Two distinct Guignardia species associated with citrus in South Africa

L. Meyer*, B. Slippers*, L. Korsten*, J.M. Kotze* and M.J. Wingfield*

The fungus Guignardia citricarpa causes a phytosanitary restrictive disease called citrus black spot (CBS). Some researchers have suggested the existence of two strains, but these cannot be distinguished by mere microscopy. South African Guignardia isolates from different lesion types, as well as from symptomless fruit, were compared by means of ribosomal DNA internal transcribed spacer sequence analysis, proving the existence of two Guignardia species on citrus. Restriction enzyme (CfoI) digestion fingerprints of the PCR products clearly distinguished the two species, providing a quick yet reliable identification tool. Growth rate in culture also corresponded with the two species. The first species, G. citricarpa, is confirmed as the causal organism of CBS and is restricted to citrus. This fungus occurs in all major citrus-producing areas of South Africa, except the Western Cape. The pathogen can be isolated from hard spots, virulent spots, freckled spots and false melanose lesions from sweet orange, grapefruit, lemon and tangerine. The second species, which is harmless to fruit, can be isolated from symptomless citrus products, but also from avocado, mango, banana, cabbage tree and kumquat, which occur in various geographical areas. Phytosanitary measures may be used against the export of citrus fruit suspected of being infected with CBS. The DNA tests we have devised are able, for the first time, to distinguish the pathogenic from the harmless endophyte of citrus and other plants.

Background
South Africa is a relatively small producer of Citrus L. in global terms, yet it exports on average 56% of its crop. This makes the country the third biggest exporter of fresh citrus fruit in the world. This important source of foreign exchange is seriously threatened by restrictive quarantine regulations linked to a fruit disease known as citrus black spot (CBS), caused by the fungus Guignardia citricarpa Kiely (asexual state: Phyllosticta citricarpa (McAlpine) Aa). Preharvest CBS lesions resulting from field infections spoil the appearance of the fruit and thus significantly reduce sales. In addition, latent infections that are not associated with symptoms at the time of harvesting can also develop on fruit in transit. CBS is most prevalent in South Africa, Argentina, Australia and Brazil, and has not been reported in Europe or the U.S.A. (on citrus). Since South Africa exports the bulk of its fruit to the countries of the European Union and is currently negotiating entry into the United States market, phytosanitary measures might restrict future exports. The typical symptom of CBS-infected fruit, hard spot, is a circular lesion on the rind. Although the rind may become extensively necrotic, it seldom causes post-harvest decay. Fruit symptoms can be classified into five categories: hard spot/shoot-hole, freckle spot, virulent spot, false melanose (in South Africa) and cracked spot (in Argentina). Except for Seville orange (Citrus aurantium L.) and its hybrids, all commercially grown Citrus spp. are susceptible to the CBS pathogen. Lemons (C. limon Osbeck) are particularly susceptible, and heavy losses may occur on late Valencia and navel oranges [C. sinensis (L.) Osbeck] and grapefruit (C. paradisi Macfadyen). Some researchers have suggested that there are two different, but morphologically indistinguishable, forms of the CBS pathogen. One of these is pathogenic, host specific and the causal agent of the disease, while the other is believed to be non-pathogenic, occurs on numerous hosts and is relatively unimportant. The aim of this investigation was to compare Guignardia isolates from the rind of apparently healthy fruit with those from CBS-spotted citrus rind and different alternative hosts based on ribosomal DNA internal transcribed spacer (ITS) sequences and certain morphological features. Nucleic acid characters, and especially the internal transcribed spacer ITS1 and ITS2, have been successfully used in resolving intra- and interspecific relationships in numerous fungi.

