CHAPTER 3

Expression profiles of *Cercospora zeina Avr4*, *Ecp2*, and *Ecp6* effectors

1. ABSTRACT

Grey leaf spot (GLS) disease is an economically important foliar disease of maize caused predominantly by *Cercospora zeina* in southern Africa. Whilst little is known about the molecular mechanisms underlying *C. zeina* infection, homologs of the effector genes *Avr4*, *Ecp2*, and *Ecp6* were previously shown to be present in *C. zeina* (Chapter 2). Phytotron inoculations of maize with *C. zeina* were established in this study providing controlled infection conditions independent of the maize growing season. The *in planta* expression profiles of *C. zeina Avr4*, *Ecp2*, and *Ecp6* were analysed by RT-qPCR. The study identified two *C. zeina* reference genes suitable for *in planta* gene expression normalisation, namely glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and cytochrome c oxidase subunit III (*Cyt III*). *GAPDH* and *Cyt III* showed constant expression across all inoculation time points analysed making them suitable reference genes for expression normalisation. It was shown that *C. zeina Avr4*, *Ecp2*, and *Ecp6* were expressed at constant levels during infection. *Ecp2* was expressed at very low levels throughout infection and *Avr4* and *Ecp6* showed constant low levels of expression. Determination of fungal quantity present at each time point by means of an optimised qPCR method enabled correlation studies between fungal quantity and effector gene expression. *Avr4* and *Ecp6* expression showed a weak positive correlation to fungal quantity. No statistically significant correlation was shown for *Ecp2* expression and fungal quantity. This study gave more insight into the molecular mechanisms of infection by showing that *C. zeina* effectors *Avr4* and *Ecp6* are expressed during fungal pathogenesis. Even though the *C. zeina Ecp2* effector was expressed during pathogenesis, the expression could only be detected when double the amount of cDNA was added to RT-qPCR reactions.

2. INTRODUCTION

Grey leaf spot (GLS) disease of maize in southern Africa is caused by the foliar pathogen *Cercospora zeina* (MEISEL et al. 2009). GLS disease is associated with major yield losses in maize production. Yield losses of up to 50% in hybrids with moderate resistance and 65% in susceptible maize hybrids have been reported in South Africa (WARD and NOWELL 1998). Initial GLS disease symptoms are chlorotic spots, which are small, irregular shaped spots that appear approximately 10 days post inoculation (dpi). The chlorotic spots can easily be seen when held against the light, but are sometimes confused with the initial symptoms of other maize foliar diseases (WARD *et al.* 1999). Mature GLS lesions caused by *C. zeae-maydis* are usually observed from 15 dpi, where after lesions will start to coalesce at about 21 dpi. In favourable conditions, which include high levels of humidity and warm temperatures, blighting of entire leaves may occur that drastically influences the photosynthetic ability (BECKMAN and PAYNE 1982) of the leaves causing low yields and even death of maize plants.

It is proposed that *C. zeina* infects maize leaves in a similar way as is described for *C. zeae-maydis* (BECKMAN and PAYNE 1982). Conidia germinate on the maize leaf surface and germ tubes penetrate stomatal openings following formation of an appressorium. An infection peg will grow out of the appressorium and through the stomatal opening into the apoplastic space. *C. zeina* has a hemibiotrophic life style and fungal hyphae will grow intercellularly without causing damage to the maize leaf during the initial biotrophic growth stage of the fungus. The necrotrophic growth stage is characterized by dying leaf material where the fungus relies on the nutrients released from the dying plant cells for survival. The fungus is able to remain alive during this second growth stage and conidiophores will grow out of the stomata and produce conidia for secondary infection of neighbouring maize plants (WARD *et al.* 1999).

Little is known about the morphological or molecular processes underlying *C. zeina* infection. Insight into the molecular mechanisms of infection would aid in the development of an effective strategy to combat this economically important disease. In the previous chapter, it was determined that *Avr4*, *Ecp2*, and *Ecp6* effector homologs are present *C. zeina*. This chapter aimed to identify if these genes are expressed during maize infection by means of RT-qPCR. *C. fulvum Avr4* plays a defensive role during infection by binding to chitin present in fungal cell walls, thereby inhibiting detection by the host (VAN DEN BURG *et al.* 2006). Joosten *et al.* (1997) showed that expression of *C. fulvum Avr4* is induced during pathogenesis and showed variable expression levels between different fungal strains (JOOSTEN *et al.* 1997). *C. fulvum Ecp6* plays a defensive role during pathogenesis by sequestering chitin oligomer molecules to prevent detection by the host (DE JONGE and

