A One-Day Sensitive Method to Detect and Distinguish Between the Citrus Black Spot Pathogen *Guignardia citricarpa* and the Endophyte *Guignardia mangiferae*

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ABSTRACT

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If South African citrus exporters wish to retain their competitive edge in the European market and access new markets such as the United States of America, it is of quarantine importance to distinguish between the citrus black spot pathogen, *Guignardia citricarpa*, and the harmless endophyte, *G. mangiferae*. The endophyte is not a sanitary or phytosanitary concern. This paper describes the design of species-specific primers that are able to detect and distinguish between these two *Guignardia* species. Application of the primer set CITRIC1 and CAMEL2 in conjunction with the ITS4 primer yielded polymerase chain reaction (PCR) amplicons of approximately 580 bp and 430 bp for *G. citricarpa* and *G. mangiferae*, respectively. Results obtained with these primers are in accordance with sequence data, and repeated tests verified accuracy and sensitivity. A BLAST search revealed no matches other than *G. citricarpa* and *G. mangiferae*, and no positive PCR results were obtained with *Colletotrichum gloeosporioides*, which is the most common contaminant in black spot lesions. We are, therefore, able to distinguish *G. citricarpa* and *G. mangiferae* unequivocally using a PCR-based method. This method was further improved to directly isolate DNA from fruit lesions by means of the DNeasy Plant Mini Kit (Qiagen). This eliminates the prior need for culturing the slow-growing organism, thereby shortening the time required to one day to test for and verify the presence or absence of the pathogenic *G. citricarpa* in export consignments.

Additional keywords: DNA extraction, *Phyllosticta*

Increased global trade in fresh fruit has resulted in setting minimum standards and harmonization of legislation dealing with import requirements and specifications. Lack of acceptance of test data for these specifications across national borders has been identified as a technical barrier to trade (8). The World Trade Organization has subsequently adopted two new agreements to ensure that technical requirements do not restrict trade viz. the agreement on Technical Barriers to Trade and the agreement on Sanitary and Phytosanitary measures (8). Despite this, restrictions on citrus consignments to the European Union (EU) and the United States of America (USA) threaten continued exports from South Africa (2-4,13).

The fungus *Guignardia citricarpa* Kiely (anamorph *Phyllosticta citricarpa* (McAlp.) Van der Aa) causes citrus black spot (CBS), a disease subject to phytosanitary restrictions (3,19,20,40). Preharvest CBS lesions resulting from field infections af-

fect the appearance of the fruit and thus significantly reduce potential export revenue (19). In addition, latent infections that are not associated with symptoms at the time of harvesting can also develop on fruit in transit during export (10,18,20).

For many years, a morphologically similar *Guignardia* sp. on citrus was often incorrectly identified as the citrus pathogen causing CBS (17,26,40). Meyer et al. (28) proved the existence of two distinct species of *Guignardia* on citrus by means of sequence analysis of the internal transcribed spacer (ITS) regions of the rRNA operon. Baayen et al. (6) subsequently designated this harmless endophyte as *Guignardia mangiferae* A.J. Roy (anamorph *Phyllosticta capitalensis* P. Hennings). *G. citricarpa* causes CBS and *G. mangiferae* does not cause symptoms, although it has been isolated from symptomatic and asymptomatic citrus fruit rind and leaves (6,28). *G. mangiferae* tends to grow faster in culture than the CBS pathogen. It may also produce perithecia, but in general, morphology is insufficient to distinguish these congeners of *Guignardia* (26,27,37,38).

Timeliness and accuracy of pathogen identification are crucial and cultivation of the slow-growing *G. citricarpa* is undesirable in time-critical diagnosis of export consignments. Appropriate quarantine de-

cisions, affecting exports, were previously only made after carefully studying cultures made from the suspect fruit. Since the differentiation between *G. citricarpa* and *G. mangiferae* was based on morphological features alone, the endophyte was often incorrectly identified as *G. citricarpa*. Isolation, culturing, and examination of isolates takes anything from 5 to 14 days, with an efficacy of less than 50% (4,5,14). In many instances, where exporters screen citrus consignments for pathogen-free validation, the fruit is kept at the harbor for extended periods of time. The value of the consignment decreases rapidly with each additional day spent in holding. The limitations of the cultivation of slow-growing microorganisms can be sidestepped with the culture-independent polymerase chain reaction (PCR) process, which has previously been applied successfully in various natural environments (7,12,22,29,30,33,36).

