Quantitative phenotyping of grey leaf spot disease in maize using real-time PCR

J. Korsman · B. Meisel · F. J. Kloppers · B. G. Crampton · D. K. Berger

Abstract Grey leaf spot is an important maize foliar disease caused by the fungal pathogens *Cercospora zeae-maydis* and *Cercospora zeina*. Although methods exist to detect these Cercospora species in maize, current techniques do not allow quantification of the fungi *in planta*. We developed a real-time SYBR® Green PCR assay for quantification of grey leaf spot disease in maize based on the amplification of a fragment of a cytochrome P450 reductase (*cpr1*) gene. *In planta* fungal DNA content was normalised to a maize glutathione S-transferase III gene (*gst3*) to yield values of ng Cercospora DNA/mg maize DNA. The assay was specific to the two *Cercospora* spp., and we observed no amplification of the *cpr1* fragment in non-target maize leaf pathogens or saprophytes. The assay was employed to quantify *C. zeina* in glasshouse inoculated maize plants and grey leaf spot infected field plants of resistant and susceptible maize lines. In both instances, *C. zeina* DNA content correlated with symptomatic leaf lesion area, and the susceptible maize line contained significantly more *C. zeina* DNA than the resistant line. Sequence differences between the *C. zeina* and *C. zeae-maydis cpr1* amplicons enabled us to perform melt curve analyses to identify the Cercospora species causing grey leaf spot at a particular location. This assay has application in the early detection and quantification of *Cercospora* spp., both of which are important tools in grey leaf spot disease management and maize breeding programmes.

Keywords *Cercospora zeae-maydis* · *Cercospora zeina* · Precision phenotyping · Quantitative PCR · Grey leaf spot · Gray leaf spot · Real-time PCR · qPCR

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Abbreviations

Ct	cycle threshold
cpr1	cytochrome P450 reductase 1 gene
dpi	days post inoculation
GLS	grey leaf spot/gray leaf spot
gst3	glutathione S-transferase III gene
ITS	internal transcribed spacer
rDNA	ribosomal DNA

Introduction

Grey/gray leaf spot (GLS) caused by fungi of the genus Cercospora is a foliar disease of maize of great economic importance in many countries. GLS can reduce grain yield by 20 to 60% depending on the level of susceptibility of the hybrid (Latterell and Rossi 1983, Ward et al. 1994). Infected maize crop debris is the source of primary inoculum. After periods of high humidity spores are wind-dispersed or rain-splashed onto bottom leaves of the plant and the disease then progresses upwards. Some infection later in the season also takes place when spores are blown in from adjacent fields. Typically, lesions develop from small spots which then become tan and rectangular. GLS has a long latent period with 14 to 28 days after infection before lesions produce conidia under humid conditions, which can be seen as a greyish cast. Lesions first run parallel with leaf veins but lesion expansion can result in blighting of entire leaves due to coalescing of lesions (Ward et al. 1999).

Depending on the geographic region, GLS disease of maize can be either caused by *Cercospora zeae-maydis* (formerly known as *C. zeae-maydis* Group I), which has been found throughout various areas of the U.S.A., Canada, Mexico and Brazil, or *Cercospora zeina* (formerly known as *C. zeae-maydis* Group II), which has been reported in the Eastern maize growing states of the U.S.A., Brazil and sub-Saharan Africa (Wang et al. 1998, Dunkle and Levy 2000, Goodwin et al. 2001, Zhu et al. 2002, Okori et al. 2003, Shim and Dunkle 2005, Crous et al. 2006, Brunelli et al. 2008, Meisel et al. 2009). Characteristics and dimensions of conidia and conidiophores produced on infected plants or nutrient media were found to be unreliable criteria for taxonomic differentiation of *C. zeae-maydis* and *C. zeina* (Wang et al. 1998). Crous et al. (2006) developed a PCR-based test to distinguish the two species based on species-specific primers designed from the histone H3 gene. Dunkle and Levy (2000) employed restriction analysis of internal transcribed spacer (ITS) and 5.8S ribosomal DNA (rDNA) regions to differentiate *C. zeae-maydis* and *C. zeina* isolates. Although these methods can distinguish the causal agents of GLS in maize, there are currently no available techniques to quantify *Cercospora* spp. levels in infected maize leaves.

Quantitative PCR (qPCR) offers a reliable and sensitive method for detecting and quantifying fungi *in planta*, and has particular application in pathogen detection prior to symptom development. Recently, De Coninck and co-workers (2011) developed a qPCR assay to quantify *Cercospora beticola* in sugar beet leaves, which will help breeders to discriminate minor differences in resistance in a segregating population. A number of recent studies have employed qPCR to quantify pathogens on maize material. *Fusarium* spp. are a major source of mycotoxin contamination in maize, and quantification of fungal biomass is essential to understanding interactions between individual species in disease development. Waalwijk et al. (2008) developed a qPCR assay based on a mycotoxin biosynthesis gene to detect mycotoxin producing *Fusarium verticillioides* isolates in maize grain from subsistence farmers in South Africa, and correlated fungal DNA content with mycotoxin levels. Nicolaisen et al. (2009) established a SYBR Green qPCR assay based on the elongation factor 1 alpha gene to detect and quantify eleven *Fusarium* sp. in maize and wheat field material. These studies demonstrate the importance of qPCR for quantification of fungal pathogens in order to select resistant plants in breeding programmes.

The objective of this study was to develop a quantitative PCR assay (qPCR) to measure the amount of *Cercospora* sp. DNA in infected maize leaves. We hypothesised that a qPCR assay based on *cpr1*, a putative cytochrome P450 reductase (van den Brink et al. 1995), would accurately quantify *Cercospora* spp. DNA content in glasshouse inoculated and field infected maize leaves. Results obtained were significant as the qPCR assay was specific to *Cercospora* spp., and showed good correlation between *C. zeina* DNA content and leaf lesion area measured by digital imaging. In addition, we could distinguish between *C. zeae-maydis* and *C. zeina*, both causal agents of GLS in maize. Quantification of *Cercospora* spp. in maize leaves has important application in the breeding and selection of GLS resistant maize lines.

