Quantitative detection of *Exserohilum turcicum* in northern leaf blight diseased sorghum and maize leaves

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Abstract

Exserohilum turcicum is the causal agent of northern leaf blight (NLB) disease in sorghum and maize. Early detection of this economically important pathogen is essential for effective disease management to limit yield losses. Here we present a real-time quantitative PCR (qPCR) assay specific for *E. turcicum* detection and biomass quantification in sorghum and maize. *In planta* fungal quantification was achieved through amplification of a cytochrome P450 oxidoreductase (*cpr1*) gene fragment and subsequent normalisation to the host glutathione S-transferase III gene (*gst3*). The assay could specifically detect *E. turcicum* in sorghum and maize, but the *cpr1* gene fragment was not amplified in non-target fungal pathogens. Application of the assay with NLB diseased sorghum and maize leaf material revealed a significant increase in *E. turcicum* DNA in leaves with lesion symptoms when compared to leaves with early stage chlorotic fleck symptoms in both hosts. Furthermore, *E. turcicum* was detected at levels as low as 1 pg in infected sorghum and maize leaves. The assay enables rapid detection and quantification of *E. turcicum* in sorghum and maize and has useful applications in crop breeding programmes and disease management where cultivar selection and early detection of the pathogen are essential to limit disease spread.

Keywords: Exserohilum turcicum; Fungal biomass; Northern leaf blight; qPCR; Real-time quantitative PCR

Introduction

Maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench) are considered the third and fifth most important cereal crops grown globally (FAOSTAT 2017). Maize is the most important grain crop in South Africa, and is a major dietary staple as well as a good source for animal feed and biofuel production (Department of Agriculture 2018). Sorghum is a drought and heat tolerant crop and therefore highly beneficial in hot, dry areas where other cereal crops do not produce sufficient yields. Sorghum is therefore a vital staple for populations in semi-arid regions such as Africa and Asia (Du Plessis 2008).

One of the major factors influencing the yield of maize and sorghum is pathogen infection. *Exserohilum turcicum* (Pass.) Leonard & Suggs (sexual stage: *Setosphaeria turcica*) is a hemibiotrophic fungal pathogen that causes northern leaf blight (NLB) disease in maize, sorghum, and other related grass species, such as Johnson grass and Sudan grass (Leonard and Suggs 1974). This economically important disease occurs in maize and sorghum producing countries worldwide and causes significant yield loss (Kloppers and Tweer 2009). NLB is regarded to be the most critical disease of maize in recent growth seasons (Kloppers and Tweer 2009). Disease symptoms start off with chlorotic spots, which develop into cigar-shaped lesions on infected maize and sorghum leaves resulting in reduced photosynthetic ability of the host (Kloppers and Tweer 2009; Wise 2011). Early *E. turcicum* infection followed by spread to the upper leaves during grain formation and filling is associated with significant yield losses (Kloppers and Tweer 2009).

The most cost-effective and environmentally friendly strategy to manage NLB is through resistant cultivars (Kloppers and Tweer 2009), however more research into the molecular mechanisms of *E. turcicum* infection is needed to aid NLB resistance breeding. Another key aspect of effective disease management is early detection, which gives crop producers the opportunity to make decisions on the use of management practices, such as fungicides. The development of a definitive assay that is sensitive enough to detect and quantify *in planta E. turcicum* across all infection stages would greatly aid in the monitoring of NLB.

There are limited strategies available to detect and quantify *E. turcicum* reliably in maize and there are no available methods to detect and quantify this pathogen in sorghum. Chung et al. (2010), developed an assay to quantify *E. turcicum* in maize leaf material and applied the assay to determine the amount of *E. turcicum* DNA present *in planta* based on the amplification of a 170 bp fragment specific to the *E. turcicum* internal transcribed spacer 1 (ITS1) region. The downfall of this quantification assay is that it can only be applied to quantify *E. turcicum* at initial stages of pathogenesis and under controlled conditions such as uniform inoculation and sampling (Chung et al. 2010).

qPCR remains a sensitive and reliable molecular method that can be applied to specifically detect and quantify fungal biomass in infected maize and sorghum leaf material. A qPCR method to quantify *Sporisorium reilianum* (the head smut fungal pathogen of maize and sorghum) relative to total plant DNA was developed for infected maize and sorghum leaf material (Poloni and Schirawski 2016). *Fusarium verticillioides* biomass was determined through a qPCR assay in maize, sorghum, rice, and beet seedlings under greenhouse conditions (Dastjerdi and Karlovsky 2015). Furthermore, a qPCR method exists that specifically quantifies and distinguishes the two grey leaf spot pathogens *Cercospora zeina* and *Cercospora zeae-maydis* in diseased maize leaf material (Korsman et al. 2012). The method is based on quantification of the fungal cytochrome P450 oxidoreductase (*cpr1*) gene and subsequent normalisation to the host glutathione S-transferase III (*gst3*) gene. This method is valuable as it can be applied to field material where other fungal pathogens are present. These studies highlight the importance of *in planta* fungal pathogen detection and quantification, which might contribute to effective disease management strategies and aid resistance breeding programs to reduce fungal infection levels.

Detection and quantification of *E. turcicum* in field material would greatly benefit breeders in selecting NLB resistant sorghum and maize lines. We therefore aimed to develop a specific and sensitive qPCR assay to detect *E. turcicum* in infected maize and sorghum leaf material as well as to quantify *E. turcicum* biomass in sorghum and maize leaf tissue at different stages of NLB disease development. Based on the qPCR method developed for *C. zeina* biomass quantification in maize (Korsman et al. 2012), it was hypothesized that the fungal *cpr1* gene, a putative cytochrome P450 oxidoreductase gene (Vandenbrink et al. 1995), would be a good target to detect and quantify *E. turcicum* biomass *in planta*. We provide a real-time SYBR green quantitative PCR assay for *E. turcicum* biomass evaluation *in planta*. The fungal primer set was designed to amplify a fragment of the *cpr1* (cytochrome P450 oxidoreductase) gene in *E. turcicum*.