A direct PCR test on fruit lesions was developed at the University of Oregon (US) and Plant Research International, Wageningen (NL) for the detection of *G. citricarpa* (9). Even though this method can be done in 2 days, a same-day test is still required for South African fruit exports, which are often rejected at the harbor due to the presence of a single fruit spot suspected of being CBS. In addition, Bonants et al. (9) did not provide any evidence of successfully amplifying *G. mangiferae*. The endophyte *G. mangiferae* can, however, be present in the peel prior to lesion formation by other pathogens, such as pathogenic *Colletotrichum* species, ever-present in the peel (6,15). Minute spots on grapefruit, lemon, and lime fruits often produce cultures of *G. mangiferae* (6,28). It is therefore necessary to accurately distinguish between the two *Guignardia* species in preference of identifying *G. citricarpa* alone.

The aim of this study was to develop a same-day test to unequivocally identify the citrus black spot pathogen and distinguish it from the omnipresent endophyte. This involved the isolation of DNA from fruit lesions and the design of a primer set based on ITS sequences, which would distinguish the two species of *Guignardia*.

MATERIALS AND METHODS

Fungal isolates. A set of 23 *Guignardia* isolates was selected for this study. These isolates were from the major citrus-producing areas in South Africa, from

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different cultivars (lemons, sweet oranges, grapefruit, tangerine [*C. reticulata* Blanco], and kumquat [*Fortunella* Swingle]), different lesion types, and alternative hosts, i.e., avocado (*Persea americana* Mill.), mango (*Mangifera indica* L.), banana (*Musa acuminata* Colla), and cabbage tree (*Cussonia* Thunb.) plants (Table 1). The fungal isolates were obtained by direct isolation from host tissue onto potato dextrose agar (PDA) (Biolab, Merck, South Africa) supplemented with 250 mg of chloramphenicol (The Premier Pharmaceutical Co., SA). Fruit were surface-sterilized for 2 min using 1.5% NaOCl and rinsed thoroughly. A thin layer of the fruit surface surrounding the lesion was removed using a scalpel, and the exposed tissue was cut into 2 × 2 mm squares and plated onto PDA plates. An ex-holotype culture of the species, *Phyllosticta citricarpa* from the Centraalbureau of Schimmelcultures (CBS111.20), was used as a comparative standard in all tests. Isolate GC172 was used throughout this study as standard for the newly named *G. mangiferae*. DNA of these isolates was extracted, the ITS regions amplified, and sequencing reactions were carried out and data processed as described previously by Meyer et al. (28).

Plant material. Asymptomatic and symptomatic lemon (cv. Eureka), oranges (cv. Valencia, Midnight, and Navel), and grapefruit (cv. Marsh) with symptoms including typical and atypical hard spot lesions, false melanose, postharvest red spots, freckle spot, and virulent spot were collected from Sandvlei, Letaba, Karino, Nelspruit, Marble Hall, Hermitage, Croc Valley Estates, Westfalia, and Burgersfort (all in South Africa) as well as fruit from Sao Paulo (Brazil) and Chiredzi (Zimbabwe) (Table 2).

DNA extractions. DNA of all the isolates mentioned in Table 1 was extracted from freeze-dried mycelium using the technique described by Raeder and Broda (34). The DNeasy Plant Mini Kit (Qiagen, Valencia, CA) was evaluated for rapid production of sufficient DNA of a suitable quality for PCR from fruit lesions (different types and sizes), fungal cultures, and freeze-dried mycelium (Table 2). Fungal growth was removed from 10- to 14-day-old cultures on PDA up to 100 mg wet weight. Freeze-dried fungal mycelium was ground to a powder, and 20 to 30 mg was used per DNA extraction. Fruits were surface-disinfested with 70% (vol/vol) ethanol and left to air dry, and lesions were carefully dissected from fruits. Half of the lesion was plated on PDA and the other half was ground to a fine powder in liquid nitrogen, and extractions were performed using the DNeasy Plant Mini Kit according to the manufacturer's instructions. The elution volume was reduced to 1 × 75 µl. Successful extractions were confirmed using a 1.0% (wt/vol) agarose gel in TBE buffer (23).