Materials and Methods

Biological material and host plant infection

The *C. zeae-maydis* ex-type culture CBS 117757 as well as other *C. zeae-maydis* cultures CBS 117755, CBS 117758, CBS 117759, CBS 117760, CBS

117761, CBS 117762, CBS 117763, the *C. zeina* ex-type culture CBS118820, and *Cercospora* sp. CPC 12062 were obtained from the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. The *C. zeina* cultures CMW 25463, CMW 25467, CMW 25445, CMW 25442, CMW 25459, CMW 25465, CMW 25462, CMW 25454, CMW 25452 and CMW 25466 are described in Meisel et al. 2009. Fungal cultures were grown on V8 medium (800 ml of distilled water, 200 ml of V8 juice, 15 g of agar, and 2 g of $CaCO_3$) in the dark at 25°C. DNA from *C. zeae-maydis* cultures WB IL and WB KS, and from *C. zeina* cultures WB VA, WB UG and WB BZ were obtained from W-B Shim, Texas A & M University, College Station, Texas, USA.

Maize leaves showing typical symptoms of GLS (*C. zeina*), northern corn leaf blight (*Exserohilum turcicum*), Phaeosphaeria leaf spot (*Phaeosphaeria maydis*), Hyalothyridium leaf spot (*Hyalothyridium maydis*), zonate leaf spot (*Gloeocercospora sorghi)* and tropical Diplodia (*Stenocarpella macrospora*) were collected from Greytown and other sites in South Africa. Maize leaves showing symptoms of GLS were collected from fields in Brookston, Indiana, USA; Argentina, Mpongwe and Gart farm, Zambia. DNA was isolated under quarantine conditions from diseased maize material collected outside South Africa.

Saprophytes were isolated from GLS lesions by scraping a needle over a lesion and streaking it out on V8 medium. Single spores of *Epicoccum* sp., *Cladosporium* sp., *Phoma* sp., *Tilletiopsis* sp., *Sporobolomyces* sp. and *Cryptococcus* sp. were placed on separate plates and grown for DNA isolation. Sequences of the ITS region amplified using primers ITS1/4 (White et al., 1990) were used for identification by BLASTN analysis against Gen-Bank (Altschul et al. 1990).

Maize plants of a *C. zeina*-susceptible hybrid PAN 6724B were planted in a glasshouse at 28 +/− 4°C under a 16 h day length. Sporulation of *C. zeina* culture CMW 25463 grown on V8 medium was achieved by incubating the plates under equal periods of light and dark (12 h of fluorescent light, 12 h of dark). Conidia were dislodged with a brush and rinsed with 0.01% Tween 20. The inoculum was diluted to 3×10^5 conidia/ml and applied onto both surfaces of all three leaves (V3-stage) of the maize plants with a small brush. Control plants were treated with 0.01% Tween 20. Plants were covered with transparent plastic bags for 5 days to increase humidity levels. Leaves developed typical lesions 13 to 19 dpi. Leaf pieces from different plants were photographed using a SLR digital camera for image analysis, and sampled at 19 days post inoculation (dpi) for DNA extraction.

Field grown maize material was collected from two inbred lines, one that is moderately resistant to grey leaf spot disease (line PEX1), and another that is susceptible (line PEX2). Leaf samples were taken 15 weeks after planting from nine individual plants from each line that were positioned randomly within a field site situated in Kwazulu-Natal Province, South Africa. Grey leaf spot disease is prevalent in this region, and therefore the plants were exposed to a natural inoculum of *C. zeina* (Meisel et al., 2009). A 12 cm length of the leaf immediately above the lowest cob from each plant was marked with a permanent black marker pen. Photographs were taken prior to sampling of the leaf piece for subsequent DNA extraction.

Digital image analysis

The proportional areas of maize leaf pieces with grey leaf spot lesions from the glasshouse and field experiments were calculated from the photographs of the 12 cm leaf pieces using ASSESS 2.7 software (L. Lamari, American Phytopathological Society, St. Paul, MN, USA), as described in De Coninck et al., 2011.

DNA extraction from fungi and plants

DNA from 50 to 100 mg of fungal cultures, as well as from 50 to 100 mg of control or diseased maize leaves was isolated according to Moller et al. (1992). Integrity of the DNA was checked on a 0.8% agarose gel and concentration was determined with a Nano-Drop^{TM} 1000 spectrophotometer (Thermo Scientific) at 260 nm.

Conventional and quantitative PCR amplification

Two cDNA sequences of a *C. zeae-maydis* putative cytochrome P450 reductase (GenBank accession numbers AF448828 and FG242129) were used to compile a composite sequence for primer design, resulting in primers CPR1_1F (5′-TCCACTCTCGCTCAATTCG-3′) and CPR1_1R (5′-GCCTTCATCGCCATATGTTC-3′). These primers, designated CPR1_1, were used to amplify a 1.1 kb product from *C. zeina* genomic DNA, that

was sequenced on an ABI PRISM™ 3100 Automated DNA sequencer (Applied Biosystems) using the ABI Prism Big Dye Terminator Cycle sequencing reaction kit v3.1 (Applied Biosystems). The internal primers, designated CPR1_2, and named and CPR1_2F (5′-TGAACTACGCGCTCAATG-3′) and CPR1_2R (5′-TCTCTCTTGGACGAAACC-3′) were designed in two short regions of 100% identity between the two species. The primers GST3F (5′-GGAGCCCTGAGTCGAATAAAAG-3′) and GST3R (5′-AACACACACGAAAGGCAACAGT-3′) were used to amplify a 106 bp-fragment of the glutathione S-transferase III gene *gst3* from maize (GenBank accession number X06755).

For conventional PCR, reaction volumes of 15 μl consisted of 1x NH$_4$ PCR reaction buffer, 2.7 mM of MgCl$_2$, 0.1 μM of each dNTP (Bioline), 0.2 μM each of the primers, 1 U of BIOTAQ™ DNA Polymerase (Bioline), 11.15 μl of sterile distilled water and 10 ng of DNA template. Cycling conditions were 2 min at 94°C followed by 35x (15 s at 94°C, 15 s at 60°C, 20 s at 72°C). A final elongation step was performed at 72°C for 5 min. Quality of all PCR products was verified by gel electrophoresis.