Materials and methods
Fungal isolates: a set of 35 Guignardia isolates obtained from the major citrus-producing areas in South Africa, from different cultivars [lemons, sweet oranges, grapefruit, tangerine (C. reticulata Blanco) and kumquat (Fortunella Swinglen)] and different lesion types from citrus fruit, twigs and leaves, as well as from avocado (Persa americana Mill.), mango (Mangifera indica L.), banana (Musa acuminata Colla) and cabbage tree (Cassonion Thunb.) were included in the study (Table 1). The fungal samples were obtained by direct isolation from host tissue onto potato-dextrose agar (Biolab Diagnostics, Midrand), supplemented with 250 mg chloramphenicol (Premier Pharmaceutical, Bryanston). An ex-halotype culture of the species, Phyllosticta citricarpa (CBS1120), was used as comparative standard in all tests.

DNA amplification and sequence analysis: DNA was extracted from freeze-dried mycelium using the technique described by Raeder and Broda, and a portion of the ITS region of the ribosomal DNA operon amplified using primers ITS1 (5'-TCC GTA GGT TGA CCA G-3') and ITS4 (5'-GCT GCG TAT TTC ATC GAT GC-3'). The amplified DNA fragments were visualized on a 1.0% (w/v) agarose gel to assess the amplification and purified using a Wizard Preps DNA purification system kit (Promega Corp., Madison, WI). Sequencing reactions were carried out using standard protocols recommended by the manufacturer. The sequence data for the Guignardia isolates were processed using Sequence Navigator version 1.0.1 (Perkin-Elmer). These DNA sequences were aligned with each other, as well as with ITS sequences of Guignardia philoprina (Berkeley & Curtis) van der Aa (GenBank accession number AF312014).

Analysis: sequences were studied using Phylogenetic Analysis Using Parsimony (PAUP) version 3.1.1. A heuristic search with tree-bisection-reconnection (TBR) was carried out and trees were rooted to the sequence of G. philoprina. The confidence limits were determined from a bootstrap analysis with 1000 replications.

RFLP analysis: amplified DNA was digested with CfoI (Roche Diagnostics, Mannheim). Digestions were prepared using 2 units of enzyme, 25 μl of the accompanying enzyme buffer (Buffer I), 2.1 μl H₂O, and 20 μl of each PCR reaction sample. The mixtures were incubated at 37°C for 1 h. The fragments were separated on a 3% agarose gel stained with ethidium bromide.
A Single DNA fragment of approximately 610 bp was obtained from the ITS region of the rDNA operon and manually aligned for all the isolates. Of the aligned data set of 610 bp, 80 sequences of approximately 610 bp were sequenced. DNA amplification: A Single DNA fragment of approximately 660 base pairs was obtained for all PCR amplification reactions.

<table>
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<tr>
<th>Isolate code</th>
<th>Host cultivar and tissue type</th>
<th>Lesion type on citrus</th>
<th>Origin</th>
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</table>

*Provinces in South Africa: KZN = Kwazulu-Natal; MP = Mpumalanga; S. Cape = Southern Cape part of the Western Cape Province; GP = Gauteng.*

**Table 1. Isolates of Guignardia and Phyllistina species compared by means of ITS sequences data.**

**Culture and conidial characteristics:** to measure growth rate, 5-mm-diameter mycelial plugs were transferred from the margin of a single, morphologically uniform colony on PDA to a 9-cm-diameter plastic Petri dish containing PDA. Three plates of each strain were incubated at 22°C under continual, wide spectrum fluorescent light. Colony diameter was measured at 5, 10 and 20 days. Colour and morphology of fungal growth were noted in the above treatments after 15 days. After sporulation was induced on PDA, the lengths and widths of 50 conidia mounted in water were measured for each isolate using bright field microscopy.

**Results**

**DNA amplification:** A Single DNA fragment of approximately 660 base pairs was obtained for all PCR amplification reactions.