Primer design and PCR conditions for detection of *G. citricarpa* and *G. mangiferae*. The design of oligonucleotide primers CITRIC1 (5'-GAA AGG TGA TGG AAG GGA G-3') and CAMEL2 (5'-AGT ATA CAA AAC TCA AGA ATT C-3') was based on the DNA sequences of previously sequenced ITS regions (28). For detection and differentiation of *G. citricarpa* and *G. mangiferae*, these two primers were used together with the ITS4 primer (42). The primers were synthesized by MWG Biotech (Germany). PCR reactions were performed in 50-µl volumes, each reaction containing 1 µl of template DNA, primers, i.e., CITRIC1

(10 pmol), CAMEL2 (60 pmol), and ITS4 (15 pmol), 5 µl of recommended 10× buffer (supplied with *Taq* polymerase), 200 µM of each dCTP, dGTP, dATP, and dTTP (TaKaRa), and 0.5 U *Taq* polymerase (TaKaRa). Following an initial denaturation step of 95°C for 2 min, 35 PCR cycles were performed on a Perkin-Elmer 2400 thermocycler using the following conditions: a denaturation step of 93°C for 30 s followed by annealing at 56°C for 45 s and extension at 72°C for 90 s, followed by a final extension of 72°C for 7 min. The amplified DNA fragments were visualized on a 1.25% (wt/vol) agarose gel in TBE buffer (23). A purified DNA extract from an avocado isolate of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. and water were included as negative controls.

Evaluation of primer sets. In order to confirm the specificity of the primers, they were analyzed using the BLAST program (1) (National Centre for Biotechnology Information). Some of the previously sequenced isolates (28) were used to evaluate the efficacy of the primer sets (Table 1). Other isolates, including *Guignardia* isolates of unknown identity and direct DNA isolations from fruit, were included in the evaluation process (Table 2). All tests were repeated five times to ensure consistency of results. In some instances, DNA originating from the same lesion, either directly isolated from the fruit lesion or first cultured for 5 days on PDA, were used. PCR analysis was conducted on all these isolates as described previously.

RESULTS

Specificity of PCR amplification. Results of PCR analyses are shown in Table 2. PCR amplification using these primer

Table 1. *Guignardia citricarpa* and *G. mangiferae* isolates used for selection and validation of the CITRIC1 and CAMEL2 primers

Species name	Isolate code	Origin ^a	Host cultivar	Lesion type	Accession no. ^b
<i>G. citricarpa</i>	GC62	Letsitele, MP	<i>Citrus sinensis</i> cv. Valencia	Red margin	AF346779
	GC63	Letsitele, MP	<i>C. sinensis</i> cv. Valencia	Freckled spot	AF346780
	GC87	Nelspruit, MP	<i>C. sinensis</i> cv. Valencia	False melanose	AF374371
	GC25	Hoedspruit, MP	<i>C. sinensis</i> cv. Valencia	Hard spot	AF374365
	GC31	Swaziland	<i>C. paradisi</i> cv. Marsh	Hard spot	AF374366
	GC154	Nkweleni, KZN	<i>C. paradisi</i> cv. Marsh	Red margin	AF346775
	GC149	Malelane, MP	<i>C. paradisi</i> cv. Star Ruby	Hard spot	AF346774
	GC4	Zebediela, MP	<i>C. limon</i> cv. Eureka	Hard spot	AF346778
	GC51	Croc Valley, MP	<i>C. limon</i> cv. Eureka	Virulent spot	AF346773
	GC66	Karino, MP	<i>C. limon</i> cv. Eureka	Hard spot	AF374367
	GC80	Croc Valley, MP	<i>C. limon</i> cv. Eureka	Hard spot	AF374370
	GC45	Alkmaar, MP	<i>C. reticulata</i> cv. Nova	Hard spot	AF346776
	CBS111.20	NSW, Australia	<i>Citrus</i> sp. Ex holotype	...	AF346772
	GC117	Nkweleni, KZN	<i>C. paradisi</i> cv. Marsh	No visible symptoms	AF346766
	GC119	Nkweleni, KZN	<i>C. paradisi</i> cv. Marsh	Red margin	AF374356
	GC146	Malelane, MP	<i>C. paradisi</i> cv. Star Ruby	No visible symptoms	AF346767
	GC41	Patensie, Scape	<i>C. limon</i> cv. Eureka	No visible symptoms	AF346764
<i>G. mangiferae</i>	GC10	Letaba, MP	<i>Fortunella</i> sp.	No visible symptoms	AF374362
	GC6	Pretoria, GP	<i>Cussonia</i> sp.	...	AF346768
	GC172	Letaba, MP	<i>Mangifera indica</i>	...	AF346769
	GC5	Nelspruit, MP	<i>Musa acuminata</i>	...	AF346771
	GC14	Port Edward, KZN	<i>M. acuminata</i>	...	AF374363
	GC18	Letaba, MP	<i>Persea americana</i>	...	AF346765

^a Provinces in South Africa: EC = Eastern Cape, GP = Gauteng Province, KZN = KwaZulu Natal, MP = Mpumalanga Province, S Cape = Southern Cape, part of the Western Cape Province.