The *cpr1*-and *gst3*-fragments amplified in conventional PCR were ligated into the pJET1.2/blunt vector from the CloneJET™ PCR Cloning Kit (Fermentas) and subsequently transformed into chemically competent *Escherichia coli* using the manufacturer's instructions. Transformed *E. coli* were grown at 37°C overnight in Luria Bertani broth supplemented with 100 μg/ml ampicillin. Purification of the plasmid DNA was performed using the Invisorb Spin Plasmid Mini Two Kit (Invitek). Plasmid DNA was used as template for sequencing reactions as described above. Sequences were checked by BLASTN (Altschul et al. 1990).

Quantitative PCR amplifications were performed in a total volume of 10 μl on a LightCycler® 480 instrument (Roche Diagnostics Corp.). Each reaction contained 1 μl of the DNA template or water in the non-template controls, 5 μl of the LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics Corp.), 0.2 μM of each primer, and 3.6 μl of sterile distilled water. Each PCR was run in triplicate. Cycling conditions consisted of 5 min at 95°C followed by 45x (10 s at 95°C, 10 s at 60°C, 5 s at 72°C). Fluorescence was detected at the end of each elongation step. The amplification specificity of the PCR was investigated by melting curve analysis after every run.

The specificity of the CPR1_2 primers was tested on 10 ng of DNA isolated from healthy maize leaves and maize leaves with typical symptoms of GLS (*C. zeae-maydis* and *C. zeina*), northern corn leaf blight (*E. turcicum*), Phaeosphaeria leaf spot (*P. maydis*), Hyalothyridium leaf spot (*H. maydis*), zonate leaf spot (*G. sorghi*), tropical Diplodia (*S. macrospora*), and a number of fungi and yeast isolated from GLS lesions (*Epicoccum* sp., *Cladosporium* sp., *Phoma* sp., *Tilletiopsis* sp., *Sporobolomyces* sp., *Cryptococcus* sp.). The specificity of the GST3 primers was tested on 10 ng of DNA isolated from healthy maize leaves, *C. zeina,* and *C. zeae-maydis* cultures and lesion samples. To exclude false negative results template DNA samples from lesions were tested for PCR amplification using primers ITS1/4 following the method of White et al. (1990).

In order to estimate the amount of fungal DNA in the infected leaf samples, DNA extracted from pure *C. zeina* and *C. zeae-maydis* cultures was diluted in maize DNA of concentration 10 ng μl^{-1}. The final fungal DNA concentrations were 5, 1, 5×10^{-1}, 2.5×10^{-1}, 1×10^{-1}, 5×10^{-2}, 2.5×10^{-2}, 1×10^{-2}, 5×10^{-3}, 2.5×10^{-3}, 1×10^{-3} ng μl^{-1}. These dilutions were used to determine the detection limits of the CPR1_2 primer pair in a simulated lesion sample. A serial dilution of DNA extracted from healthy leaves (2×10^1, 1.5×10^1, 1.25×10^1, 1×10^1, 5, 1, 5×10^{-1}, 1×10^{-1}, 5×10^{-2} ng μl^{-1}) was prepared to measure detection limits of the GST3 primers.

Standard curves were prepared by plotting log$_{10}$ of the DNA concentration of known standards against the cycle number at which the fluorescent signal from the amplified PCR products surpassed the detection threshold. Regression curves were drawn and the qPCR efficiency was calculated as equal to $10^{(-1/\text{slope})}$, the optimal efficiency thus equalling two. The amount of target DNA for unknown samples was extrapolated from the respective standard curves. To normalise gene quantification between different samples the amount of fungal *cpr1* was divided by the amount of plant *gst3* quantified in infected leaves.

Results

### Design of a specific PCR assay for *Cercospora* spp. in infected maize leaves

CPR1_1 primers were designed to the sequence of the *C. zeae-maydis cpr1* gene, which encodes a

