiferae*. The latter fungus has not been associated with typical black spot, but is considered a harmless endophyte of citrus leaves and fruits. The present study identified *G. mangiferae* as an incidental member of the mycoflora of lesions, along with *Colletotrichum* sp., *Alternaria* sp., and *Penicillium* sp. The single three cases where *G. mangiferae* was detected were lesions that also carried pycnidia of *G. citricarpa*. The reason that *G. mangiferae*, and possibly also other endophytic or saprophytic fungi, colonize such lesions may be the ample availability of nutrients. Given the slow growth of *G. citricarpa*, the presence of this quarantine fungus may be easily overlooked. Since mixed infection can occur, direct amplification from mycelium without isolating DNA is a fatally flawed procedure for quarantine purposes and therefore direct isolation of DNA from citrus peels is much more preferred.

Application of the presently developed PCR method for the detection of *G. citricarpa* will enable citrus producing as well as importing countries to prevent further spread of this harmful organism.

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