^b GenBank accession numbers.

sets showed a clear distinction between *G. citricarpa*, with a PCR amplicon of approximately 580 bp, and *G. mangiferae*, approximately 430 bp (Fig. 1A and B). PCR results obtained for previously sequenced isolates confirmed their identities based on sequence data with no exceptions. Isolates described in Table 2 also gave similar results, with all isolates from other hosts or asymptomatic material producing a band of approximately 430 bp, indicating that these isolates were *G. mangiferae* (Fig. 1A and B). All symptoms, regardless of the type, shape, and size on fruit produced positive results for *Guignardia*. Isolates from typical CBS symptoms all produced a PCR amplicon of approximately 580 bp, indicating that they were *G. citricarpa*. The only exceptions were an isolate from a red margin hard spot lesion on Marsh grapefruit, which were shown to be *G. mangiferae*. The ex-holotype culture of *G. citricarpa* (CBS111.20) tested positive for *G. citricarpa*.

Isolates of unknown identity were tested and PCR results compared to the source from which they were isolated. In all cases, the isolates were *G. citricarpa* (Figs. 2 and 3), which was consistent with the cultural morphology and growth rate characteristics for this organism. In all assays, no reactions were obtained from the *C. gloeosporioides* and water controls. The same results were obtained for all five replicates (data not shown).

The BLAST analysis done using the primer specific for *G. citricarpa* (CITRIC1) showed several matches, all of which were *G. citricarpa* sequences depos-

ited by Meyer et al. (28). The only other matches obtained were those of *G. citricarpa* sequences deposited by Baayen et al. (6). There were no other matches for

Fig. 1. DNA amplification products from primers ITS4, CITRIC1, and CAMEL2. **A**, Predominantly *Guignardia mangiferae* isolates, with *G. citricarpa* isolates included for comparative purposes. **B**, Predominantly *G. citricarpa* isolates, with *G. mangiferae* isolates included for comparative purposes. CG in both cases denotes a *Colletotrichum gloeosporioides* control, and [N] is a negative water control. The first lane in both cases is a 100-bp ladder.

Table 2. Isolates of unknown identity retrieved from fruit lesions or freeze-dried mycelium, and/or direct potato dextrose agar (PDA) isolations, using the DNeasy Plant Mini Kit, and identified with ITS4, CITRIC1, and CAMEL2 primers