Materials and methods

Fungal isolates and maintenance

Fungal cultures of *E. turcicum* sorghum isolate 73 and *E. turcicum* maize isolate 2 were obtained from M. P. Haasbroek (Haasbroek et al. 2014). Pure *E. turcicum* cultures were maintained in Petri plates containing 0.5 X potato dextrose agar (PDA) and kept at room temperature and in the dark to promote conidiation. Conidia were harvested by flooding the surface of the cultures with 0.02% Tween 20, scraping off the conidia with a glass spreader and filtering the suspension through a double layer of sterile cheesecloth. Conidial concentrations were determined with a Neubauer Haemocytometer.

Host plant infection

Disease trials were performed by inoculating five-week-old maize and sorghum plants with conidial suspensions of *E. turcicum* in a phytotron facility (University of Pretoria, South Africa) under controlled levels of temperature and humidity. Conditions in the phytotron were kept at day/night temperatures of 28 °C/22 °C, while at 60–100% relative humidity. NLB susceptible B73 maize plants were inoculated with maize *E. turcicum* isolate 2 (Kotze et al. 2019) and susceptible sorghum NS5511 hybrid plants were inoculated with the sorghum specific *E. turcicum* isolate 73. Inoculations were made by adding 3×10^5 conidia ml^{-1} *E. turcicum* conidial suspension to the whorls of maize and sorghum plants. Whole leaves with chlorotic spot and lesion symptoms were harvested in triplicate from separate plants for each host and flash frozen.

DNA extraction

DNA from both healthy and infected plant material and fungal cultures was extracted following a modified CTAB method (Cullings 1992; Doyle and Dickson 1987). DNA was isolated from 40 to 50 mg of frozen fungal or leaf material. A CTAB-Urea buffer was used to aid isolations from melanised fungal cultures (Stefania Lagonigro et al. 2004). The amount of CTAB buffer used per sample, for fungal and plant material, was increased to 500 µl. Modifications also included the addition of 0.08 volumes of cold 7.5 M ammonium acetate that was added together with the isopropanol to the aqueous phase. Furthermore, the first wash step was done with cold 70% ethanol and the second wash step was done with cold

95% ethanol. The extracted DNA from plant and fungal material was resuspended in 50 µl sterile distilled water. DNA from all samples was quantified with a Nano-Drop™ 1000 spectrophotometer (ThermoFisher Scientific, Waltham, USA) and the quality and integrity of each DNA sample was determined by electrophoresis through a 0.8% agarose gel prepared in 1X Tris acetate buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA). DNA was stained with ethidium bromide (0.5 µg ml^{-1}) and visualised under ultra-violet light using the Molecular Imager® Gel Doc™ XR System equipped with Image Lab™ software.

E. turcicum quantification assay primer design

The gene targets for the *E. turcicum* detection and quantification assay were adapted from the quantification method developed for *C. zeina* in maize (Korsman et al. 2012). The *C. zeina cpr1* gDNA sequence (Korsman et al. 2012) was used as a query sequence in a BLASTn (Altschul et al. 1990) search against the *Setosphaeria turcica* (*E. turcicum*) Et28A v1.0 genome sequence hosted on the Joint Genomes Institute website (JGI, https://mycocosm.jgi.doe.gov/Settu1/Settu1.home.html). The *E. turcicum cpr1* gene homolog (accession number: XM008026943.1) was identified and a reciprocal BLAST was performed to confirm the identity of the *E. turcicum cpr1* gene. Primer sequences were designed to an *E. turcicum*-specific fragment spanning the intron region of the *cpr1* gene, resulting in primers EtCPR1QF (5'-TCTTCTTTGGATGCCGGAAG-3') and EtCPR1QR (5'-CACGCTAGGTTAGCAACAGT-3').

A BLASTn search was performed with the *Z. mays gst3* gene sequence (Genebank accession: X06755.1) against the *S. bicolor* v2.1 genome available on Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Thereafter, the sorghum *gst3* gene homolog (accession number: XM_021455931.1) was aligned to the maize *gst3* gene and primers were designed to a commonly conserved region in both *gst3* genes to yield SbGST3F (5'-CACCACTTCTACCCGAAC-3') and SbGST3R (5'-GTAGACGTCGAGCACCTTG-3').

Specificity of the qPCR assay for *E. turcicum*

Primer specificity was further investigated through conventional PCR amplification with the EtCPR1Q and SbGST3 primer sets and amplification of the ITS1/4 primers (White et al. 1990) was included as a control. DNA template used to test primer specificity included *E. turcicum* (sorghum-specific isolate 73 and maize-specific isolate 3), *Exserohilum rostratum*, *Cercospora zeina*, *Cercospora zeae-maydis*, *Fusarium verticillioides*, *Fusarium graminearum*, *Nigrospora oryzae*, *Alternaria alternata*, *Curvularia borreriae*, *Chaetomium globosum, Fusarium udum, Phaeosphaeria maydis, Kabatiella zeae, Sorghum bicolor,* and *Zea mays.* DNA from *E. rostratum* was acquired from M. P. Human (University of Pretoria, South Africa). DNA for *C. zeina* and *C. zeae-maydis* was acquired from V. Swart (University of Pretoria, South Africa). Fungal DNA from *F. verticillioides* was obtained from S. L. Murray (University of Cape Town, South Africa). *F. graminearum*, *N. oryzae*, *A. alternata*, *C. borreriae*, *C. globosum, and F. udum* were isolated from maize (K. de Ridder) and DNA was obtained from K. de Ridder (University of Pretoria, South Africa). DNA was isolated form maize leaves with eyespot (causative agent: *K. zeae*) and leafspot (causative agent: *P. maydis*) symptoms.

