Phyllosticta species on citrus: Risk estimation of resistance to QoI fungicides and identification of species with cytochrome b gene sequences

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Abstract

Isolates of three fungal species associated with citrus, Phyllosticta citricarpa, Phyllosticta citriasiana and Phyllosticta capitalensis, collected from different citrus growing countries of the world, were investigated for their sensitivities to the QoI fungicides pyraclostrobin and azoxystrobin. Isolates were highly sensitive in microtiter tests and EC50 values were in narrow ranges, which indicate no acquired adaptation to QoIs. The resistance risk of P. citricarpa to QoIs is considered low since an intron was found immediately after codon 143 in the cytochrome b gene. The presence of an intron is known to reduce the risk of the G143A mutation, the mutation which causes QoI resistance with high resistance factors. The other two species had no intron and therefore are considered having a higher resistance risk. Impact of these two species is rather low, since P. citriasiana is restricted in its regional and host distribution and P. capitalensis is non-pathogenic. Furthermore, the development of a rapid and reliable assay for species detection and identification was made possible based on an analysis of the cytochrome b gene.

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1. Introduction

Citrus black spot (CBS), caused by Phyllosticta citricarpa (McAlpine) van der Aa (syn. Guignardia citricarpa Kiely), is responsible for substantial losses to citrus farmers in countries of Africa, Asia, South America and Australia (Baayen et al., 2002; Kotze, 1981) and affects all commercial citrus cultivars in the summer rainfall regions of the world. Recently, the disease was also reported from Florida (Schubert et al., 2012). P. citricarpa has been added to the A1 quarantine list of EPPO, causing restrictions of importation of citrus fruits to the EPPO region and US to avoid that P. citricarpa is established in these regions. Fruits for import to EPPO countries and US from infested countries should be disease free. Two other Phyllosticta spp. were recently isolated from citrus and newly described, namely Phyllosticta citriasiana on Citrus maxima from China, Thailand and Vietnam (Wulundari et al., 2009), and Phyllosticta citrichinaensis on four Citrus spp. in China (Wang et al., 2011). Further studies are required, however, to establish host ranges, geographic distribution, disease status and biosecurity significance of these species. Phyllosticta capitalensis Henn. (syn. Guignardia mangiferae A.J. Roy) is often isolated from citrus fruits, but represents an endophytic, wide host range, non-pathogenic species on citrus (Baayen et al., 2002; Gliemke et al., 2011).

Control of the disease in the southern hemisphere is entirely dependent on the application of fungicidal sprays during the critical period of infection from onset of the first summer rain and subsequent ascospore release in October to January/February when the fruit becomes resistant (Kotzé, 1981, 2000; McOnie, 1964). Initially, protective fungicides such as copper and dithiocarbamates were recommended for the control of CBS (McOnie and Smith, 1964), but spray applications had to be carefully timed to coincide with the critical infection period. For this, spore trapping with an Interlock volumetric spore trap and sampler (Truter et al., 2004) are used in South Africa to determine the first onset of ascospore release. A four-spray programme of copper fungicides commonly used for CBS control can, however, result in rind stippling and darkening of blemishes (Brodrick, 1970; Schutte et al., 1997).

Protective fungicides like copper became less popular with the use of benzimidazole fungicides such as benomyl that have post-infection activity. In 1971, the introduction of a single benomyl application in a tank mixture with mancozeb and mineral spray oil came as a breakthrough as it replaced copper and dithiocarbamates
that must be applied in a four-spray protective schedule (Kellermann and Kotze, 1977). However, since the detection of CBS resistance to benomyl in South Africa in 1981 (De Wet, 1987), emphasis has shifted back to the use of contact fungicides for disease control. Field evaluations using quinoxylin outside inhibitor fungicides (QoIs, also known as strobilurins) for the control of CBS in 1993 came as another breakthrough. Two applications of kresoxim-methyl or azoxystrobin at respective rates of 0.10 and 0.075 g a.i./liter in tank mixtures with mancozeb (1.2 g a.i./liter) and mineral oil (0.5% [vol/vol]/liter of water) were initially recommended (Schutte et al., 2006a; b) and no G143A has been detected in these species (Miessner and Stammler, 2010).