THOMMA 2009). *C. fulvum Ecp6* had low, but steady levels of expression during an incompatible reaction and induced expression levels during a compatible reaction (BOLTON *et al.* 2008). The exact function of *Ecp2* still needs to be elucidated, but it was proposed that *Ecp2* induces necrosis that favours the necrotrophic growth stage of hemibiotrophic fungi (STERGIOPOULOS *et al.* 2010). C*. fulvum Ecp2* exhibited low expression levels when analysed *in vitro* conditions and high expression levels at *in planta* conditions (VAN DEN ACKERVEKEN *et al.* 1993). We therefore hypothesised that *C. zeina Avr4* and *Ecp6* are expressed at constant levels during infection and that *C. zeina Ecp2* is expressed at high levels during the necrotrophic fungal growth stage as *Ecp2* was proposed to function during this stage.

No reference genes have been identified for *C. zeina in planta* gene expression normalisation. Therefore, we aimed to identify and validate possible reference genes for *C. zeina in planta* expression normalisation. It was hypothesised that the *glyceraldehyde-3-phosphate dehydrogenase*, *40S ribosomal protein*, and *cytochrome c oxidase subunit III* reference genes (VIEIRA *et al.* 2011) would be suitable candidates for *in planta C. zeina* expression normalisation.

A method for *in planta* quantification of *C. zeina* in infected maize leaf material has recently been published (KORSMAN *et al.* 2010). The method involves quantitative expression analysis of the *C. zeina* cytochrome P450 reductase (*cpr1*) gene that is normalised by the expression of the maize glutathione S-transferase III (*gst3*) gene. Another aim of this study was to determine if there is a correlation between fungal quantity and effector gene expression during infection. The hypothesis was that there would be a positive correlation between fungal quantity and effector gene expression. In other words, as fungal quantity increases, the effector expression also increases.

Elucidating the expression profiles of *C. zeina Avr4, Ecp2* and *Ecp6* and determining if there is a correlation between fungal quantity and effector gene expression, will give more insight into the molecular interactions between *C. zeina* and maize during infection. If the *C. zeina Avr4*, *Ecp2*, and *Ecp6* genes are expressed during infection it will indicate that these genes play a role in fungal pathogenesis. The effector genes would then be candidates for target specific control mechanisms of GLS disease.

For a successful infection, prolonged and frequent periods of high humidity and warm temperatures are needed (WARD *et al.* 1999). Up until now, maize inoculation trails have been undertaken in greenhouses and inoculations have therefore been limited to maize growing seasons. This study aimed to establish maize inoculation in the phytotron. The phytotron provides a controlled environment for infection as factors such as temperature and

humidity can be regulated. Establishing inoculation in the phytotron would mean that maize inoculation trials could be undertaken at times independent of maize growing seasons.

3. MATERIALS & METHODS

All reagents used in this study were obtained from Sigma (Aston Manor, South Africa) unless otherwise stated. Primers were synthesized by IDT (Coralville, Iowa) and sequencing reactions were done by Inqaba Biotec (Pretoria, South Africa). All centrifugation steps were performed using a bench top MiniSpin® centrifuge (Eppendorf, Hamburg, Germany) unless stated otherwise.

3.1 Inoculum preparation

Virulent *C. zeina* (Mkushi) (CMW25467) (MEISEL *et al.* 2009) V2A (Virulent strain 2A) cultures were grown *in vitro* on V8 media (200 ml V8 juice, 15 g Agar, 2 g $CaCO_3$, and 800 ml distilled water) at room temperature and in the dark to promote conidiation. Fungal growth was maintained until densely grown cultures were obtained. Conidia from densely grown cultures were harvested by flooding the plates with 0.01% Tween 20 solution. Concentrations of approximately $3x10^6$ conidia per ml were used for the maize inoculations.

3.2 Plant material

Maize B73 seeds were planted in pots containing a 1:1 ratio of soil and sand. The B73 maize line has been shown to be susceptible to *C. zeina* in field trials in South Africa (Crampton, unpublished) and is also susceptible to *C. zeae-maydis* in the USA (MAROOF *et al.* 1996). The plants were kept in a phytotron with day/night temperatures of 35˚C/22˚C and 60-100% relative humidity. Maize plants were watered daily and Nutrifeed fertilizer (Starke Ayres, Bredell, Johannesburg) was applied once a week. Maize plants were grown to the six leaf stage (L6) before they were inoculated. After inoculation, plants were covered with plastic bags to form canopies (Figure 1) for the first five days in order to obtain high humidity levels necessary for infection. The relative humidity and temperature in the phytotron were measured by a HOBO® Pro v2 external temperature and relative humidity data logger.