Reactions were set up in a total volume of 12.5 µl and consisted of 6.3 µl 2X KAPA2G Robust Hotstart readymix (Kapa Biosystems, Wilmington, USA), 0.6 µM of each primer, 1 µl of DNA template, and sterile distilled water. DNA template was added at amounts of 30 ng for the *cpr1* primer set and 10 ng for the *gst3 and* ITS1/4 primer reactions. The PCR amplification conditions for the *cpr1* gene fragment included an initial denaturation step at 95 °C for 3 min, followed by 35 amplification cycles of (95 °C for 15 s, 64 °C for 15 s, and 72 °C for 15 s) and a final extension step at 72 °C for 1 min. PCR cycling conditions for the *gst3* gene fragment included denaturation at 95 °C for 3 min, followed by 30 amplification cycles of (95 °C for 15 s, 62 °C for 15 s, and 72 °C for 15 s) and a final extension step at 72 °C for 1 min. All PCR products were electrophoresed through a 2.5% agarose gel stained with ethidium bromide (0.5 µg ml^{-1}) and visualised as described previously.

Quality of the DNA was tested through amplification of the ITS2 region using ITS1/4 primers as outlined by White et al. (1990). These fragments were used as templates for forward and reverse sequencing reactions on an ABI3500xl Genetic Analyzer (Applied Biosystems, Foster City, USA) using the BigDye® Terminator Cycle Sequencing kit V3.1 (Applied Biosystems). The *E. turcicum* 73 isolate amplified with the EtCPR1Q primer set as well as *S. bicolor* NS5511 and *Z. mays* IMP50-10B products that were both amplified with the SbGST3 primer set, were also sequenced as outlined above. Identity of the sequenced products was confirmed through BLASTn analysis of the Genebank database (Altschul et al. 1990).

Optimisation of the *E. turcicum* qPCR assay

The amplification efficiency of the EtCPR1Q primer set was initially tested on *E. turcicum* DNA to determine if the assay could amplify different haplotypes of *E. turcicum*. DNA from the *E. turcicum* sorghum isolates 72 and 73, as well as DNA from *E. turcicum* maize isolates 2, 3, 4, 5,10, 23, 30, 44, 54, 58, 59, 60, 61, 62, 95, 96, 98, 100, 101, 103, 107, 11, and 113 were obtained from M. P. Haasbroek (Haasbroek et al. 2014). DNA from the *E. turcicum* sorghum isolates S050, DPS5, DPS7b, S0339, DPS9b, and DPSS7b was provided by A. Nieuwoudt (Nieuwoudt et al. 2018). Robustness of the *cpr1* primers to amplify various *E. turcicum* isolates was tested through qPCR and melting curve analysis of 30 ng *E. turcicum* DNA of each sorghum and maize isolate.

The qPCR reactions were performed on the Bio-Rad CFX96 Touch™ real-time PCR detection system (Bio-Rad, Hercules, USA) in total reaction volumes of 10 µl. Each reaction was performed in triplicate and each reaction consisted of 5 µl SYBR green - KAPA2G Robust HotStart ReadyMix (Kapa Biosystems), 0.5 µM of each primer, 1 µl DNA template and sterile distilled water. The cycling conditions consisted of a pre-incubation step of 95 °C for 5 min, followed by 45 cycles of (95 °C for 10 s, 64 °C for 15 s, and 72 °C for 10 s). Fluorescence was detected continuously at the end of each elongation step and the amplification specificity was investigated through melting curve analysis after every run.

Sensitivity of the qPCR assay for low concentrations of *E. turcicum*

qPCR reactions were set up to draw standard curves for the *cpr1* and *gst3* primer sets to determine the detection limits of the assay. Standard DNA samples for the EtCPR1Q primer set were prepared by diluting DNA extracted from pure *E. turcicum* (isolate 73) cultures in

100 ng pure maize (IMP50-10B) or sorghum (NS5511) DNA. The final *E. turcicum* DNA concentrations used to determine the detection limits of *cpr1* in sorghum and maize were 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}, 5×10^{-4}, 2.5×10^{-4}, and 1×10^{-4} ng μl^{-1}. These dilutions simulated infected leaf samples with known concentrations of *E. turcicum* DNA. qPCR reactions for *cpr1* and *gst3* were set up as outlined previously. Standard curves for each gene were prepared by plotting the $\log_{10}$ value of the known standards against the respective Cq (threshold cycle) values to obtain a regression curve.

Quantification of *E. turcicum* infected sorghum and maize leaf material

Reaction standards were included to draw standard curves to determine the amount of *E. turcicum* biomass present in infected sorghum and maize leaf samples. Reaction standards for *cpr1* contained final *E. turcicum* DNA concentrations of 1. 5×10^{-1}, 1×10^{-1}, 5×10^{-2}, 2.5×10^{-2}, 1×10^{-2}, and 1×10^{-3} ng μl^{-1} in sorghum or maize carrier DNA. Reaction standards for the *gst3* sorghum and maize fragments were prepared by diluting pure maize or sorghum DNA with sterile distilled water to obtain final concentrations of 150, 120, 100, 80, 40, 20, and 10 ng μl^{-1}. Standard curves were drawn as outlined previously.