QoIs are generally classified by the Fungicide Resistance Action Committee (FRAC) as fungicides with a high resistance risk. QoI resistance was soon found in some pathogens such as powdery mildews in cereals and cucurbits 1–3 years after market introduction (Kuck and Russell, 2006). Resistance to QoIs is conferred by mutations in the target protein cytochrome b, where the most important amino acid substitution is G143A, which leads to high resistance to all QoI fungicides. Interestingly, this mutation has not yet been found in some plant pathogenic fungi, even after many years of QoI fungicide use and high selection pressure. Grasso et al. (2006a; b) reported that an intron directly after codon 143 in the cytochrome b gene may prevent the occurrence of the G143A mutation. The glycine codon is hypothesized to play a role in splicing of the mRNA, and changes in codon 143 (from glycine codon GCT, GCC, GCA or GCG to the alanine codon GCT, GCC, GCA or GCG) would lead to incorrect splicing during mRNA maturation and consequently to a non-functional cytochrome b protein (Vallieres et al., 2011). Many plant pathogenic fungi have been sequenced and introns directly after or even within codon 143 have been found in Alternaria solani (Grasso et al., 2006a), Guignardia bidwellii (Miessner et al., 2011), Monilinia laxa, M. fructicola (Miessner and Stammler, 2010), Pyrenopeziza brassicae (Sierotzki et al., 2007), various Puccinia species, and Phakopsora pachyrhizi (Grasso et al., 2006a; b) and no G143A has been detected in these species thus far. For A. solani and Pyr. teres the mutations F129L and or G137R have been reported (www.frac.info) as mechanisms for QoI tolerance. Both mutations are of minor importance because they generally lead to lower resistance factors (www.frac.info) than G143A and have in case of Pyr. teres no or only a limited impact on field efficacy of the QoIs pyraclostrobin and picoxyystrobin (Drobsny et al., 2008; Semar et al., 2007). A stronger decrease in efficacy of QoIs has been described for A. solani with the F129L mutation (Pasche et al., 2005).

The aim of this study was to clarify the risk of occurrence of the mutation G143A as the main resistance mechanism to QoIs but also of the F129L and G137R mutations in some Phylosticta spp. isolated from citrus. Therefore, Qol sensitivities of strains isolated in the last seasons were determined in microtiter tests using two QoI fungicides (pyraclostrobin and azoxystrobin) and compared with a reference isolate to identify if an adaptation has already occurred. Additionally, the cytochrome b gene was analysed for the presence and position of intron sequences close to the three relevant codons (143, 129, and 137). Various isolates of the pathogenic species P. citricarpa from different regions worldwide, as well as isolates of P. citroniana and of the non-pathogenic species P. capitalensis were included in the analysis.

2. Materials and methods

2.1. Origin of isolates

Seven P. citricarpa isolates (four from Citrus sinensis in South Africa, one from C. sinensis in Swaziland, two from Citrus paradisi in Swaziland) were isolated at Citrus Research International in Nelspruit, South Africa. Two P. citricarpa and three P. capitalensis isolates from PR China were kindly provided by H.Y. Li. One P. citricarpa, two P. citroniana and three P. capitalensis isolates were obtained from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. Species identification was confirmed by morphological characteristics and by molecular genetic analysis as described earlier (Peres et al., 2007). Isolates details are given in Table 1.

2.2. Morphological studies

Isolates were maintained on 2% malt extract agar and morphological studies were made with structures which had developed on pine needle agar (Crous et al., 2006) after incubation at 23 °C under near-ultraviolet and white fluorescent light to promote sporulation. Fungal structures were mounted on glass slides in water for microscopic examination after 14 and 28 days of incubation. Thirty measurements were determined, where possible, for each structure.

Table 1

Phylosticta sp. isolates investigated in this study.