Fig. 1 Alignment of a 164 bp fragment of *cpr1* from *C. zeae-maydis* CBS 117757 (Czm_cpr1) and *C. zeina* CBS 118820 (Cz_cpr1). The CPR1_2F and CPR1_2R primers used for the qPCR assay that are identical between the species are shown. *Nla*VI sites are underlined. Consensus nucleotides are indicated with an asterisk

```
                    5' TGAACTACGCGCTCAATG 3' CPR1_2F primer
                    1                                                          60
Czm_cpr1   TGAACTACGCGCTCAATGGTCCACGAAACAAGTACGATGGCATCCACGTCCCGGTCCACA
Cz_cpr1    TGAACTACGCGCTCAATGGTCCACGAAACAAATACGATGGCATCCACTTCCCGGTTCACA
           ************************************ *************** ****** ****

                    61                                                         120
Czm_cpr1   TTCGACACTCGAACTTCAAGCTCCCCTCGGATCCAAGCAAGCCGATCATCATGGTTGGCC
Cz_cpr1    TTCGACACTCGAACTTCAAGCTCCCCTCAGACCCAAGCAAGCCCATTATCATGGTTGGTC
           *************************** ** ********** ** ********** *

                    121                                  164
Czm_cpr1   CTGGTACCGGCGTTGCTCCATTCCGCGGTTTCGTCCAAGAGAGA
Cz_cpr1    CTGGCACCGGCGTTGCTCCATTCCGCGGTTTCGTCCAAGAGAGA
           **** ***************************************

                    CPR1_2R primer  3'CCAAAGCAGGTTCTCTCT 5'
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cytochrome P450 reductase first described in *A. niger* (van den Brink et al. 1995). These primers were used to amplify 967 bp of this gene from *C. zeae-maydis* and 1,024 bp from *C. zeina*. Alignment of the *cpr1* gene sequences from *C. zeae-maydis* and *C. zeina* enabled us to design internal primers (designated CPR1_2; see Materials and Methods) to conserved areas of the gene sequence (Fig. 1). Conventional and quantitative PCR produced a 164 bp fragment in *C. zeina* and *C. zeae-maydis* isolates, but no amplicon was obtained from uninfected maize leaves or leaves infected with non-target maize pathogens

(*Exserohilum turcicum, Phaeosphaeria maydis, Hyalothyridum maydis, Gloeocercospora sorghi, Stenocarpella macrosporum*) (Fig. 2a). In addition, the CPR1_2 primer set did not produce an amplification product from saprophytic organisms (*Epicoccum* sp, *Cladosporium* sp., *Phoma* sp., *Tilletiopsis* sp., *Sporobolomyces coprosmae, Cryptococcus flavescens*) isolated from maize leaves. As a check for DNA quality, all samples from infected leaves and saprophytic organisms were successfully subjected to conventional PCR analysis using the ITS1 and ITS4 primers (Fig. 2c).
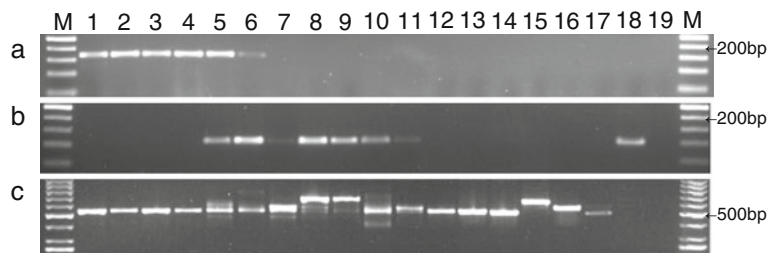
Fig. 2 PCR amplification of *cpr1* **a**, *gst 3* **b** and ITS1/4 **c** gene fragments from *C. zeae-maydis* and *C. zeina* cultures, DNA extracted from grey leaf spot lesions, other maize foliar pathogens, and saprophytic organisms isolated from grey leaf spot lesions. **a** CPR1_2 primers amplified fragments of 164 bp from *C. zeae-maydis* and *C. zeina*, but not from other fungal pathogens and saprophytes isolated from grey leaf spot lesions. **b** GST primers amplified the *gst3* fragment only from samples derived from lesions on maize leaves. **c** The ITS1/4 region was amplified from all samples containing fungi/yeast and indicates DNA quality is adequate for DNA amplification. Lanes M, 50 bp molecular marker (Fermentas). Templates used for each reaction were as follows: lanes 1–4, *C. zeae-maydis* CBS117755, *C. zeae-maydis* CBS117757, *C.* *zeina* CBS118820, *C. zeina* CMW25467 respectively; lanes 5–6, DNA isolated from lesions containing *C. zeae-maydis* from Argentina, and *C. zeina* from Greytown, RSA, respectively; lanes 7–11, DNA isolated from maize foliar diseases caused by *Exserohilum turcicum* (Northern corn leaf blight), *Phaeosphaeria maydis* (Phaeosphaeria leaf spot), *Hyalothyridum maydis* (Hyalothyridum leaf spot), *Gloeocercospora sorghi* (zonate leaf spot), *Stenocarpella macrosporum* (tropical diplodia), respectively; lanes 12–17, grey leaf spot saprophytes *Epicoccum* sp., *Cladosporium* sp, *Phoma* sp., *Tilletiopsis sp., Sporobolomyces coprosmae* and *Cryptococcus flavescens*, respectively; lane 18, non-diseased maize tissue; lane 19, no template PCR control

Since *cpr1* appears to be conserved in many fungal species, the specificity of the primers was verified by comparison to corresponding regions of the *cpr1* orthologues from other fungi available from GenBank (Supplementary Figure 1). The closest *cpr1* orthologue is from the non-plant pathogen *Podospora anserina*, and this shows five and four mismatches with the CPR1_2F and CPR12R primers, respectively (Supplementary Figure 1). Other close orthologues from plant pathogens such as *Pyrenophora* spp. pathogenic on wheat, as well as *Alternaria brassicicola*, show increasing numbers of mismatches with the primers. It is therefore unlikely that amplification would occur from related pathogens on maize under the stringent conditions of the qPCR assay. This was borne out by the empirical evidence that shows no amplification from other fungi from maize (Fig. 2a), whose *cpr1* sequences are not yet available on GenBank.

Conventional PCR with GST3F/GST3R primers produced specific amplicons of the expected 106 bp when templates contained DNA from maize (Fig. 2b). Sequencing of *gst3* amplicons showed a nucleotide sequence exhibiting 100% identity with the *gst3* gene from *Zea mays* (GenBank accession number X06755). No PCR products were amplified from pure fungal cultures of *C. zeae-maydis* or *C. zeina* using the GST3F/GST3R primers (Fig. 2b).