PCR amplification efficiencies were calculated as $10^{(-1/\text{slope})}$ and unknown DNA biomass from target samples could be extrapolated from the curve. Normalized amounts of fungal biomass between different infected leaf samples were obtained by dividing the *E. turcicum cpr1* quantification value by the corresponding plant *gst3* quantification value for each infected leaf sample. Student's T-tests were performed to compare differences in fungal biomass between samples, with statistically significant differences determined at $p < 0.05$.

Results

In planta E. turcicum quantification assay design and specificity

For the *E. turcicum* quantification assay, primers (EtCPR1QF and EtCPR1QR) were designed to amplify a cytochrome P450 oxidoreductase (*cpr1*) gene fragment of 109 bp (Fig. 1). The *E. turcicum cpr1* gene was predicted to be 2084 bp in length and consists of two exons and a single 56 bp intron. In order to ensure specificity of the primers to the *E. turcicum cpr1* gene, the *cpr1* amplicon includes the intron region, as all other regions of the *cpr1* gene are highly conserved in other fungi (Supplementary Fig. 1).

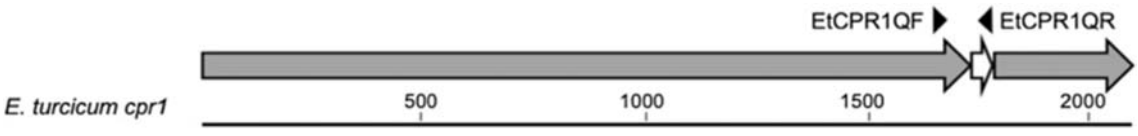

Fig. 1. The predicted *E. turcicum* cytochrome P450 oxidoreductase *(cpr1)* gene is 2084 bp in size, which includes two exons of 1731 bp and 297 bp respectively (grey arrows) as well as a single 56 bp intron (*white arrow*). Primers EtCPR1QF and EtCPR1QR (*black arrows*) were designed to exclusively amplify a 109 bp amplicon, that includes the intronic region, from *E. turcicum*

The plant target for normalization of the *in planta* fungal biomass values, was the glutathione S-transferase III (*gst3*) gene. The *gst3* target could be amplified from sorghum or maize using a single primer set (Fig. 2). The sorghum and maize *gst3* amplicons are both 129 bp in size but differ in base composition at 16 different positions.

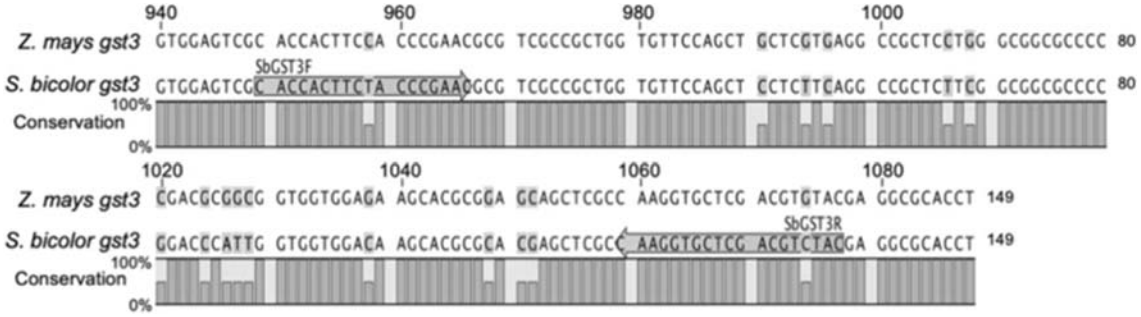

Fig. 2. Sequence alignment of the *gst3* amplicon from sorghum and maize. The percentage of conservation for each nucleotide is indicated by the bar graph and nucleotides that differ between the two hosts are indicated by the shaded blocks. The expected *gst3* amplicon in both sorghum and maize is 129 bp in size