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Original ID</th>
<th>Origin</th>
<th>Species</th>
<th>Citrus cultivar</th>
<th>Country</th>
<th>Year of isolation</th>
<th>Collector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gc2</td>
<td>CBS 102373</td>
<td>CBS</td>
<td>P. citricarpa</td>
<td>Citrus aurantium</td>
<td>Brazil</td>
<td>1999</td>
<td>–</td>
</tr>
<tr>
<td>Gc4</td>
<td>38/1</td>
<td>CRI</td>
<td>P. citricarpa</td>
<td>Citrus sinensis</td>
<td>Swaziland</td>
<td>2010</td>
<td>G.C. Schutte</td>
</tr>
<tr>
<td>Gc5</td>
<td>46/1</td>
<td>CRI</td>
<td>P. citricarpa</td>
<td>Citrus paradisi</td>
<td>Swaziland</td>
<td>2010</td>
<td>G.C. Schutte</td>
</tr>
<tr>
<td>Gc6</td>
<td>46/2</td>
<td>CRI</td>
<td>P. citricarpa</td>
<td>C. sinensis</td>
<td>Swaziland</td>
<td>2010</td>
<td>G.C. Schutte</td>
</tr>
<tr>
<td>Gc8</td>
<td>2</td>
<td>CRI</td>
<td>P. citricarpa</td>
<td>C. sinensis</td>
<td>South Africa</td>
<td>2010</td>
<td>G.C. Schutte</td>
</tr>
<tr>
<td>Gc9</td>
<td>3</td>
<td>CRI</td>
<td>P. citricarpa</td>
<td>C. sinensis</td>
<td>South Africa</td>
<td>2010</td>
<td>G.C. Schutte</td>
</tr>
<tr>
<td>Gc10</td>
<td>4</td>
<td>CRI</td>
<td>P. citricarpa</td>
<td>C. sinensis</td>
<td>South Africa</td>
<td>2010</td>
<td>G.C. Schutte</td>
</tr>
<tr>
<td>Gc11</td>
<td>5</td>
<td>CRI</td>
<td>P. citricarpa</td>
<td>C. sinensis</td>
<td>South Africa</td>
<td>2010</td>
<td>G.C. Schutte</td>
</tr>
<tr>
<td>Gc14</td>
<td>G105</td>
<td>ZJU</td>
<td>P. citricarpa</td>
<td>P.R. China</td>
<td>H.Y. Li</td>
<td>2007</td>
<td>J. de Gruyter</td>
</tr>
<tr>
<td>Gc16</td>
<td>G111</td>
<td>ZJU</td>
<td>P. citricarpa</td>
<td>P.R. China</td>
<td>H.Y. Li</td>
<td></td>
<td>J. de Gruyter</td>
</tr>
<tr>
<td>Gm1</td>
<td>CPC 17442</td>
<td>CBS</td>
<td>P. capitalensis</td>
<td>C. sinensis</td>
<td>Brazil</td>
<td>2006</td>
<td>C. Glienke</td>
</tr>
<tr>
<td>Gm2</td>
<td>CPC 17443</td>
<td>CBS</td>
<td>P. capitalensis</td>
<td>C. sinensis</td>
<td>Brazil</td>
<td>2006</td>
<td>C. Glienke</td>
</tr>
<tr>
<td>Gm3</td>
<td>CPC 17444</td>
<td>CBS</td>
<td>P. capitalensis</td>
<td>C. sinensis</td>
<td>Brazil</td>
<td>2006</td>
<td>C. Glienke</td>
</tr>
<tr>
<td>Gm4</td>
<td>G103</td>
<td>ZJU</td>
<td>P. capitalensis</td>
<td>P.R. China</td>
<td>H.Y. Li</td>
<td>2005</td>
<td>J. de Gruyter</td>
</tr>
<tr>
<td>Gm5</td>
<td>G104</td>
<td>ZJU</td>
<td>P. capitalensis</td>
<td>P.R. China</td>
<td>H.Y. Li</td>
<td>2005</td>
<td>J. de Gruyter</td>
</tr>
<tr>
<td>Gm6</td>
<td>G110</td>
<td>ZJU</td>
<td>P. capitalensis</td>
<td>P.R. China</td>
<td>H.Y. Li</td>
<td>2005</td>
<td>J. de Gruyter</td>
</tr>
<tr>
<td>Pc1</td>
<td>CBS 120.427</td>
<td>CBS</td>
<td>P. citroniana</td>
<td>Citrus maxima</td>
<td>P.R. China</td>
<td>2005</td>
<td>J. de Gruyter</td>
</tr>
<tr>
<td>Pc2</td>
<td>CBS 123.393</td>
<td>CBS</td>
<td>P. citroniana</td>
<td>C. maxima</td>
<td>Vietnam</td>
<td>2007</td>
<td>J. de Gruyter</td>
</tr>
</tbody>
</table>
2.3. Characterisation of fungal growth on oatmeal agar

Isolates were grown on oatmeal agar as described earlier (Baayen et al., 2002). A mycelial plug was transferred to Petri dishes with oatmeal agar. After incubating at 25 °C and 12 h light/12 h dark for 6 days the dishes were evaluated for the presence of a diffuse yellow halo around the colonies. This yellowing is characteristic for *P. citricarpa* and indicative of pathogenicity (Baayen et al., 2002; Baldassari et al., 2008; Eppo, 2003).