qPCR method to quantify *Cercospora* spp. in infected maize tissue

To quantify the pathogen in biological samples, a dilution series of genomic DNA from *C. zeina* and *C. zeae-maydis* was added to 10 ng of DNA extracted from healthy maize creating progressively lower fungal DNA concentrations from 5 to 1×10^{-3} ng μl^{-1} to generate standard curves for *cpr1* (Fig. 3a and b, respectively). Efficiency of amplification of the 164 bp *cpr1* fragment in maize carrier DNA was 1.84, with a minimum detection limit of 5 pg *C. zeina* or *C. zeae-maydis* DNA. Quantifications of the pathogens showed a linear relationship between $\log_{10}$ values of the amount of DNA and cycle threshold values, indicating that the method is suitable as a quantitative assay over the concentration range tested. The qPCR efficiency of maize *gst3* was 1.68 (Fig. 3c). Both melt curve analyses and agarose gel electrophoresis of qPCR reactions indicated that a single product was
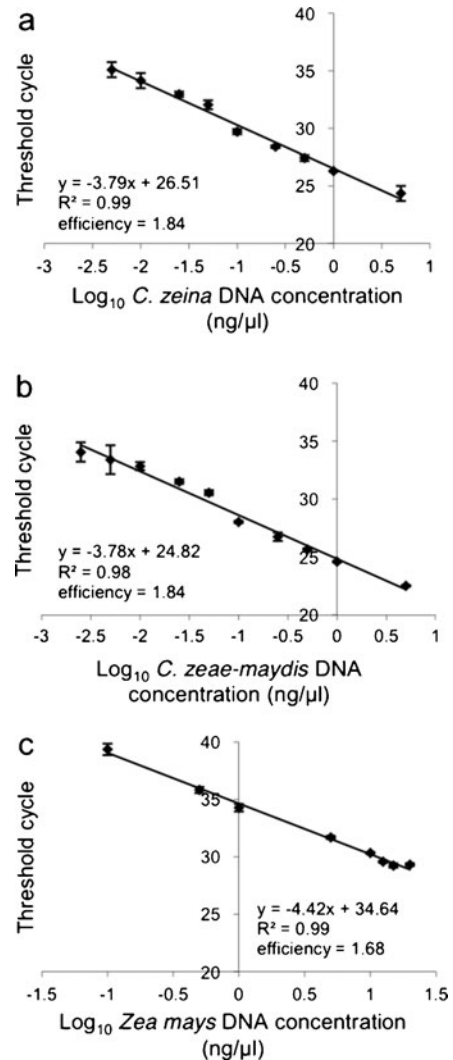
Fig. 3 Standard curves used for qPCR assay to quantify *C. zeina* and *C. zeae-maydis in planta*. The graphs illustrate the efficiency and sensitivity of *cpr1* and *gst3* fragment amplification using the CPR1_2 primers and GST primers, respectively. Figures a and b represent standard curves of *C. zeina* and *C. zeae-maydis* DNA diluted in maize carrier DNA to a total of 10 ng DNA, respectively. Figure c indicates a standard curve for *gst3* amplification from serial dilutions of maize genomic DNA

obtained for *C. zeina* and *C. zeae-maydis cpr1* and for maize *gst3*.

qPCR quantification of *C. zeina* DNA in glasshouse inoculated maize leaves before and after lesion development

Amplification of the *cpr1* gene by qPCR was undertaken in order to assess *C. zeina* content in maize

leaves after inoculation with *C. zeina* in the glasshouse. DNA extractions were performed on three leaf segments (Fig. 4 a, b and c) with approximately 2, 4 and 16% infected leaf area, respectively, and subjected to *cpr1* and *gst3* qPCR. The amount of *C. zeina* DNA in each leaf sample was determined from a *cpr1* standard curve (Fig. 3a), and normalised to the amount of maize DNA as calculated from a *gst3* standard curve (Fig. 3c). In this manner, leaf samples in Fig. 4 a-c were estimated to contain 4.7 ± 0.3, 7.8 ± 1.1 and 46.0 ± 9.2 ng *C. zeina* DNA/mg maize DNA, which was proportional to 2, 4 and 16% infected leaf area, respectively. In addition, we were able to detect *C. zeina* DNA in inoculated maize leaves prior to the development of GLS symptoms (results not shown). These results verified that the qPCR assay using *cpr1* and *gst3* genes was an effective method for quantifying grey leaf spot disease in glasshouse experiments.

C. zeina fungal content in GLS resistant and susceptible maize lines

The qPCR assay was applied to quantify *C. zeina* in field infected maize leaves. Lesion area was determined for nine biological replicates from each of two maize inbred lines PEX1 and PEX2 (resistant and susceptible, respectively) (Fig. 4d and e) using digital image analysis. Differences in the number and area of lesions were observed between the two maize lines (Fig. 4d and e). DNA was subsequently extracted from these samples and subjected to *cpr1* and *gst3* qPCR. The amount of *C. zeina* DNA was normalised relative to the amount of maize DNA (*gst3* qPCR), and a comparison was made to leaf lesion area (Fig. 5). There was good correlation between amount of *C. zeina* DNA and leaf lesion area (Pearson correlation= 0.76), and the quantification of *C. zeina* in the resistant maize line was significantly less than the susceptible line as determined by ng *C. zeina*/mg maize tissue (Student's *T*-test; $p < 0.005$) and scanned lesion area (Student's *T*-test; $p < 5 \times 10^{-5}$).

Implementation of qPCR assay to differentiate between *C. zeae-maydis* and *C. zeina*

GLS in maize is caused by both *C. zeae-maydis* and *C. zeina*. Sequence differences between *C. zeina* and *C. zeae-maydis* in the 164 bp *cpr1* amplicon (Fig. 1: nine nucleotides differ) allowed us to detect which of the two species was the causal agent of GLS on a symptomatic maize leaf. Melting curve analysis of known *C. zeae-maydis* and *C. zeina* isolates yielded significantly different single peaks of 85.28 ± 0.07 and 84.53 ± 0.07 respectively (two-tailed *T*-test, $p < 0.05 \times 10^{-15}$) (Table 1; Supplementary Figure 2). Importantly, this differentiation was clear for a wide range of isolates tested, namely ten isolates of *C. zeae-maydis* from different sites in the USA, and 14 isolates of *C. zeina* from diverse sites in the USA and Africa, including samples amplified directly from GLS lesions (Table 1).

Fig. 4 Photographs of leaves with symptoms of GLS from glasshouse and field grown maize that were used for GLS disease quantification shown in Figure 5. Figures **a**, **b** and **c** indicate leaf pieces with different areas of GLS lesions from glasshouse grown maize plants (hybrid PAN 6724B) at 19 days post inoculation with *C. zeina*. Figures **d** and **e** show leaf pieces of nine biological replicates each from two field grown maize lines PEX1 and PEX2 that are resistant and susceptible to grey leaf spot, respectively
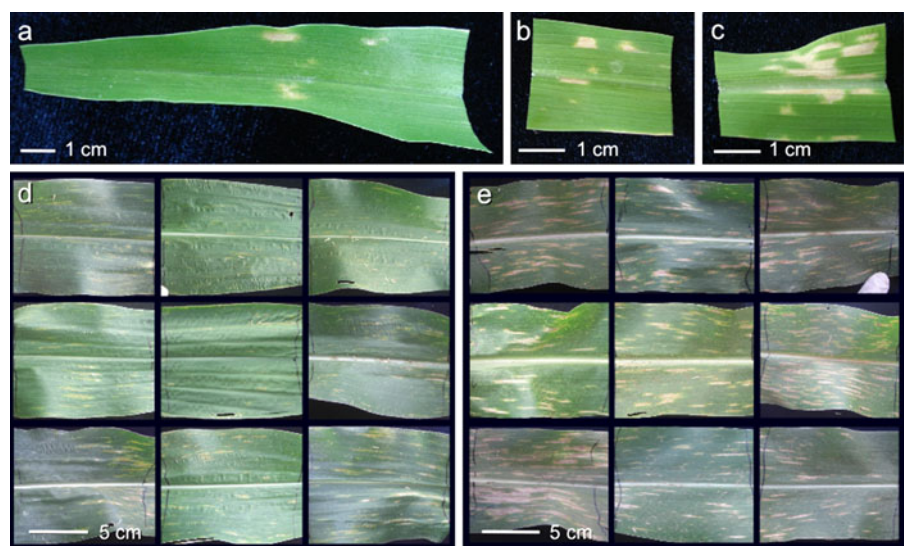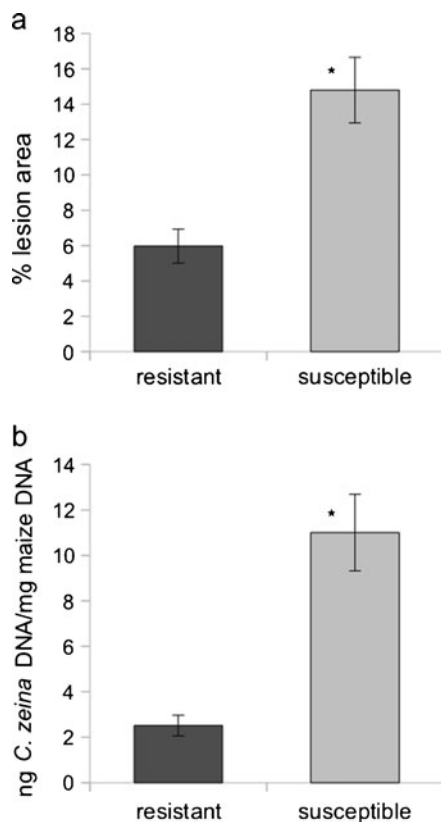
