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

G. Stammler [a,*], G.C. Schutte [b], J. Speakman [a], S. Miessner [a], P.W. Crous [c]

[a] BASF SE, Agricultural Centre, D-67117 Limburgerhof, Germany
[b] Citrus Research International, P.O. Box, 28, Nelspruit 1200, South Africa
[c] CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

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 EC_{50} 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; Glienke 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 quinone 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., 1996, 2003). The possibility that CBS may develop resistance to the QoIs justified the incorporation of two additional mancozeb sprays, in October and January, before and after the QoI applications (Nel et al., 2003).

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 GGT, GGC, GGA or GGG 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), Pyrenophora teres (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 picoxystrobin (Drobny 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 Phyllosticta spp. isolated from citrus. Therefore, QoI 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. citriasiana 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. citriasiana 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
Phyllosticta sp. isolates investigated in this study.

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

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
CRI: Citrus Research International, Nelspruit, South Africa.
ZJU: Institute of Biotechnology, College of Agriculture and Biotechnology, Zhejiang University, Zhejiang, P. R. China.

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. capitalensis* 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. capitalensis* 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. capitalensis* 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′-TGGCTACAGCTTTCTTGGGTTAT-3′) and KES 1288 (5′AATGTAG AGCGAAGAATCT-3′) were selected from sequences of cytochrome *b* known to be conserved for ascomycetes and the resulting PCR product covered codons 143 and 137, which are of interest for QoI sensitivity. PCR was performed with 2× HotStart-IT FideliTaq Mastermix (USB, Staufen, Germany) with an initial heating step for 2 min at 95 °C followed by 35 cycles of 15 s at 95 °C, 30 s at 48 °C and 10 min at 68 °C. PCR products were sequenced by the Sanger method and primer walking sequencing. Alignments of DNA and corresponding cDNA sequences were used for identification of the exon/intron gene structure for each isolate.

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′-GAGGTTTATATTACGGATCATATA GAG-3′) and KES 1288 for *P. citricarpa* and *P. capitalensis* and with primer pair KES 124 (5′-CAGCTTCAGCTTTCTTTTTCTTAG-3′) and KES 1288 for *P. citriasiana* were done to identify codon 129 in all isolates. KES 126 was not usable for *P. citriasiana* because of several mismatches. Therefore, primer KES 124 (providing a longer PCR product) was used.

2.6. Species-specific detection of Phyllosticta species

PCR amplifications for species-specific detection of *P. citricarpa*, *P. capitalensis* and *P. citriasiana* 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 *Phyllosticta ampelicida* (syn. *Guignardia bidwellii*), *Penicillium digitatum*, *Penicillium italicum*, *Geotrichum candidum*, *Rhizopus stolonifer*, *Aspergillus niger*, *Alternaria alternata*, *Alternaria solani*, *Botrytis cinerea*, *Fusarium graminearum*, *Colletotrichum gloeosporioides*, *Monilinia fructigena*, *Monilinia laxa*, *Peronophythora litchii*, *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, 1.0, 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). EC$_{50}$ 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 QoIs.

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. capitalensis* 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. capitalensis: Conidia (8−)10−12 × (5−)6−7 µm, solitary, hyaline, aseptate, thin- and smooth-walled, coarsely guttule, 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. Asci 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 ellipsoidal 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 300 bp for all isolates. Bands were weak for *P. capitalensis* isolates but clearly visible and reproducible in various independent trials in two different laboratories (Citrus Research International, Nelspruit and BASF, Limburgerhof) using different PCR equipment (Table 2). PCR using primer pair "PB-N-ITS-Gc" (Peres et al., 2007) resulted in an amplification product of 490 bp for *P. citricarpa* isolates and the

Table 2
Overview of properties of isolates of *Phyllosticta citricarpa*, *P. capitalensis* and *P. citriasiana*.