Isolate code	Origin ^a	Host cultivar	Lesion type	DNA isolation source	PCR result
GC1	Sandvlei, NW	<i>Citrus sinensis</i> cv. Valencia	Hard spot	Freeze-dried mycelium	GC ^b
GC12	Letaba, MP	<i>C. sinensis</i> cv. Valencia	Hard spot	Freeze-dried mycelium	GC
GC13	Sao Paolo, Brazil	<i>C. sinensis</i> cv. Valencia	Hard spot	Freeze-dried mycelium	GC
GC71	Croc Valley, MP	<i>C. sinensis</i> cv. Valencia	False melanose	PDA culture	GC
GC85	Nelspruit, MP	<i>C. sinensis</i> cv. Valencia	Hard spot	PDA culture	GC
GC86	Nelspruit, MP	<i>C. sinensis</i> cv. Valencia	Virulent spot	PDA culture	GC
GC89	Nelspruit, MP	<i>C. sinensis</i> cv. Valencia	Hard spot	PDA culture	GC
GC158	South Zimbabwe	<i>C. sinensis</i> cv. Valencia	Hard spot	PDA culture	GC
TR02/06/01.1	Marble Hall, MP	<i>C. sinensis</i> cv. Valencia	False melanose	Fruit lesion + PDA culture	GC
TR02/06/01.3	Marble Hall, MP	<i>C. sinensis</i> cv. Valencia	Red margin hard spot	Fruit lesion + PDA culture	GC
TR02/06/01.4	Marble Hall, MP	<i>C. sinensis</i> cv. Valencia	Freckled spot	Fruit lesion + PDA culture	GC
TR03/09/03.1	Unknown	<i>C. sinensis</i> cv. Valencia	Hard spot	Fruit lesion + PDA culture	GC
CV6	Croc Valley, MP	<i>C. sinensis</i> cv. Midnight	Virulent spot	Fruit lesion + PDA culture	GC
CV25	Croc Valley, MP	<i>C. sinensis</i> cv. Navel	Virulent spot	Fruit lesion + PDA culture	GC
CV29	Croc Valley, MP	<i>C. sinensis</i> cv. Navel	False melanose	Fruit lesion + PDA culture	GC
GC57	Croc Valley, MP	<i>C. limon</i> cv. Eureka	Hard spot	PDA culture	GC
GC73	Karino, MP	<i>C. limon</i> cv. Eureka	Hard spot	PDA culture	GC
GC170	Sondagsriver Valley, SCape	<i>C. limon</i> cv. Eureka	No lesion	PDA culture	GC
TR03/03/03.1	Unknown	<i>C. limon</i> cv. Eureka	No lesion	Fruit	GC
TR03/03/03.2	Unknown	<i>C. limon</i> cv. Eureka	Small hard spot lesions	Fruit lesion + PDA culture	GC
TR03/03/03.3	Unknown	<i>C. limon</i> cv. Eureka	Small hard spot lesions	Fruit lesion + PDA culture	GC
TR03/03/03.4	Unknown	<i>C. limon</i> cv. Eureka	Big hard spot lesions	Fruit lesion + PDA culture	GC
C9.1	Chiredzi, Zimbabwe	<i>C. limon</i> cv. Eureka	False melanose	PDA culture	GC
K10.1	Chiredzi, Zimbabwe	<i>C. limon</i> cv. Eureka	FM and VIR ^c	Fruit lesion + PDA culture	GC
K9	Karino, MP	<i>C. limon</i> cv. Eureka	FM and VIR	Fruit lesion	GC
GC81	Croc Valley, MP	<i>C. paradisi</i> cv. Marsh	Hard spot	PDA culture	GC
GC167	K. Naidoo, KZN	<i>C. paradisi</i> cv. Marsh	Hard spot	PDA culture	GM
GC7	Tzaneen, MP	<i>Mangifera indica</i>	No symptoms	Freeze-dried mycelium	GM
GC9	Tzaneen, MP	<i>M. indica</i>	No symptoms	PDA culture	GM

^a Provinces in South Africa: KZN = Kwazulu Natal, MP = Mpumalanga Province, NW = North Western Province, SCape = Southern Cape, part of the Western Cape Province.

^b GC = *Guignardia citricarpa*, GM = *Guignardia mangiferae*.

^c FM = false melanose, VIR = virulent spot.

this sequence. The analysis done with the primer specific for *G. mangiferae* (CAMEL2) showed several matches. These included those deposited by Meyer et al. (28), designated *Guignardia* sp. (endophyte), and those deposited by Baayen et al. (6), which were the newly described *G. mangiferae*. Other matches included a *Phyllosticta* sp. MSR1 (32) and a *Guignardia endophyllicola* (31). There were no other matches for this sequence.

Evaluation of DNeasy Plant Mini Kit. A high-concentration pure DNA of PCR quality (without any RNA or cellular contaminants) was successfully and consistently extracted from cultures, freeze-dried mycelium, and fruit lesions. A very small amount of lesion tissue, regardless of the symptom, is required to produce a sufficient DNA concentration for PCR detec-

tion. The extraction process is completed in approximately 2 to 3 h.

DISCUSSION

A set of two primers was designed to distinguish between *G. citricarpa* and *G. mangiferae* in conjunction with the ITS4 primer in a PCR test. PCR-based techniques for diagnosis and differentiation of pathogens of quarantine importance has proved to be very useful, as in the case of *Xanthomonas axonopodis* pv. *citri*, the causal agent of bacterial canker of citrus (11). Furthermore, the application of the information contained within the ITS region of rDNA for diagnostic purposes is well documented (16,25,35,39,41). The positive PCR identification of all previously sequenced isolates (28) proved that this assay is robust and reliable, since the PCR results obtained using this primer set were in ac-

cordance with the described sequence data. This was further confirmed by the positive identification of the ex-holotype culture (CBS 111.20) of *G. citricarpa*.