Fig. 5 Comparison of *C. zeina* infection in resistant and susceptible maize lines using **a** digital image analysis and **b** qPCR. **a** Nine biological replicate leaf pieces from each resistant (PEX1) and susceptible (PEX2) maize line were photographed and GLS lesion area relative to total leaf area was determined for the two lines. **b** Thereafter, DNA was extracted from these maize leaf pieces and subjected to the qPCR assay to determine ng Cercospora DNA per mg maize DNA. Bars on graphs indicate standard error. Asterisks indicate that the susceptible cultivar shows significantly different values to the resistant cultivar (Student's *T*-test; $p < 0.005$)

The qPCR assay was also successful in amplification of the 164 bp *cpr1* product from a distinct Cercospora isolate from maize in South Africa, named *Cercospora* sp. CPC 12062 (Crous et al. 2006), which yielded a Tm=84.59±0.14, which is not significantly different from *C. zeina* (Table 1; Supplementary Figure 2). This was to be expected since the sequences corresponding to the CPR1_2 primers were identical, and there was only one base pair difference between the 164 bp *cpr1* amplicons of these two species (Supplementary Figure 1).

An alternative differentiation between the two species known to be pathogens was evident from PCR-RFLP analysis of the 164 bp *cpr1* product, since the *C. zeae-maydis* amplicon has two *Nla*IV sites whereas *C.*

zeina (as well as Cercospora sp. CPC 12062; Supplementary Figure 1) has one *Nla*IV site. *Nla*IV digestion of the *C. zeae-maydis* amplicon yielded three fragments of 91, 39 and 34 bp, whereas the *C. zeina* amplicon yielded two fragments of 125 and 39 bp (Supplementary Figure 3).

Discussion

In this study, we developed a qPCR method to detect and quantify *C. zeae-maydis* and *C. zeina* DNA in maize leaves. Primers were designed to a cytochrome P450 reductase gene (*cpr1*) (Fig. 1), and the specificity was confirmed by testing against uninfected maize leaves and leaves infected with non-target maize pathogens (Fig. 2). Primers designed to the glutathione S-transferase III gene (*gst3*) from maize proved to be plant specific by the absence of cross-reaction with fungal DNA. Normalisation with the maize *gst3* was performed to compensate for differences in the amount of maize DNA in each sample.

The functional role of the product of the *cpr1* gene in *Cercospora* spp. is not yet known, although sequence similarity indicates that it has cytochrome p450 reductase activity. One of the ESTs from which the CPR1_1 primers were designed (AF448828) was initially annotated as *ctb4*, one of the cercosporin biosynthesis genes (Shim and Dunkle 2002). However, this EST does not correspond to any of the three oxido-reductases of the well-characterized cercosporin biosynthetic cluster in *Cercospora nicotianae* (Chen et al. 2007). This was supported by the fact that the second EST (FG242129) was cloned from *C. zeae-maydis* growing vegetatively, which are conditions when cercosporin biosynthesis is repressed (Bluhm et al. 2008). CPR1 was first described in *Aspergillus niger* (GenBank accession number Z26938), and appears to be conserved in many fungal species (Supplementary Figure 1). Importantly, the gene regions corresponding to the primers used in the qPCR assay for quantifying *Cercospora* spp. in this study are sufficiently variable in other fungal species (Supplementary Figure 1), and were shown in our study to be specific to *Cercospora* spp. (Fig. 2).

De Coninck and co-workers (2011) recently developed a qPCR assay to detect and quantify *Cercospora beticola* in sugar beet cultivars. These authors utilised a Taqman® probe and associated primers to detect the

Table 1 Melting temperatures of 164 bp qPCR products obtained with CPR1_2 primers from *Cercospora* spp. isolated from maize

Species	Isolate no./code[a]	Location[b]	Year[c]	Tm[d]	aveTm[e]
C. zeae-maydis	CBS 117755	Indiana, USA	2003	85.27±0.05	
	CBS 117757*	Wisconsin, USA	2000	85.31±0.03	
	CBS 117758	Iowa, USA	2004	85.22±0.05	
	CBS 117759	Tennessee, USA	1999	85.24±0.03	
	CBS 117760	Pennsylvania, USA	1999	85.33±0.02	85.28±0.07
	CBS 117761	Indiana, USA	1999	85.33±0.02	
	CBS 117762	Missouri, USA	2000	85.32±0.02	
	CBS 117763	Iowa, USA	1999	85.33±0.03	
	WB IL	Illinois, USA	–	85.12±0.05	
	WB KS	Kansas, USA	–	85.32±0.06	
C. zeina	CBS 118820*	KZN, South Africa	2005	84.48±0.05	
	CMW 25442	Art Farm, Zimbabwe	2007	84.56±0.04	
	CMW 25445	Gart Farm, Zambia	2007	84.52±0.02	
	CMW 25452	Karkloof, KZN, SA	2007	84.49±0.03	
	CMW 25454	Karkloof, KZN, SA	2007	84.45±0.02	
	CMW 25459	Winterton, KZN, SA	2007	84.53±0.00	
	CMW 25462	Cedara, KZN, SA	2007	84.44±0.01	
	CMW 25463	Greytown, KZN, SA	2007	84.48±0.02	
	CMW 25465	Cedara, KZN, SA	2007	84.49±0.06	84.53±0.07
	CMW 25466	Karkloof, KZN, SA	2007	84.56±0.03	
	CMW 25467	Mkushi, Zambia	2007	84.51±0.02	
	lesion DNA GF	Gart Farm, Zambia	2010	84.65±0.01	
	lesion DNA Mp	Mpongwe, Zambia	2010	84.64±0.01	
	lesion DNA Gtn	Greytown, KZN, SA	2010	84.68±0.06	
	WB VA	Virginia, USA	–	84.49±0.01	
	WB UG	Kamengo-Mpigi, Uganda	–	84.49±0.04	
	WB BZ	Goias, Brazil	–	84.57±0.07	
Cercospora sp.	CPC 12062	KZN, South Africa	–	84.59±0.14	

* ex-type cultures

[a] CBS = culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands

CMW = culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

CPC: Culture collection of Pedro Crous, housed at CBS

WB = isolates from W-B. Shim

[b] Location where the diseased maize material was collected

[c] Year of GLS lesion collection

[d] Melting temperature analyses were performed on the three technical replicates for each sample to obtain an average melting temperature and standard deviation