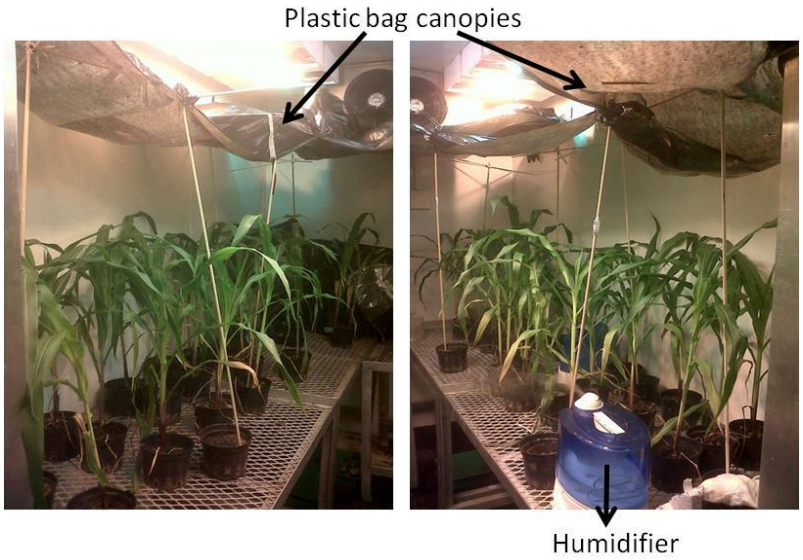


Figure 1. Plastic bag canopies used to obtain increased humidity levels. Inoculated maize plants were covered in plastic bag canopies, as shown in the photographs, during the first five days to increase humidity levels. A high level of humidity is needed during the initial stages for successful *C. zeina* infection, and therefore extra humidifiers were also placed in the phytotron.

3.3 Maize inoculation

At least one leaf per maize plant was inoculated with a *C. zeina* conidial suspension ($3x10^6$ conidia per ml) by brushing the conidia onto both sides of the leaf with a paint brush. Tween 20 (0.01%) solution without fungal conidia was used for control plants. Three biological replicates at each time point (0, 3, 5, 10, 12, 15, and 23 days post inoculation (dpi)) were harvested. Harvested leaf material for all samples was similar in size and region harvested from each plant with each plant only harvested once. No disease symptoms were seen prior to 23 dpi. Two samples were harvested in triplicate at 23 dpi, namely 23C dpi (representing chlorotic disease symptoms) and 23L dpi (representing necrotic disease symptoms). The disease symptoms that were observed are summarized in figure 2. Eight time points in total were harvested in triplicate and therefore 24 samples in total. Harvested leaf material was immediately frozen away at -80˚C till further use. No conidia could be re-isolated from infected maize leaves.

3.4 DNA extraction

DNA extractions were done for three replicates of each time point harvested during the inoculation trial. Ground-up leaf material was used for genomic DNA extraction purposes. A mini CTAB DNA extraction protocol modified from a method described by Möller *et al.* (1992) (MOLLER *et al.* 1992) as well as a method used by IIlustra Nucleon Phytopure genomic DNA extraction kit (GE Healthcare UK limited, UK, Buckinghamshire) was optimized in our laboratory was used for the extractions. In short, approximately 100mg of infected maize

material was incubated at 65˚C for one hour with shaking in the presence of 1 ml Cetyltrimethyl ammonium bromide (CTAB) buffer and 16 µl β-Mercaptoethanol. After the first incubation step, 900 µl chloroform and isoamylalcohol in the ratio 24:1 was added and the mixture was incubated again at 65˚C for 30 minutes with shaking. The mixture was then centrifuged for 10 minutes at 12000 rpm (18192 g) and 5 µl RNase was added to the supernatant before the third incubation step at room temperature for 30 minutes. After the incubation step, 900 µl of the chloroform and isoamylalcohol mixture (24:1) was again added before the fourth incubation step at 28˚C for 90 minutes. The mixture was then centrifuged for 10 minutes at 12000 rpm (18192 g), where after the aqueous phase was discarded and 700 µl ice-cold isopropanol was added to the supernatant and mixed by careful inversion. The mixture was then incubated on ice for 30 minutes and then centrifuged again for 10 minutes at 12000 rpm (18192 g). The supernatant was discarded without disturbing the pellet and 300 µl 70% ethanol was added to the DNA pellet. This was followed by an incubation step at 4˚C for 30 minutes, where after the 70% ethanol was replaced before incubating overnight at 4˚C. The overnight incubation step was followed by a centrifugation step at 12000 rpm (18192 g) for 10 minutes. Ethanol was removed by air-drying the sample. The DNA pellet was re-dissolved in 50 µl double distilled water. The concentrations and purity of DNA extracted for each time point was measured spectrophotometrically with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts). The DNA purity of each sample was analysed by the 260/280 (protein contamination) and 260/230 (organic compounds) ratios. The integrity of the extracted DNA was determined by analysing 3 µl of each sample on a 0.8% agarose gel.