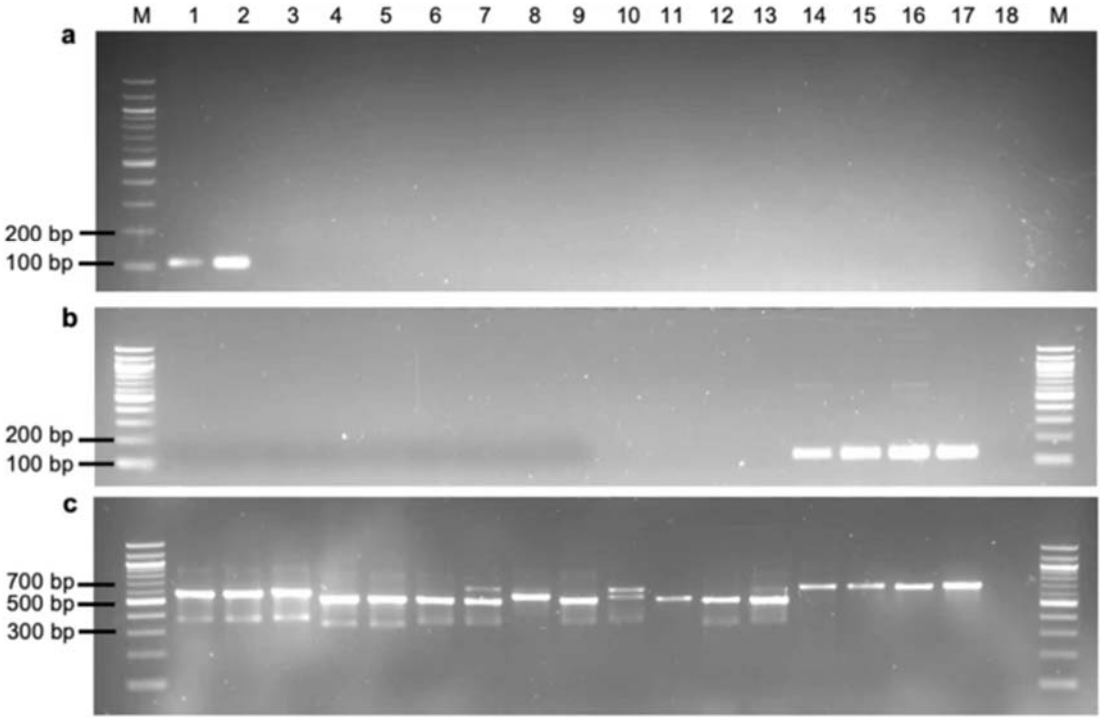

Fig. 3. PCR amplification with *cpr1* (**a**), *gst3* (**b**) *and* ITS (**c**) primers to establish primer specificity and DNA quality. Primers were tested on DNA isolated from *E. turcicum* (sorghum isolate 73) (lane 1), *E. turcicum* (maize isolate 3) (lane 2), *E. rostratum* (lane 3), *C. zeina* (lane 4), *C. zeae-maydis* (lane 5), *F. verticillioides* (lane 6), *F. graminearum* (lane 7), *N. oryzae* (lane 8), *A. alternata* (lane 9), *C. borreriae* (lane 10), *C. globosum* (lane 11), *F. udum* (lane 12), *N. oryzae* (lane 13), *P. maydis* (lane 14), *K. zeae* (lane 15), *S. bicolor* (lane 16) and *Z. mays* (lane 17). Lane 18 consisted of the non-template control and lane M is the size standard (100 bp ladder, New England Biolabs). Products were separated on a 2.5% agarose gel. **a** The *cpr1* amplicon of 109 bp was amplified in both *E. turcicum* isolates and no amplification was seen in the non-target DNA samples. **b** The *gst3* amplicon of 129 bp was amplified in the *P. maydis* and *K. zeae* DNA samples that were derived from maize lesions as well as from *S. bicolor* and *Z. mays* samples that were derived from maize and sorghum seedling material. **c** All DNA samples showed amplification with the ITS1/4 primer set, suggesting the integrity of DNA samples

Conventional PCR reactions were performed to test the specificity of the *cpr1* and *gst3* primer sets on DNA templates isolated from various sorghum and maize fungal pathogens and endophytes. The *cpr1* primer set specifically amplified the desired 109 bp product from the two *E. turcicum* isolates and no amplification was seen in the other samples (Fig. 3a). Similarly, the *gst3* primer set only amplified the desired 129 bp amplicon from leaf samples whether the DNA was extracted from maize leaves with eyespot and leafspot symptoms or healthy sorghum and maize plants (Fig. 3b). Furthermore, as a false negative control, all the above-mentioned samples were also subjected to amplification with ITS1/4 primers (Fig. 3c). All the samples showed amplification with the ITS1/4 primers and the DNA samples were therefore adequate for DNA analysis.

Furthermore, the *cpr1* primer set was able to amplify a number of different *E. turcicum* haplotypes that were isolated from sorghum or maize. qPCR reactions with melting curve analysis showed that the desired 109 bp *cpr1* fragment amplified from eight *E. turcicum* sorghum isolates and from 23 *E. turcicum* maize isolates (Supplementary Fig. 2). The *cpr1* amplicon from all these isolates produced a single melt-peak at 82.5–83 °C (Supplementary Fig. 2a) and a single products of the desired size were visualised after agarose gel electrophoresis (Supplementary Fig. 2b).

Detection limits of the qPCR assay

Dilutions were made with pure *E. turcicum* DNA in 100 ng healthy sorghum or maize carrier DNA to obtain a concentration range from 1 to 1×10^{-3} ng μl^{-1}, which was used to generate standard curves for *cpr1* (Supplementary Fig. 3). The detection limit of the *cpr1* amplicon was 1 pg *E. turcicum* DNA in both sorghum and maize simulated lesion samples. The linear relationship between the Log_{10} *E. turcicum* DNA concentration and threshold cycle values indicates that the assay is suitable for quantification across the tested concentration ranges within DNA samples of both host plants.

Quantification of *E. turcicum* biomass in infected sorghum and maize leaf samples

To quantify *E. turcicum* biomass in infected sorghum and maize leaf samples, standard curve dilutions with a concentration range from 1 to 1×10^{-3} ng μl^{-1} were used to generate standard curves for *cpr1* from sorghum and maize (Fig. 4a). The amplification efficiency was 102% for *cpr1* in infected sorghum samples and 96.3% in infected maize samples. Pure maize or sorghum DNA was diluted in sterile distilled water to obtain dilutions ranging from 10 to 150 ng μl^{-1} to be able to generate standard curves for the *gst3* amplicon in sorghum and maize (Fig. 4b). The amplification efficiencies were 93.6% and 86.3% for sorghum and maize, respectively.