[e] Melting temperatures for isolates from each species were averaged to obtain a species melting temperature

C. beticola calmodulin gene in infected sugarbeet material. Fungal DNA levels were normalised to levels of an endogenous sugar beet DNA sequence. As was the case with our study, these authors found a significant correlation between fungal DNA levels and leaf lesion area, as well as a significant difference between *C. beticola* levels in susceptible and resistant sugar beet cultivars. Their assay is of particular value in glasshouse screening of sugar beet in which the inoculum is limited to *C. beticola*. Although the

authors state that the qPCR assay did not amplify from two other *Cercospora* species, it was not tested in field experiments. In contrast, we have demonstrated our qPCR assay to be specific and effective in maize field-grown material (Figs. 2, 4 and 5), conditions under which screening for quantitative resistance to this disease is most desirable.

Our qPCR assay was sensitive, and could detect the target pathogen in as little as 5 pg DNA for both *C. zeae-maydis* and *C. zeina* (Fig. 3). This represents an improvement in sensitivity compared to other DNA based detection methods such as dot blots that were able to detect 160 pg of DNA from *Sporisorium reiliana*, the cause of head smut in maize (Xu et al., 1999). Alternative approaches that are not based on DNA are less sensitive, such as ELISA-based assays (Ward et al., 2004) or measurement of the fungus-specific lipid ergosterol, which has been used for quantification of *Cercospora kikuchii* in soybean (Xue et al., 2006). Ergosterol is present in many fungal species, and thus its use is limited to controlled inoculations. In contrast, our qPCR assay is specific to *Cercospora* spp. from maize, and therefore can be applied in field experiments.

A detection limit of 5 pg for both C. zeae-*maydis* and *C. zeina* in our qPCR assay corresponds to approximately 125 genome equivalents. This is based on the assumption that both species have a genome size of ~40 Mb. This was the average genome size for ten of twelve sequenced Dothidiomycete genomes (March 2011; www.osti.gov/bridge/purl.cover.jsp?purl=/1012481-8TJMwv/). Nicolaisen et al. (2009) achieved a lower detection limit of 2.5 genome equivalents (0.1 pg) for *Fusarium* spp. in maize using primers to the elongation factor 1 alpha gene. A TaqMan® PCR assay based on *cpsD* from the maize pathogen *Pantoea stewartii* was able to detect 1 pg of purified *P. stewartii* DNA, the equivalent of 10^4 colony forming units per millilitre (Tambong et al. 2008). We were unable to compare the sensitivity of our qPCR assay with that for *C. beticola*, as De Coninck et al. (2011) based their detection limits on ΔCt values calculated by subtracting the Ct value of an endogenous sugar beet control from the Ct value of the *C. beticola* calmodulin gene.

C. zeae-maydis and *C. zeina* are closely related species, which both cause GLS in maize, but occur in different geographic regions. We have recently confirmed that *C. zeina* remains the causal agent of GLS of maize in southern Africa and that absence of *C. zeae-maydis* is highly likely (Meisel et al. 2009). Since

occurrence of both *Cercospora* species in one field is rare and has only been reported for some locations in Ohio and New York in the U.S.A. (Wang et al. 1998), it is not crucial that the quantitative assay presented here cannot quantify *C. zeina* and *C. zeae-maydis* separately within a sample. Sequence analysis of the 164 bp *cpr1*-amplicon revealed nine bp differences between the two *Cercospora* species (Fig. 1). These sequence differences allowed us to employ melt curve analyses to distinguish between species, and could be utilised to detect a mixed infection within a maize leaf (Table 1; Supplementary Figure 2). There was only one base pair difference between the *cpr1* amplicon from *C. zeina* and *Cercospora* sp. CPC 12062, a distinct isolate from maize in South Africa (Crous et al., 2006)(Supplementary Figure 1), and thus these two species could not be distinguished based on Tm (Table 1; Supplementary Figure 2). It is not known if this isolate is a pathogen on maize, however it would be quantified by the qPCR assay if present.

Resistance to grey leaf spot disease is generally quantitative in nature (Balint-Kurti et al., 2008), and it has been suggested that an understanding of the molecular basis of quantitative disease resistance requires exploitation of maize genetic diversity combined with improved phenotyping approaches (Poland et al., 2009). The qPCR assay reported here represents a tool that can be applied in this type of "precision phenotyping" approach for assessment of both QTL and association mapping populations of maize for quantitative resistance to GLS (Rafalski 2010).

We have demonstrated the use of a qPCR assay based on the *cpr1* gene to quantify GLS disease in maize. In this manner, we were able to show good correlation between *C. zeina* DNA levels and GLS lesion area on maize leaves in the glasshouse and field. Because of its specific, precise, cost-effective and fast nature, the assay presented here will be a useful tool in grey leaf spot disease management, breeding programmes and plant-pathogen interaction studies, and thus mitigate the threat of this pathogen to food security in maize producing countries of the world.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*, 403–410.