**Sequence analysis:** sequences of approximately 610 bp were obtained from the ITS region of the rDNA operon and manually aligned for all the isolates. Of the aligned data set of 610 bp, 80 characters were parsimony informative. Four most parsimonious trees of 133 steps were obtained (CI = 1.000, RI = 1.000). The topography of these trees was the same, with rearrangements occurring only within the main groups. Isolates were grouped into two main clades, which were supported by 100% bootstrap values at the branching points (Fig. 1). All isolates from CBS lesions grouped with the pathogen P. citricarpa (CBS111.20) (group B), whereas all the endophytic isolates grouped separately from this species (group A).

**RFLP analysis:** CfoI digestion of the ITS PCR products resulted in fragment patterns that clearly distinguished the two groups (Fig. 2). The sizes of fragments for group B, were similar to that of the ex-holotype of P. citricarpa (CBS111.20).

**Cultural and conidial characteristics:** two distinct colony types were produced on PDA at 22°C.

**Guignardia citricarpa isolates:** colony on PDA black, sometimes with a thin yellow margin, deeply lobed, mycelium dense, felt, appressed, reverse black. Conidiomata pyrcidal in culture, globose, grey to black, hairy, in dense clusters embedded in the mycelium and formed over the whole colony, conidial mass white to cream. Conidia 8–11 × 6.5–8 μm. Spermata present.

**Endophytic isolates:** colony on PDA black to olive green, margin less lobed than G. citricarpa isolates, never yellow. Mycelium less dense and woolier. Otherwise, cultural and conidial characteristics were the same as for the G. citricarpa isolate group.

**Growth rate:** G. citricarpa isolates (including CBS111.20) grew at 2.7–3.2 mm/day, whereas isolates from the endophytic group grew at 4.5–5.3 mm/day on PDA (22°C).

**Discussion**

This study confirms the existence of two Guignardia taxa on citrus, and suggests that they are two distinct species. Growth rate and colony morphology correspond with the two main clades of the ITS phylogeny, but the species could not be
The pathogen is thus restricted to citrus, but occurs in all the main citrus-producing areas of South Africa except the Western Cape. The second fungus (group A) represents another species of Guignardia, which is apparently not pathogenic and was isolated from the above cultivars, as well as from avocado, banana, cabbage tree, mango and kumquat. Isolates were obtained from plant material from various geographic areas.

Kiely first suggested that CBS, latent infection of citrus fruit, and infection of other semi-tropical non-citrus plants, might all be caused by G. citricarpa. The CBS pathogen, G. citricarpa, was subsequently described on 21 plant families worldwide. The latent nature of the pathogen in citrus contributed to even greater uncertainty.

Van der Aa studied selected species of Phyllosticta Pers. and stated that P. citricarpa could not be distinguished on morphological grounds alone, as morphologically similar isolates were obtained from different substrates or hosts. He recommended that host specificity be used to delimit species. Various new names have therefore been given to species of Guignardia and Phyllosticta occurring on hosts where none has been identified previously. Sometimes species described from closely related host species or genera have been disregarded. Since morphologically, physiologically and genetically identical isolates from citrus, mango, avocado, banana and cabbage tree were retrieved in this study, the practice of applying new names to these fungi from new hosts is clearly not advisable. A similar conclusion was reached by Okane et al., who studied isolates of Guignardia from various ericaceous hosts. It is clear, therefore, that a detailed molecular taxonomic study of this genus is required, as this would eliminate the establishment of unnecessary names resulting from the practice of naming fungal isolates solely according to host.

Conclusion

The fungus causing CBS is Guignardia citricarpa (anamorph Phyllosticta citricarpa); another, morphologically similar but distinct species of Guignardia, which has not yet been named, is a harmless endophyte of citrus and several other plants. In the past, these two fungi were treated as a single entity responsible for CBS. There are, however, two distinct species, and therefore appropriate quarantine decisions, affecting exports for example, can only be made after careful study of isolates from the fruit. These studies must rely on DNA tests, which provide the only robust approach to distinguish the two species. Failure to discriminate correctly between these fungi may severely threaten future international trade in citrus fruit from South Africa.
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