Properties	*P. citricarpa* (n = 9)	*P. capitalensis* (n = 6)	*P. citriasiana* (n = 2)
Yellowing of oat meal agar	Yes	No	No
PCR product with "PB-N-ITS-Gc"	++	−	++
PCR product with "NP-BR-ITS-Gc"	++	+	++
PCR product with "PB-N-ITS-Gm"	−	+	−
PCR product with "NP-BR-ITS-Gm"	−	+	
PCR product with KES 556 + KES 1288 (size)	++	++	++
Amplicon size (bp) with KES 556 + KES1288	7634 bp	1419 bp	4221 bp
Intron after codon 129/137/143	No/No/Yes	No/No/No	No/No/No
Mean EC$_{50}$ pyraclostrobin [μg ml^{-1}]/DF	0.0033/1.3	0.0422/18.6	0.0037/1.3
Mean EC$_{50}$ azoxystrobin [μg ml^{-1}]/DF	0.0069/2.2	0.2237/11.5	0.0092/2.3

"++" means strong, "+" weak, "-" no PCR product visible under described conditions. Mean EC$_{50}$ values were calculated as geometric means, diversity factors (DF) by dividing maximum value by minimum value.

two *P. citriasiana* isolates. No isolate of *P. capitalensis* produced any band (Table 2).

PCRs 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.
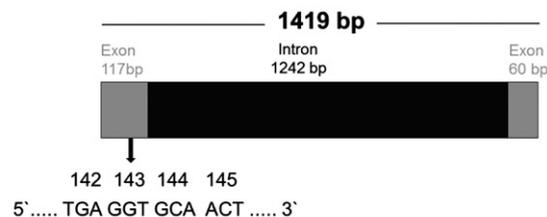
3.5. Species-specific detection of Phyllosticta species

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),
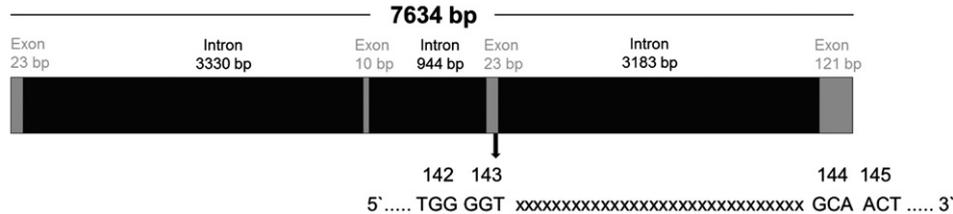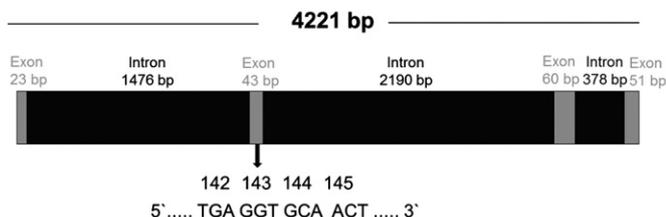

Fig. 1. Intron/exon organization of the partial cytochrome *b* gene of three *Phyllosticta* species as derived by PCR amplification of DNA and cDNA with primer pair KES 556 + KES 1288. Amplification products with DNA as template resulted in different sizes among species. Amplicons using cDNA template were of same size (177 bp) for all isolates. Alignments show gene structures for each species. Codon 143 of *P. citricarpa* borders directly an intron sequence.

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                   P  R  T  L  V  W  T  I  G  T  F  I  L  I
P. citricarpa      CTCCTAGAACACTTGTTTGAACTATAGGTACAATTATCTTAAT