Balint-Kurti, P. J., Wisser, R., & Zwonitzer, J. C. (2008). Use of an advanced intercross line population for precise mapping of quantitative trait loci for gray leaf spot resistance in maize. *Crop Science, 48*, 1696–1704.

Bluhm, B., Dhillon, B., Lindquist, E., Kema, G., Goodwin, S., & Dunkle, L. (2008). Analyses of expressed sequence tags from the maize foliar pathogen *Cercospora zeae-maydis* identify novel genes expressed during vegetative, infectious, and reproductive growth. *BMC Genomics, 9*, 523.

Brunelli, K. R., Dunkle, L. D., Sobrinh, C. A., Fazza, A. C., & Camargo, L. E. A. (2008). Molecular variability in the maize grey leaf spot pathogens in Brazil. *Genetics and Molecular Biology, 31*, 938–942.

Chen, H., Lee, M. H., Daub, M. E., & Chung, K. R. (2007). Molecular analysis of the cercosporin biosynthetic gene cluster in *Cercospora nicotianae*. *Molecular Microbiology, 64*, 755–770.

Crous, P. W., Groenewald, J. Z., Groenewald, M., Caldwell, P., Braun, U., & Harrington, T. C. (2006). Species of *Cercospora* associated with grey leaf spot of maize. *Studies in Mycology, 55*, 189–197.

De Coninck, B. M. A., Amand, O., Delaur, S. L., Lucas, S., Hias, N., Weyens, G., Mathys, J., De Bruyne, E., & Cammue, B. P. A. (2011). The use of digital image analysis and real-time PCR fine-tunes bioassays for quantification of *Cercospora* leaf spot disease in sugar beet breeding. *Plant Pathology.* doi:10.1111/j.1365-3059.2011.02497.x.

Dunkle, L. D., & Levy, M. (2000). Genetic relatedness of African and United States populations of *Cercospora zeae-maydis*. *Phytopathology, 90*, 486–490.

Goodwin, S. B., Dunkle, L. D., & Zismann, V. L. (2001). Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. *Phytopathology, 91*, 648–658.

Latterell, F. M., & Rossi, A. E. (1983). Gray leaf spot of corn: a disease on the move. *Plant Disease, 67*, 842–847.

Meisel, B., Korsman, J., Kloppers, F. J., & Berger, D. K. (2009). *Cercospora zeina* is the causal agent of grey leaf spot disease of maize in southern Africa. *European Journal of Plant Pathology, 124*, 577–583.

Moller, E. M., Bahnweg, G., Sandermann, H., & Geiger, H. H. (1992). A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Research, 20*, 6115–6116.

Nicolaisen, M., Suproniene, S., Nielsen, L. K., Lazzaro, I., Spliid, N. H., & Justesen, A. F. (2009). Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. *Journal of Microbiological Methods, 76*, 234–240.

Okori, P., Fahleson, J., Rubaihayo, P. R., Adipala, E., & Dixelius, C. (2003). Assessment of genetic variation among East African *Cercospora zeae-maydis*. *African Crop Science Journal, 11*, 75–85.

Poland, J. A., Balint-Kurti, P. J., Wisser, R. J., Pratt, R. C., & Nelson, R. J. (2009). Shades of gray: the world of quantitative disease resistance. *Trends in Plant Science, 14*, 21–29.

Rafalski, J. A. (2010). Association genetics in crop improvement. *Current Opinion in Plant Biology, 13*, 174–180.

Shim, W. B., & Dunkle, L. D. (2002). Identification of genes expressed during cercosporin biosynthesis in *Cercospora zeae-maydis*. *Physiological and Molecular Plant Pathology, 61*, 237–248.

Shim, W. B., & Dunkle, L. D. (2005). *Malazy*, a degenerate, species-specific transposable element in *Cercospora zeae-maydis*. *Mycologia, 97*, 349–355.

Tambong, J. T., Mwange, K. N., Bergeron, M., Ding, T., Mandy, F., Reid, L. M., & Zhu, X. (2008). Rapid detection and identification of the bacterium *Pantoea stewartii* in maize by TaqMan real-time PCR assay targeting the *cpsD* gene. *Journal of Applied Microbiology, 104*, 1525–1537.

Van Den Brink, H. M., Van Zeijl, C. M. J., Brons, J. F., Van den Hondel, C. A. M. J. J., & Van Gorcom, R. F. M. (1995). Cloning and Characterization of the NADPH Cytochrome P450 Oxidoreductase Gene from the Filamentous Fungus *Aspergillus niger*. *DNA and Cell Biology, 14*, 719–729.

Waalwijk, C., Koch, S., Ncube, E., Allwood, J., Flett, B., de Vries, I., & Kema, G. (2008). Quantitative detection of *Fusarium* spp. and its correlation with fumonisin content in maize from South African subsistence farmers. *World Mycotoxin Journal, 1*, 39–47.

Wang, J., Levy, M., & Dunkle, L. D. (1998). Sibling species of *Cercospora* associated with gray leaf spot of maize. *Phytopathology, 88*, 1269–1275.

Ward, E., Foster, S. J., Fraaije, B. A., & McCartney, H. A. (2004). Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *Annals of Applied Biology, 145*, 1–16.

Ward, J. M. J, Birch, E. B. & Nowell, D. C. (1994). Grey leaf spot on maize. Pietermaritzburg, South Africa. Coordinated extension, Maize in Natal, Cedara Agricultural Development Institute.

Ward, J. M. J., Stromberg, E. L., Nowell, D. C., & Nutter, F. W., Jr. (1999). Gray leaf spot. *A disease of global importance in maize production. Plant Disease, 83*, 884–895.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis et al. (Eds.), *PCR Protocols: a guide to methods and applications* (pp. 315–322). California: Academic.

Xu, M. L., Melchinger, A. E., & Lnbberstedt, T. (1999). Species-specific detection of the maize pathogens *Sporisorium reiliana* and *Ustilago maydis* by dot blot hybridization and PCR-based assays. *Plant Disease, 83*, 390–395.

Xue, H. Q., Upchurch, R. G., & Kwanyuen, P. (2006). Ergosterol as a quantifiable biomass marker for *Diaporthe phaseolorum* and *Cercospora kikuchii*. *Plant Disease, 90*, 1395–1398.

Zhu, X., Reid, L. M., & Woldemariam, T. (2002). First report of gray leaf spot caused by *Cercospora zeae-maydis* on corn in Ontario, Canada. *Plant Disease, 86*, 327.