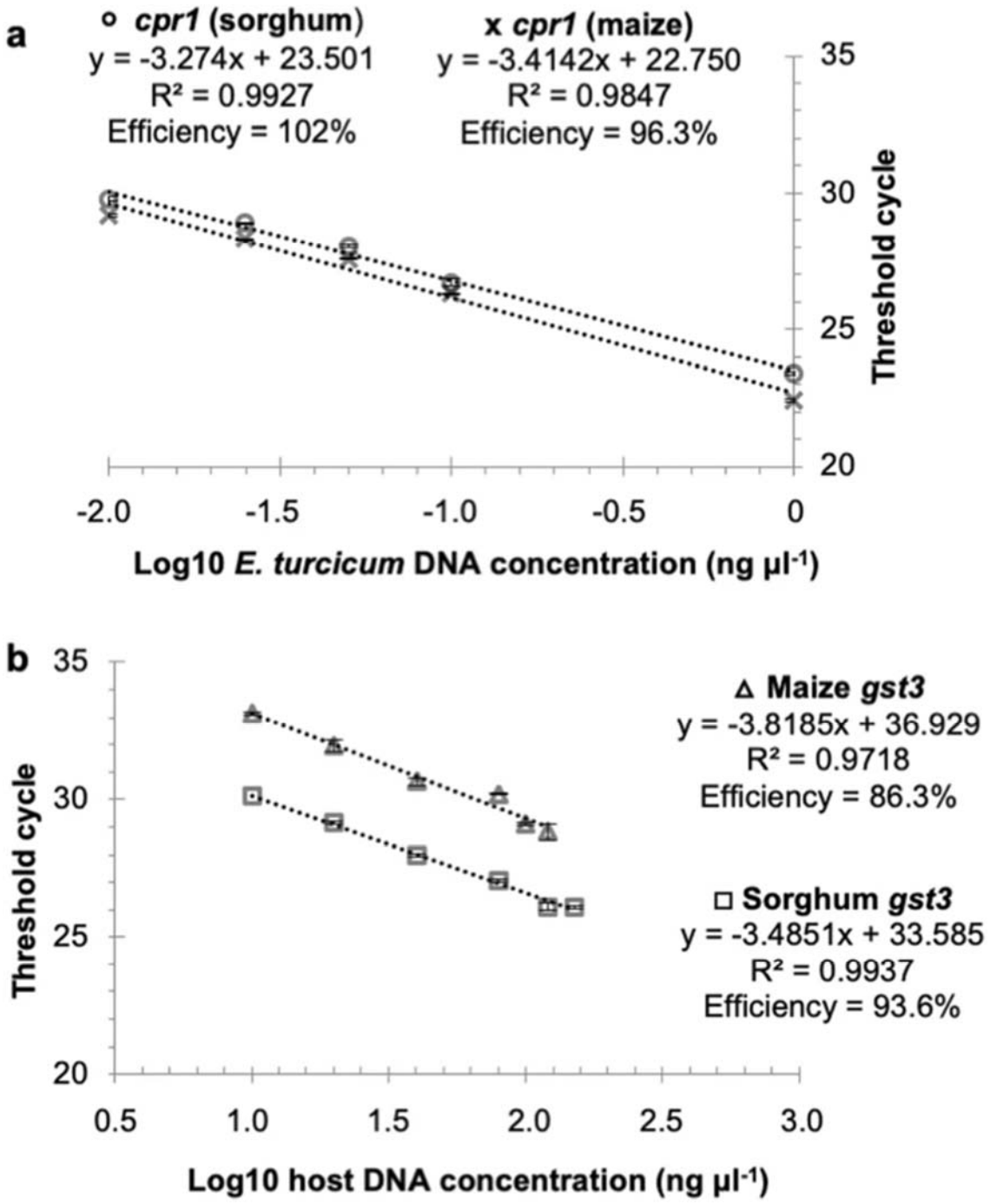

Fig. 4. Standard curves for the quantification of *E. turcicum* in *S. bicolor* (sorghum) and *Z. mays* (maize) samples with chlorotic spot and lesion symptoms. The standard curves indicate the efficiency of the *cpr1* and *gst3* primer sets designed to quantify *E. turcicum in planta*. Figure **a** shows the standard curves of *E. turcicum* DNA diluted in sorghum and maize carrier DNA, respectively. Figures **b** shows the standard curves as a result of serial dilutions made from sorghum and maize genomic DNA, respectively. The bars on the graphs represent standard error

The amplification specificities of the *E. turcicum cpr1* as well as the sorghum and maize *gst3* targets were further investigated through melt-curve analysis (Supplementary Fig. 4) and agarose gel electrophoresis, which showed that single *cpr1* and *gst3* products of the desired

size were amplified (results not shown). The *E. turcicum cpr1* amplicon showed a single melting peak at 82.5–83 °C (Supplementary Fig. 4a) and no differences were seen in the melting temperatures between samples from the two different *E. turcicum* isolates. The *gst3* amplicon from the sorghum samples showed a single melting peak at 88.5 °C, while the *gst3* amplicon from the maize samples showed a single melting peak at 90–90.5 °C (Supplementary Fig. 4b). The *gst3* amplicon form sorghum and maize differ slightly in sequence composition and thereby produce different melting temperatures.