2.4. Molecular genetic species identification

DNA of all isolates was isolated using the NucleoSpin® Plant II Kit (Machery and Nagel, Düren, Germany). Detection of *P. citricarpa* and *P. capitansis* was performed using a previously published protocol (Peres et al., 2007) with primer pairs “PB-N-ITS-Gc” and “NP-Br-ITS-Gc” for *P. citricarpa* identification and “PB-N-ITS-Gm” and “NP-Br-ITS-Gm” for *P. capitansis* identification. PCR products were visualized in ethidium bromide-stained agarose gel electrophoresis (1% agarose). PCR product sizes for *P. citricarpa* are 490 bp (PB-N-ITS-Gc) and 300 bp (NP-Br-ITS-Gc) and for *P. capitansis* 210 bp (PB-N-ITS-Gm) and 290 bp (NP-Br-ITS-Gm) (Peres et al., 2007).

2.5. Analysis of the cytochrome b gene

RNA and DNA of fungal isolates were isolated using the NucleoSpin® RNA Kit and NucleoSpin® Plant II Kit, respectively (Machery and Nagel). RNA was reverse transcribed for cDNA synthesis with the Verso cDNA kit (Thermo, Ulm, Germany) according to the manufacturer’s instructions. PCR was carried out with cDNA or DNA as templates for sequence analysis and comparison of mRNA and the cytochrome *b* gene, respectively. Primers KES 556 (5’-TGGCATACGGTTTTGCTAT-3’) and KES 1288 (5’-AATGTAAGCGAAGAATCT-3’) were selected from sequences of cytochrome *b* known to be conserved for ascomycetes and the resulting PCR product were visualized in ethidium bromide-stained agarose gel electrophoresis (1% agarose). PCR product sizes for *P. citricarpa* are for 6 days the dishes were evaluated for the presence of a diffuse yellow halo around the colonies. This yellowing is characteristic for *P. citricarpa* and indicative of pathogenicity (Baayen et al., 2002; Baldassari et al., 2008; Eppo, 2003).

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Analysis of codon F129L required additional PCR reactions and subsequent sequencing because the sequence of codon 129 is within primer KES 556. PCR amplifications covering codon 129 using a primer pair KES 126 (5’-GAGGTTTATATACGAGGATATATA GAG-3’) and KES 1288 for *P. citricarpa* and *P. capitansis* and with primer pair KES 124 (5’-CAGCTTCAGGTTTTCTTTCTTAG-3’) and KES 1288 for *P. citriciana* were done to identify codon 129 in all isolates. KES 126 was not usable for *P. citriciana* because of several mismatches. Therefore, primer KES 124 (providing a longer PCR product) was used.

2.6. Species-specific detection of *Phylllosticta* species

PCR amplifications for species-specific detection of *P. citricarpa*, *P. capitansis* and *P. citriciana* were carried out using the described conditions with primer pair KES 556 + KES 1288 and a gradient of annealing temperatures. DNA extracts from other fungal species such as *Phylllosticta ampelicida* (syn. Guignardia bidwellii), *Penicillium digitatum*, *Penicillium italicum*, *Geotrichum candidum*, *Rhizopus stolonifer*, *Aspergillus niger*, *Alternaria alternata*, *Alternaria solani*, *Botrytis cinerea*, *Fusarium graminearum*, *Coleotroctrichum gloeosporioides*, *Monilinia fructigena*, *Monilinia laxa*, *Peronosphythora itichii*, *Pythium aphanidermatum*, *Rhizoctonia solani*, *Zymoseptoria tritici* were used as templates.

2.7. Analysis of sensitivity to pyraclostrobin and azoxystrobin

Sensitivities of isolates towards pyraclostrobin and azoxystrobin were determined by microtiter assays in 96 well plates at different concentrations (0, 0.01, 0.03, 0.1, 0.3, 3.0, 10 μg ml⁻¹) of the fungicides in YBG-medium (1% yeast extract, 1% Bacto peptone, 2% glycerol). Fungicides stock solutions were 10,000 μg ml⁻¹ in dimethyl sulfoxide. The medium in each well was inoculated with approximately 5000 spores. The final total volume was 100 μl (50 μl double concentrated YBG-medium + 50 μl spore suspension in water) and plates were packed in plastic bags and incubated without any shaking for 14 days at 18 °C in the dark before growth evaluation using a photometer (405 nm). EC₅₀ values (i.e., the concentration of the compound at which fungal development is inhibited by 50%) were calculated by probit analysis (Finney, 1971). With this relatively poor medium the addition of the alternative oxidase inhibitor SHAM is not necessary to achieve complete inhibition of fungal development with Qols.