P. capitalensis    CTCCTAGAACACTTGTTTGAACTATAGGTACAATTATCTTAAT

P. citriasiana     CTCCTAGAACACTTGTTTGAACTATAGGTACAATTATCTTAAT


  L  M  M  A  T  A  F  L  G  Y  V  L  P  Y  G  Q  M  S  L
CTTAATGATGGCAACAGCATTCTTGGGTTATGTTTTACCTTATGGTCAAATGAGTTTAT

CTTAATGATGGCTACAGCTTTCTTGGGTTATGTATTACCTTATGGTCAAATGAGTTTAT

CTTAATGATGGCAACAGCATTCTTGGGTTATGTGTTGCCCTATGGTCAAATGTCACTTT


  W  G  A  T  V  I  T  N  L  M  S  A  I  P  W  I  G  Q  D  I
GGGGTGCAACTGTTATTACAAACTTAATGAGTGCTATACCTTGAATTGGACAAGATATA

GAGGTGCAACTGTTATTACAAACTTAATGAGTGCTATACCTTGAATAGGACAAGATATA

GAGGTGCAACTGTTATTACAAACTTAATGAGTGCTATTCCTTGAATAGGTCAGGACATA


  V  E  F  I  W  G  G  F  S  V  N  N  A  T  L  N
GTCGAATTTATCTGAGGTGGCTTTTCAGTTAATAATGCTACTTTAAAT

GTTGAGTTTATTTGAGGTGGGTTTTCAGTTAATAATGCTACATTAAAT

GTTGAATTTATTTGAGGTGGGTTTTCAGTTAATAATGCTACATTAAAT
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Fig. 2. Sequence alignment of a 209 bp fragment of the cytochrome *b* gene of *Phyllosticta citricarpa*, *P. capitalensis* and *P. citriasiana*. Fragments were obtained by PCR amplification of cDNA with primers KES 126 and KES 1288 for *P. citricarpa* and *P. capitalensis*, and with KES 124 and KES 1288 for *P. citriasiana*. Codons of interest for QoI sensitivity (F129, G137 and G143) are bold. No mutations were found in any isolate at these positions. Single nucleotide polymorphisms were not detected intraspecific but interspecific. Differences to the *P. citricarpa* sequence are underlined. Amino acid sequence is given above. No SNP caused amino acid exchanges.

P. capitalensis (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

EC$_{50}$ 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 EC$_{50}$ values were measured for *P. capitalensis*, which spanned EC$_{50}$ 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 EC$_{50}$ 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
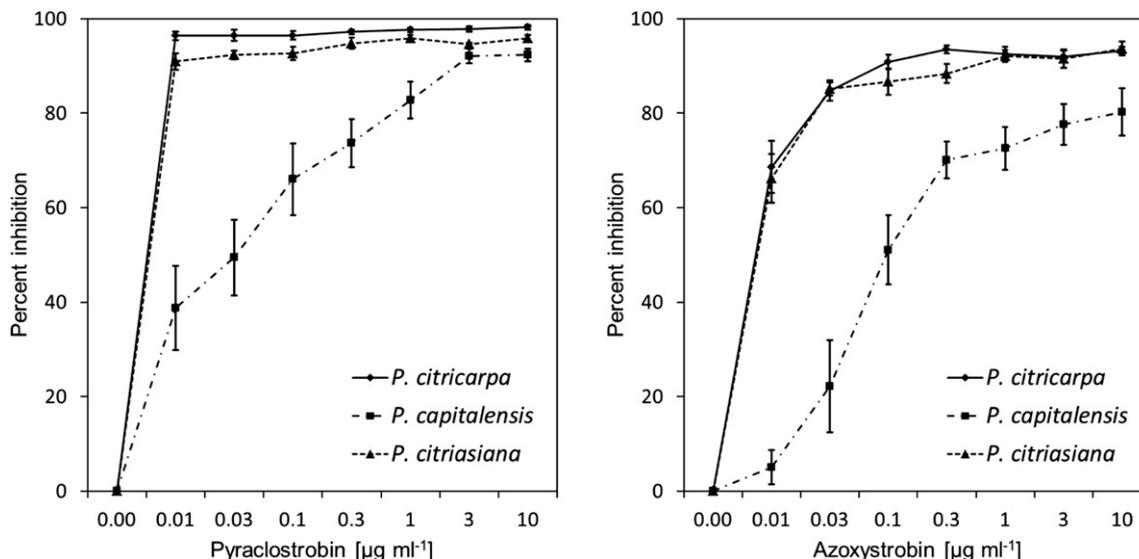
Fig. 3. Inhibition of *Phyllosticta citricarpa*, *P. capitalensis* and *P. citriasiana* by pyraclostrobin (left) and azoxystrobin (right). Curves are made by mean values, standard deviation is provided by vertical lines.

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, EC_{50} 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 mRNA 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 (Miessner 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 QoI is considered low, citrus growers currently using tank mixtures 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 QoIs may promote resistance development in *P. capitalensis* towards QoIs. 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 QoIs.

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 QoIs, resistance management strategies should be followed also for *P. citricarpa*. This can be done by limitation the number of QoI 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 antiresistance management strategies. The knowledge of the DNA sequences 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; Stammler 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* (Miessner and Stammler, 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. tritici*. Additionally no products were amplified with the oomycetes *Per. litchii* and *Pythium aphanidermatum,* nor with the basidiomycete *Rhizoctonia solani* and the zygomycete *R. 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.

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