3.5 RNA extraction

Total RNA was extracted from infected maize leaves from all time points harvested. The RNA extraction was done by using the RNeasy Plant Mini RNA Extraction kit (Qiagen, Valencia, California). RNA was extracted according to the manufacturer's specifications. Approximately 100 mg of the frozen leaf material was ground to a fine powder by using liquid nitrogen and used for RNA extraction purposes. On-column DNase digestion was performed according to the manufacturer's specifications. RNA was eluted in 30 µl RNase-free water. The spin column was centrifuged twice for one minute at 10000 rpm (12633 g) to elute the RNA. The RNA concentration of each sample was determined spectrophotometrically with a Nano-Drop™ 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts). The RNA purity of each sample was analysed by the 260/280 and 260/230 ratios. The RNA integrity of each sample was determined through gel electrophoresis by analysing the samples on a denaturing 0.9% formaldehyde agarose gel (0.5 g agarose, 5 ml 10X MOPS,

3.5 ml formaldehyde, and 37.5 ml DEPC water). RNA samples were stored away at -80˚C until cDNA synthesis.

3.6 cDNA synthesis

Purified RNA was used for cDNA synthesis using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, Massachusetts) according to the manufacturer's specifications. The maximum amount of RNA template (5 µg) was used for cDNA synthesis to ensure high amounts of cDNA produced. The cycling conditions were 10 min at 25˚C, 30 min at 65˚C followed by 5 min at 85˚C. The cDNA samples were aliquoted and stored at -80˚C until needed. As cDNA is unstable and can easily degrade after only a few freeze-thaw cycles, aliquots provided an effective way to limit freeze-thaw cycles and to maintain cDNA integrity.

3.7 Identification of reference genes

No reference genes have been identified for gene expression studies in *C. zeina*. A study done on expressed sequence tags (ESTs) in *C. zeae-maydis* identified β-tubulin (GenBank accession number: EU402967) as an endogenous reference for normalisation (BLUHM *et al.* 2008). Reference genes for RT-qPCR were also validated in a study of the biotrophic fungal pathogen *Hemileia vastatrix* that causes coffee leaf rust. These genes included β-tubulin (*β-TUB*) (GenBank accession number: FR720600), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (FERNANDEZ *et al.* 2012), elongation factor (*EF-1*) (GenBank accession number: FR720601), cytochrome c oxidase subunit III (*Cyt III*) (FERNANDEZ *et al.* 2012), cytochrome b (*Cyt b*) (GRASSO *et al.* 2006), and 40S ribosomal protein (*40S*) (FERNANDEZ *et al.* 2012). The study showed that *40S*, *GAPDH*, and *Cyt III* were most stable for *in planta* expression studies. The *β-TUB*, *GAPDH*, *EF-1*, *Cyt III*, *Cyt b*, and *40S* sequences were used to identify the corresponding gene sequences in the *C. zeina* genome through BLAST searches. The predicted gene regions (Appendix, Table S1) were then put through gene prediction programmes FGENESH (http://linux1.softberry.com), and Augustus (http://bioinf.uni-greifswald.de/augustus/) to predict the gene structures. The predicted reference gene sequence information that includes cDNA and protein sequences for each putative reference gene is outlined in the appendix (Table S1). The predicted *C. zeina* reference gene protein sequences were subjected to tBLASTx analysis against GenBank to identify homologs in other fungal species. The tBLASTx results were summarised in the appendix (Table S2). Protein alignments of each *C. zeina* reference gene with at least three fungal homologs, as listed in Table S2, were made to determine if the correct *C. zeina*

reference genes were identified. Protein alignments were made for GAPDH, β-TUB, EF-1, Cyt III, Cyt b, and 40S (Figures S7-S12, Appendix).