3. Results

3.1. Growth on oatmeal agar

All *P. citricarpa* isolates showed a yellow halo around the edge of the colony, which was easy to identify and clearly distinguishable from isolates lacking such a zone, such as *P. capitansis* and *P. citriasiana*.

3.2. Morphological characterization

*P. citricarpa*: Conidia (9–11)–12–(14) × 7–8 μm, solitary, hyaline, aseptate, thin- and smooth-walled, coarsely guttulate, ellipsoid to obovoid, tapering to a narrow truncate base and bearing a hyaline, mucoid appendage 4–11(–20) × 1–1.5 μm, straight to flexible, unbranched, tapering to an acute apex. No teleomorph developed on PNA.

*P. capitansis*: Conidia (8–)10–12 × (5–)6–7 μm, solitary, hyaline, aseptate, thin- and smooth-walled, coarsely guttulel, ellipsoid to obovoid, tapering towards a truncate base and bearing a hyaline, mucoid apical appendage, 6–14 × 1–1.5 μm, straight to flexible, unbranched, tapering towards an acute apex. Ascomata erumpent, unilocular with a central ostiole. Ascii attached to the basal peridium, clavate, 8-spored, 60–80 × 8–12 μm. Ascospores limoniform, aseptate, hyaline, thick-walled, guttulate 14–18 × 5–6 μm.

*P. citriasiana*: Conidia (8–)10–13(–15) × 6–7(–8) μm, solitary, hyaline, aseptate, thin- and smooth-walled, coarsely guttulate ellipsoidial to obovoid, tapering to a broad truncate base and bearing a hyaline, mucoid appendage 20–50 × 1–1.5 μm straight to flexible, unbranched, tapering to an acute apex. No teleomorph developed on pine needle agar.

3.3. Molecular biological species identification

PCR using the primer pair “NP-Br-ITS-Gc” (Peres et al., 2007) resulted in an amplification product of 490 bp for *P. citricarpa* isolates and the...
two *P. citriasiana* isolates. No isolate of *P. capitalensis* produced any band (Table 2).

PCR products with primer pair PB-N-ITS-Gm and primer pair NP-BR-ITS-Gm were specific for *P. capitalensis*, i.e., the expected bands (210 bp and 290 bp, respectively) were only visible with *P. capitalensis* and not with *P. citricarpa* nor *P. citriasiana* isolates, but product signals were weak (Table 2).

### 3.4. Analysis of the cytochrome b gene

PCR products using primer pair KES 556 + KES 1288 were 7634 bp for *P. citricarpa*, 1419 bp for *P. capitalensis* and 4221 bp for *P. citriasiana* when DNA was used as template and 177 bp long for all isolates when cDNA was used as template. The alignment of DNA and cDNA and the resulting intron/exon structure is shown in Fig. 1. Sequences of DNA and cDNA were identical within the species but different among the three species as shown in Figs. 1 and 2 (alignment of cDNA sequences of partial mRNA of the three species). A pairwise comparison of a 209-bp cDNA fragment showed 18 single nucleotide polymorphisms (SNPs) for *P. citricarpa* and *P. citriasiana*, 10 SNPs for *P. citricarpa* and *P. capitalensis*, and 15 SNPs for *P. citriasiana* and *P. capitalensis* (Fig. 2).

For *P. citricarpa* alignments of DNA with their corresponding cDNA identified an intron sequence directly after codon 143. No intron directly following codon 143 was found in isolates of *P. citriasiana* or *P. capitalensis*. No introns bordered directly at codons 129 or 137 in any of the three species. No mutations conferring resistance to QoIs in codons 129, 137, or 143 were found in any isolate.

### 3.5. Species-specific detection of Phyllosticta species

High specificity and strong PCR product signals using the primer pair KES 556 + KES 1288 were obtained at an annealing temperature of 54 °C. PCR products were detected for *P. citricarpa* (7634 bp),

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**Table 2**

Overview of properties of isolates of *Phyllosticta citricarpa*, *P. capitalensis* and *P. citriasiana*.