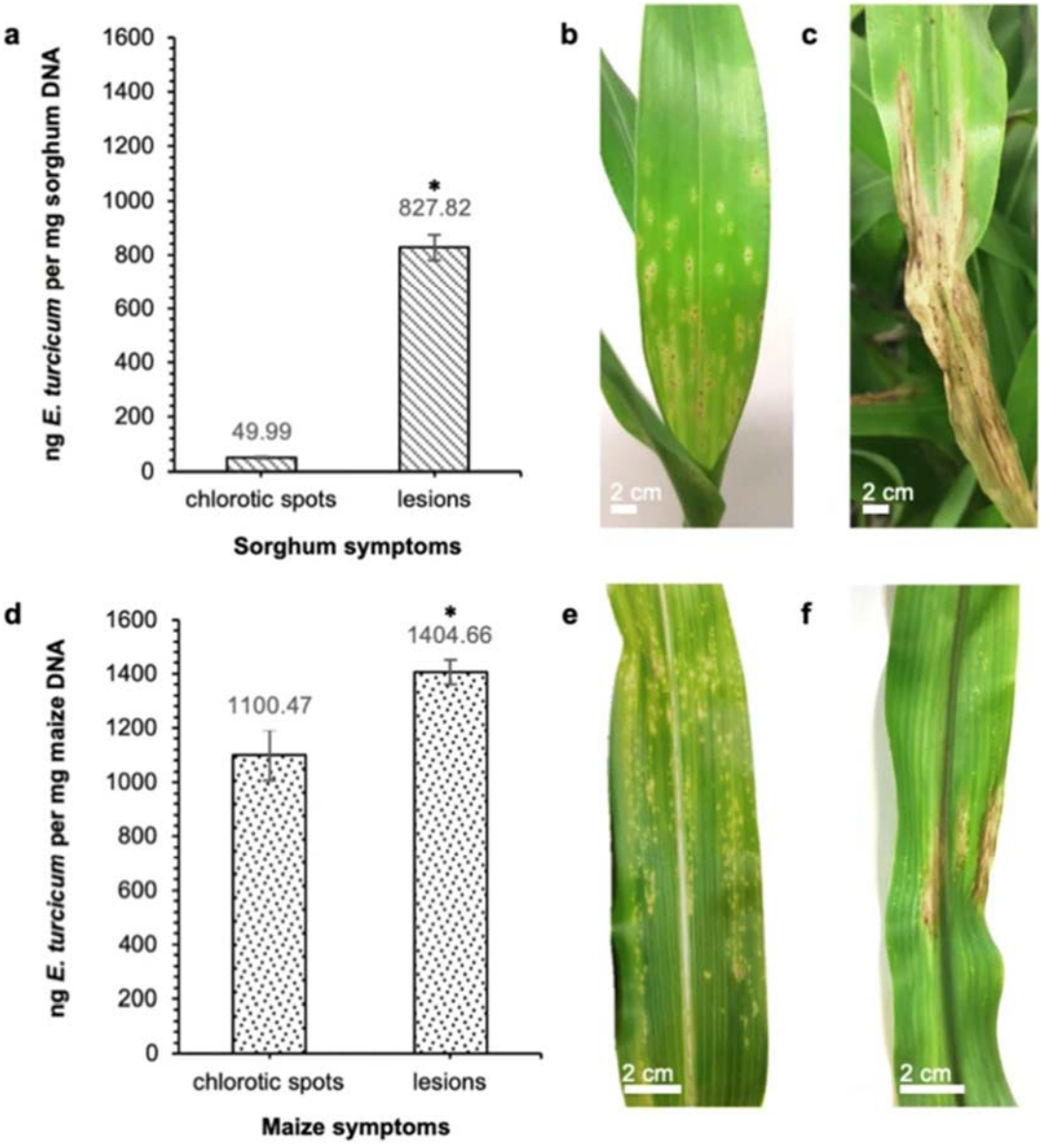

Fig. 5. *E. turcicum* biomass in terms of ng *E. turcicum* DNA per mg sorghum DNA (**a**), and per mg maize DNA (**d**) in different diseased leaf samples. DNA was extracted from three biological replicates per symptom in sorghum and maize and subsequently subjected to the qPCR assay to determine the fungal biomass present in each sample. Photographic representations of the different symptoms: chlorotic spots (**b**) and lesions (**c**) in sorghum as well as chlorotic spots (**e**) and lesions (**f**) in maize. The error bars on the graphs represent standard error and the asterisk indicate that the lesion samples in both sorghum and maize show significantly different values when compared to the chlorotic spot symptoms in sorghum and maize, respectively (Student's T test; $p < 0.05$)

The *E. turcicum* quantification assay was applied to determine the amount of fungal biomass present in infected sorghum samples showing NLB chlorotic spot symptoms and lesions (Fig. 5b and c) as well as maize leaf samples showing chlorotic spot symptoms and lesions (Fig. 5e and f). DNA extracted from these samples were subjected to qPCR analysis with the *cpr1* and *gst3* primer sets. The *E. turcicum* biomass present in each sample, as determined by the *cpr1* target, was normalized to the amplification values of the respective host *gst3* target to obtain *E. turcicum* biomass values in terms of ng *E. turcicum* DNA per mg host (sorghum or maize) DNA. Each sample was analysed in triplicate. The average relative amount of *E. turcicum* DNA present was 49.99 ng mg^{-1} sorghum DNA (samples with chlorotic spot symptoms) and 827.82 ng mg^{-1} sorghum DNA (samples with lesion symptoms) (Fig. 5a); 1100.47 ng mg^{-1} maize DNA (chlorotic spot symptoms) and 1404.66 ng mg^{-1} maize DNA (lesion symptoms) (Fig. 5d). It was subsequently determined that the *E. turcicum* biomass increased significantly with disease progression in both sorghum (Fig. 5a) and maize (Fig. 5d) as determined by ng *E. turcicum* per mg host DNA (Student's T test; $p < 0.05$).