Currently, CBS is diagnosed from classical hard spot symptoms with *Phyllosticta citricarpa* pycnidia within these lesions. This type of diagnosis is complicated by the presence of less distinctive symptoms such as false melanose and freckle spot, which are the most common type of intercepted symptoms (6). Identification was primarily done by culturing and visual identification with a very low efficacy rate. It has also been shown that despite efforts to exclude contaminants, up to 60% of isolates are identified as *C. gloeosporioides* and only 27% as *Guignardia* spp. (15). The presence of contaminants such as *C. gloeosporioides* and the long time required for sufficient growth of the fungus resulted in the search for another more efficient technique.

When various symptoms and lesions on fruit were analyzed in this study using the DNeasy kit and the designed primers, *Guignardia* spp. were consistently positively identified, also with the use of single lesions. This was furthermore confirmed when corresponding PDA cultures and direct DNA fruit lesion isolations (originating from the same lesion) tested positive for *G. citricarpa*. The consistency of positive identification found in this study proves the sensitivity of these primers in detecting the presence of *Guignardia* in plant tissue. This method reduces the required testing time significantly. This is one of the most important benefits resulting from the implementation of molecular based techniques coupled with increased sensitivity (24).

Two of the isolates obtained from symptomatic citrus material were identified as *G. mangiferae* (Table 1 and 2). This could be ascribed either to a multiple infection of both *G. citricarpa* and *G. mangiferae* within those particular lesions or to the formation of lesions due to infection by *G. mangiferae*. Baayen et al. (6) found that upon isolation, particularly from minute spots, *G. mangiferae* could readily be isolated.

Most importantly, it has been shown in our study that these primers do not react with any fungal genera. This was confirmed by PCR and BLAST searches. Some of the matches obtained in the BLAST search for CAMEL2 were an ascomycetous fungal endophyte MS3 ISI109 (21) and a *G. endophyllicola* (31). Since these sequences were published before the designation of *G. mangiferae* (6), it is highly probable that these sequences were from isolates currently known as *G. mangiferae*.

Extracting good quality DNA directly from any fruit lesion, using the DNeasy Plant Mini DNA extraction kit, makes routine diagnosis of *G. citricarpa* in export consignments more cost and time effective.

Fig. 2. Use of species-specific primers on direct fruit lesion DNA extracts of unknown identity. Samples on the gel are direct DNA extracts of: lane 1, false melanose lesion (TR02/06/01.1); lane 2, hard spot lesion (TR03/09/03.1); lane 3, virulent spot (CV6); lane 4, freckled spot (TR02/06/01.4); lane 5, hard spot lesion (TR03/03/03.4); lane 6, virulent spot (C10.1); lane 7, false melanose (C10.1). Isolates #80 and #172 serve as *Guignardia citricarpa* and *G. mangiferae* controls; (NEG) denotes a negative water control; M denotes a 100-bp ladder.

Fig. 3. Use of species-specific primers on DNA from cultures ex fruit lesions of unknown identity. Samples on the gel are as follows: lane 1, false melanose isolate (TR02/06/01.1); lane 2, hard spot isolate (TR03/09/03.1); lane 3, virulent spot isolate (CV6); lane 4, freckled spot isolate (TR02/06/01.4); lane 5, hard spot isolate (TR03/03/03.4); lane 6, virulent spot isolate (C10.1); lane 7, false melanose isolate (C10.1). Isolates #80 and #172 serve as *Guignardia citricarpa* and *G. mangiferae* controls; (NEG) denotes a negative water control; M denotes a 100-bp ladder.

This is critical, particularly in the case of South African fruit exports, where delays can result in missing the reefer's scheduled departure date with subsequent rescheduling and divergence to alternative markets. It is now possible to extract DNA directly from the citrus fruit and to perform the PCR successfully with very specific and robust *Guignardia* primers all within a day. The verification of questionable consignments can therefore be streamlined by rapid and accurate diagnosis of citrus fruit spots.

The EU and USA consider the pathogenic strain, *G. citricarpa*, to be a serious phytosanitary risk and have therefore imposed regulations on the import of fruit consignments in terms of the presence of CBS on fruit. However, these restrictions are limited to *G. citricarpa* and exclude *G. mangiferae* (4,6). The study by Baayen et al. (6) confirmed that *G. mangiferae* has a very wide host range and occurs worldwide. They therefore concluded that the exclusion of *G. mangiferae* from quarantine legislation was justified. The primer sets and test method described in this paper provide an effective, rapid, accurate, and validated protocol for one-day analysis of fruit in questionable rejected export consignments.

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