<table>
<thead>
<tr>
<th>Properties</th>
<th><em>P. citricarpa</em> (n = 9)</th>
<th><em>P. capitalensis</em> (n = 6)</th>
<th><em>P. citriasiana</em> (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellowing of oat meal agar</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>PCR product with “PB-N-ITS-Gc”</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PCR product with “NP-BR-ITS-Gm”</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PCR product with “PB-N-ITS-Gm”</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PCR product with “NP-BR-ITS-Gm”</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PCR product with KES 556 + KES 1288 (size)</td>
<td>7634 bp</td>
<td>1419 bp</td>
<td>4221 bp</td>
</tr>
<tr>
<td>Amplicon size (bp) with KES 556 + KES 1288</td>
<td>7634 bp</td>
<td>1419 bp</td>
<td>4221 bp</td>
</tr>
<tr>
<td>Intron after codon 129/137/143</td>
<td>No/No/Yes</td>
<td>No/No/No</td>
<td>No/No/No</td>
</tr>
<tr>
<td>Mean EC&lt;sub&gt;50&lt;/sub&gt; pyraclostrobin [µg ml&lt;sup&gt;–1&lt;/sup&gt;]</td>
<td>0.0033/1.3</td>
<td>0.0422/18.6</td>
<td>0.0037/1.3</td>
</tr>
<tr>
<td>Mean EC&lt;sub&gt;50&lt;/sub&gt; azoxystrobin [µg ml&lt;sup&gt;–1&lt;/sup&gt;]</td>
<td>0.0069/2.2</td>
<td>0.2237/11.5</td>
<td>0.0092/2.3</td>
</tr>
</tbody>
</table>

“++” means strong, “+” weak, “-” no PCR product visible under described conditions. Mean EC<sub>50</sub> values were calculated as geometric means, diversity factors (DF) by dividing maximum value by minimum value.
P. citricarpa (1419 bp), P. citriasiana (4221 bp), and P. ampelicida (7017 bp). No PCR products were obtained with any other fungal species tested under the described conditions.

3.6. Sensitivity of isolates to QoIs

EC50 values of P. citricarpa isolates were in a narrow range of 0.003–0.004 µg ml⁻¹ for pyraclostrobin and of 0.005–0.011 µg ml⁻¹ for azoxystrobin. Higher EC50 values were measured for P. capitalensis, which spanned EC50 values from 0.007 to 0.130 µg ml⁻¹ for pyraclostrobin and 0.040–0.459 µg ml⁻¹ for azoxystrobin. Isolates of P. citriasiana were comparable to P. citricarpa with EC50 values of 0.003 and 0.004 µg ml⁻¹ for pyraclostrobin and 0.006 and 0.014 µg ml⁻¹ for azoxystrobin. Inhibition curves show that P. capitalensis is less sensitive to pyraclostrobin and azoxystrobin than the two other species and that pyraclostrobin has a higher intrinsic activity on all three species than azoxystrobin. Inhibition curves and standard deviations are shown in Fig. 3.

4. Discussion

The aim of the present study was to identify the risk of P. citricarpa becoming resistant to QoIs, by analysing the cytochrome b gene. A previous study with a related species, P. ampelicida (syn. Guignardia bidwellii), had shown that this species has an intron at this position (Miessner et al., 2011). The finding that introns may occur in different species within a genus, as shown for Puccinia (Grasso et al., 2006a; b) and Monilinia (Miessner and Stammler, 2010), encouraged us to expand this analysis to P. citricarpa and other Phyllosticta spp. isolated from citrus. Isolates were obtained from several Citrus hosts from various citrus producing countries to achieve a high heterogeneity of isolate origin.

Species were identified by previously described morphological characteristics (Baayen et al., 2002; Wulundari et al., 2009), PCR assays (Peres et al., 2007), and a newly developed cytochrome b gene analysis. All isolates of this collection identified as P. citricarpa were identical to the reference isolate of the Centraalbureau voor Schimmelcultures (CBS 102373) regarding morphological and genetic analysis, independent of their origin. The same was found for the recently isolated strains of P. capitalensis from P.R. China and the three isolates of the same species from the Centraalbureau voor Schimmelcultures collection. The two isolates allocated to P. citriasiana were identical in their cytochrome b gene sequences and their morphological profile, but differed from the other two species. These analyses showed a high intraspecific homogeneity but interspecific differences on the genetic level. Isolates included in this study represent a relative high diversity. For P. citricarpa this can be stated regarding geographic distribution of origin (Brazil, Swaziland, South Africa, P.R. China), host origin (Citrus aurantium, Citrus paradise, C. sinensis) and years of isolation (1999, 2010), for P. capitalensis for geographical distribution (Brazil and P.R. China) and the two isolates of P. citriasiana derived from two different
countries (P.R. China and Vietnam) and were isolated in 2005 and 2007, respectively.