Discussion

In this study, a real-time SYBR Green PCR assay was developed to quantify *E. turcicum* biomass in sorghum and maize leaf material. The assay relies on the specific quantification of *E. turcicum* biomass through a region of the fungal cytochrome P450 oxidoreductase (*cpr1)* gene, which is then normalised to the host glutathione S-transferase III gene (*gst3)* gene levels. The *cpr1* gene primer set was designed to exclusively amplify a gene fragment from *E. turcicum* isolates and no amplification was seen from other closely related fungal targets that are able to infect sorghum and/or maize (Fig. 3). The EtCPR1Q *cpr1* amplicon was a good choice for *E. turcicum* quantification *in planta* when considering the specificity and sensitivity of the assay and is sensitive enough to be applied to field samples. The *cpr1* gene region is specific to *E. turcicum*, however it cannot distinguish between sorghum and maize *E. turcicum* haplotypes, as no obvious differences in the amplified *cpr1* melting curves were observed (Supplementary Fig. 2). Future studies on differentiation between *E. turcicum* isolates from sorghum and maize could focus on the nucleotide diversity of a less conserved gene, such as an effector gene or even full genome sequencing of the isolates. The *gst3* qPCR assay is an appropriate tool to normalise *E. turcicum* DNA quantities from maize and sorghum backgrounds and the *gst3* amplicon has distinct melting temperatures in each host.

E. turcicum can also infect other related grass species such as Johnson grass and Sudan grass (Leonard and Suggs 1974). The genomes of the above-mentioned grass species have not been sequenced yet, however, the 129 bp *gst3* amplicon shared at least 80% identity to *gst3* orthologs from related grass species available on Phytozome namely, *Brachypodium distachyon* (purple false brome), *Oryza sativa* (Asian rice), *Panicum virgatum* (switchgrass), and *Setaria italica* (foxtail millet) (results not shown). Therefore, it appears that the qPCR assay might also be useful to detect if NLB-like symptoms in other grass species are caused by *E. turcicum*.

Previously, *E. turcicum* biomass present in maize was detected as a ratio in terms of fungal DNA divided by the total amount of DNA present in the sample (Chung et al. 2010). One of the limitations of this qPCR assay is that it can only be applied to samples with early stage

NLB symptoms, as fungal DNA content cannot accurately be normalised when lesion samples come from necrotic tissue. Normalising total DNA present in the sample also requires controlled conditions and a uniform inoculation to maintain the accuracy and discrimination power of the assay. The assay would therefore not be suitable for *E. turcicum* quantification in field samples. Our assay provided the first measure of *E. turcicum* biomass present in sorghum and determines fungal biomass present in each sample, across all infection stages, in terms of ng *E. turcicum* biomass per mg host DNA. The fungal biomass is normalised to the host *gst3* DNA levels that allow a neutralised sampling size of the leaf showing NLB symptoms. Our quantification assay can also detect *E. turcicum* biomass in as little as 1 pg *E. turcicum* DNA in both sorghum and maize leaf samples, which is very sensitive and will support early detection of NLB.

Examples of detection limits of qPCR assays quantifying fungal DNA in host material are 5 pg DNA for both *C. zeina* and *C. zeae-maydis* in maize (Korsman et al. 2012), 12 pg *Polystigma amygdalinum* DNA in almond tree leaves (Zuniga et al. 2018), and 40 pg *Fusarium* spp. DNA in field pea roots (Zitnick-Anderson et al. 2018). The assay was also able to quantify fungal biomass in infected sorghum and maize leaf samples with distinguishable severity in disease symptoms.

Host disease resistance to fungal pathogens is the most reliable assay to improve food security of important staple food crops (Fisher et al. 2012). But, resistance to fungal pathogens is not always straightforward and often based on quantitative differences in fungal biomass between resistant, susceptible and tolerant cultivars (Ayliffe et al. 2013). Basing resistance or susceptibility of a cultivar on visible disease symptoms alone can be misleading as symptom severity does not necessarily reflect the extent of fungal growth in infected tissue (Weihmann et al. 2016). This qPCR assay provides a simple and reliable assay to quantify *E. turcicum* biomass in sorghum and maize that could aid in NLB resistance breeding by selection of resistance to *E. turcicum* infection as indicated by fungal biomass. A qPCR assay was effectively applied to maize cultivars infected with the ear rot pathogen, *Aspergillus flavus,* that produce aflatoxins that are known to be harmful to human and animal health. These studies found a highly significant correlation between aflatoxin accumulation and fungal biomass in maize infected with *A. flavus* when evaluating maize for resistance (Mideros et al. 2009; Williams et al. 2011). The qPCR assay developed by Korsman et al. (2012) showed a positive correlation between *C. zeina* biomass within maize lesions which was substantially reduced in resistant maize lines compared to susceptible ones (Korsman et al. 2012). This assay for *E. turcicum* could have application in plant breeding programmes to correlate fungal biomass to resistant and susceptible cultivars.

The *E. turcicum* real-time quantification assay is also a valuable molecular tool for research on the NLB disease cycle. Detailed microscopic analysis of the *E. turcicum* infection strategy in maize, revealed a sudden expansion of fungal hyphae in the xylem tissue post-infection (Kotze et al. 2019). The sudden increase in fungal biomass could be detected with the *E. turcicum* quantification assay to determine at which stage after infection this occurs. The *E. turcicum* quantification approach will be more sensitive and less time consuming than microscopic analysis at multiple time points during infection.

In conclusion, we have successfully developed a real-time quantitative PCR assay to reliably detect and quantify *E. turcicum* causing NLB in maize and sorghum. Early detection of NLB infection is the key to the development of rapid and more reliable diagnostic measures for assessing NLB resistance and, in turn, will aid resistance-breeding strategies to effectively evaluate and control NLB disease. The qPCR assay developed in this study is specific to *E. turcicum* and can therefore be applied to field samples where more than one disease is present.

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Contributions

Bridget Crampton, Brigitte Langenhoven, and Shane Murray contributed to the experimental design of the study. Brigitte Langenhoven conducted the experimental work and analyses and also wrote the manuscript that was revised by Bridget Crampton and Shane Murray.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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