All isolates of *P. citricarpa* and *P. citriasiana* showed a similar high sensitivity to the QoIs pyraclostrobin and azoxystrobin, higher than the isolates of *P. capitalensis*. If the lower sensitivity of *P. capitalensis* is caused by enhanced activity of alternative oxidase (AOX) or other mechanisms (uptake, efflux, metabolisation, Qo binding site) has not been investigated, but the addition of SHAM or propyl gallate as AOX inhibitors to the medium could elucidate if AOX plays a role here. However, EC50 values were within narrow ranges for the three species, which indicates that an adaptation to a lower QoI sensitivity has not yet occurred for these isolates. Additionally, the sensitivities of all recently isolated *P. citricarpa* isolates were comparable to that of the *P. citricarpa* reference strain isolated in Brazil in 1999. Since this Brazilian isolate has experienced no or only limited QoI selection pressure (introduction of first QoIs in Brazil in 1997), it can be suggested as an isolate with a natural, “wild-type” sensitivity. Analysis of the QoI target gene, cytochrome b, revealed that no isolate had amino acid exchanges at positions which are known to reduce QoI sensitivity (codons 129, 137, 143) or at any other position in the analysed partial mRNAs fragment. Additionally, an intron sequence directly after codon 143 is present in all isolates of *P. citricarpa*, which means from the experience of other “intron-pathogens” that the occurrence of the G143A mutation, the mutation with the highest impact on QoI sensitivity, is rather unlikely. In fact, there is still no example of an isolate of any fungal species with G143A mutation and presence of an intron immediately after codon 143. It is interesting to note that such a gene structure seems to be conserved within a species, even for isolates with heterogeneous regional origin, host or isolation year (Miessler et al., 2011). Thousands of *Pyr. teres* strains (also an “intron-pathogen”) which have been analysed for the codon 143 and surrounding nucleotides for QoI sensitivity monitoring were all identical in these gene regions (BASF internal studies 2003–2012, data not shown). The finding that no G143A mutated isolates showed up in *Pyr. teres*, *Puccinia triticina* or *Pha. pachyrhizi* after many years of QoI selection pressure confirms that the gene sequence around codon 143 with its intron/exon is highly conserved within a species. Therefore, it can be expected with high probability that the whole population of a species contain the intron at the same position when it has been detected for some isolates and rearrangements may not take place. In this regard there is for plant pathogenic fungi one exception described: Two types of *B. cinerea* and *Botrytis pseudocinerea*, one with and one without an intron have been reported (Banno et al., 2009; Jiang et al., 2009; Leroux et al., 2010; Yin et al., 2012) and the “intron story” is here also confirmed, since all isolates containing the G143A mutation have no intron directly after codon 143 and all isolates with an intron after codon 143 did not show the G143A mutation. An explanation for this unique finding in this species might be that *B. cinerea* is a relatively heterogeneous fungal species. No intron was found for *P. citriasiana* and *P. capitalensis* suggesting that the G143A mutation could be expected after continuous selection pressure as it has been found in many examples of other plant pathogens. However, from our sensitivity and genetic studies up to now there is no indication of an adaptation of any isolate used in this study to QoI fungicides.

The number of isolates of *Phyllosticta* spp. in this study is limited and therefore an adaptation can of course not be excluded in general, but there are no reports on field failure of QoI fungicides in CBS control or on less sensitive isolates of the relevant *Phyllosticta* species available.

Because the resistance risk of *P. citricarpa* to QoIs is considered low, citrus growers currently using tank mixes containing QoI fungicides, Mancozeb, and mineral spray oil may start using QoI by themselves to save on spray costs. However, as *P. citricarpa* and *P. capitalensis* can coexist in the same lesion on citrus fruit (Baldassari et al., 2008), and exclusion of mancozeb from tank mixtures with Qols may promote resistance development in *P. capitalensis* towards Qols. At present, we cannot distinguish between ascospores of *P. citricarpa* and *P. capitalensis* on spore trap discs, future research should focus on a technique on how this can be done. If one finds for instance that the ratio of ascospore of *P. capitalensis* towards *P. citricarpa* captured on spore trap discs is low, only then can mancozeb be excluded from tank mixtures with Qols.

*P. citricarpa* is the causative agent of CBS in all countries where this disease occurs, whereas *P. citriasiana* so far is only reported on *Citrus maxima* in Asia in apparent association with a lesion type referred to as tan spot. The presence of an intron after codon G143 in the cytochrome b gene of *P. citricarpa* significantly reduces the resistance risk to QoI fungicides. The fact that *P. citricarpa* has in most citrus growing regions a monocyclic life cycle (one generation per season), the possibility of resistance development and spread is further reduced. However, no introns were found after codons 129 and 137. Therefore such mutations may also occur, but these mutations influence QoI sensitivity with much lower resistance factors than the G143A mutation (Semar et al., 2007). As precautionary measures and to assure a sustainable use of Qols, resistance management strategies should be followed also for *P. citricarpa*. This can be done by limitation the number of Qol applications per season, alternation with fungicides with a different mode of action and/or by using efficacious mixing partners. This is even more important in regions in Asia where *P. citriasiana* may be present, to avoid the development of the G143A mutation in the cytochrome b gene in strains of this species.

Monitoring of QoI sensitivity is an essential part of anti-resistance management strategies. The knowledge of the DNA next to codons 129, 137 and 143 of *P. citricarpa* makes the development of molecular genetic monitoring assays for QoI sensitivity possible. A sensitive and reliable method for monitoring of the G143A mutation would be real-time PCR using the amplification/refractory mutation system (ARMS), which discriminates wild type and mutated DNA by special design of one of the primers (Newton et al., 1989). Since different codons for the F129L and G137R mutation have been found to occur on isolates of the same species, e.g. *Pyr. teres* (Semar et al., 2007), a set-up of a pyrosequencing protocol would be more appropriate and much easier and faster to develop and perform for these mutations than the ARMS-PCR technique. Such methods are currently under development and will be soon available for a reliable, rapid and species-specific monitoring of a high number of samples for mutations reducing QoI sensitivity.

According to current knowledge, several *Phyllosticta* species are known to occur on citrus, which include *P. citricarpa*, *P. citriasiana*, *P. capitalensis*, *P. citribraziliensis* and *P. citrichinaensis*. Only *P. citricarpa* has been proved to be pathogenic, while others are non-pathogenic endophytes on citrus, or in the case of *P. citriasiana* has been reported to have an apparent association with a lesion described as tan spot. Although it was not the aim of the study to contribute to a further elucidation of the taxonomy of *Phyllosticta* spp. isolated from citrus, it seems that the cytochrome b gene might be a valuable tool for species differentiation and identification. One advantage of this gene is its high copy number, since it is located on mitochondrial DNA (mtDNA); this high copy number significantly reduces the detection limit, which is favourable for development of reliable PCR assays. Previous studies have shown that mtDNA offers an appropriate tool for species and subspecies identification in various fungal genera (Foerster and Coffey, 1992; Stammiller et al., 1993; Wang et al., 2002; Zhou and Stanosz, 2001). In particular the intron sequences associated with the cytochrome b gene in...
**Phyllosticta** might provide interesting approaches for identification, differentiation and detection of *Phyllosticta* species as it has been shown for the genus *Monilinia* (Miessler and Stammmer, 2010). Since identification and differentiation of non-pathogenic and pathogenic *Phyllosticta* species on citrus fruits is important, the findings of this study can also be helpful in developing efficient diagnostic tools for phytosanitary purposes. The PCR assay with the primer pair KES 556 and 1288 provided accurate differentiation of *Phyllosticta* species based on PCR product length. The specificity of the assay is high since no products were observed with other fungal species which can be found on citrus fruits, such as *P. digitatum, P. italicum, G. candidum*, *A. niger, A. alternata*, *Citrus gloeosporioides* and *B. cinerea*. Similarly, no products were observed with other ascomycetes such as *A. solani, F. graminearum, M. fructigena, M. laxa*, and *Z. triticum*. Additionally no products were amplified with the oomycetes *Per. litchii and Pythium aphanidermatum*, nor with the basidiomycete *Rhizoctonia solani* and the zygomycete *K. stolonifer*. Since only one single reaction is needed for sensitive and reliable detection the assay offers a rapid and simple approach for specific detection of *P. citricarpa* on citrus fruits.

**References**


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