3.8 RT-qPCR primer design

Primers were designed using PrimerQuest (http://eu.idtdna.com/PrimerQuest/Home/) and synthesized by Integrated DNA Technologies (IDT) (Coralville, Iowa). For RT-qPCR purposes, designed primers all conformed to the following specifications: primer lengths between 16-22 nucleotides, amplicon lengths between 80-150 bp, GC content of 30-80%, melting temperatures (T_m) within 58-61˚C, avoidance of runs of more than four nucleotides at the 3'-end as well as avoidance of two or more G's or C's in the last five nucleotides of the 3'-end. Primer sequences and associated information are summarized in Table 1. After primers were designed, reciprocal BLAST searches against the maize genomic sequence DNA database (http://www.maizesequence.org/BLAST) were made to make sure primers were specific to *C. zeina* and that maize genes would not be amplified.

Table 1. A summary of the primer sequences used for *in planta* expression analysis and fungal quantification. The gene target and melting temperature (T_m) for each primer is indicated. The expected amplicon sizes were noted as cDNA/gDNA or only as gDNA amplicon size. Primer sets with different cDNA and gDNA amplicon sizes can be used to test for gDNA contamination.

Name	Gene Target	Sequence (5'-3')	T_m (˚C)	cDNA/gDNA Amplicon (bp)
Avr4Q F	*C. zeina Avr4*	AGACTGTCTCTACCCACTAC	60	148/148
Avr4Q R	*C. zeina Avr4*	CGGATAATCGCACCACTT	59	
Ecp2Q F	*C. zeina Ecp2*	GTCCCACAGAGGAAGAATG	59	137/137
Ecp2Q R	*C. zeina Ecp2*	TTGTCGAGGCTCTGAATG	59	
Ecp6Q F	*C. zeina Ecp6*	GCCAAGGGTGATCTCTTT	59	143/143
Ecp6Q R	*C. zeina Ecp6*	GTGATGTTGTGGCAGTTC	59	
TUB F	*C. zeina* Beta-Tubulin	TACCTGCTCGGCTATCTA	59	93/93
TUB R	*C. zeina* Beta-Tubulin	ACGAAGTAGGCAGTGTTC	59	
GAPDH F	*C. zeina* glyceraldehyde-3-phosphate dehydrogenase	TTCATCGAGCCACACTAC	59	103/161*
GAPDH R	*C. zeina* glyceraldehyde-3-phosphate dehydrogenase	TGACGATCAGGCCTTTG	59	
EF-1 F	*C. zeina* elongation factor	GTGCTCGACAAGCTGAA	59	99/154*
EF-1 R	*C. zeina* elongation factor	GTCGATGACGGTGACATAG	59	
40S F	*C. zeina* 40S ribosomal protein	GGTCCTCAAGGTCATTCTC	59	102/102
40S R	*C. zeina* 40S ribosomal protein	TTGACACCCTTTCCAGTC	59	
Cyt III F	*C. zeina* cytochrome c oxidase III	GAGCTCTTTATGGTGCTCTA	59	108/108
Cyt III R	*C. zeina* cytochrome c oxidase III	CGTAGGCTCCATCTGATAAA	59	
Cyt b F	*C. zeina* cytochrome b	GTAGAAGCAGAGGGTTACAG	59	111/111
Cyt b R	*C. zeina* cytochrome b	AAGGCGATTCAACGTGT	59	
GST3 F	*Z. mays* glutathione S- transferase III (KORSMAN *et al.* 2010)	GGAGCCCTGAGTCGAATAAAAG	60	106
GST3 R	*Z. mays* glutathione S- transferase III (KORSMAN *et al.* 2010)	AACACACACGAAAGGCAACAGT	60	
CPR1 2F	*C. zeina* cytochrome P450 reductase (KORSMAN *et al.* 2010)	TGAACTACGCGCTCAATG	60	164
CPR1 2R	*C. zeina* cytochrome P450 reductase (KORSMAN *et al.* 2010)	TCTCTCTTGGACGAAACC	60	

*Can be used to indicate gDNA contamination

3.9 RT-PCR optimization of effector and reference genes

The specificity of each primer pair to be used for relative expression analysis was further investigated through conventional PCR methods prior to RT-qPCR analysis. First, the effector gene primers as well as the reference gene primers were tested on *C. zeina* (Mkushi) gDNA to determine if the gene products were amplified and if the expected product sizes were obtained. The primer sets were then tested on infected cDNA and maize cDNA

(control) to determine if the genes were expressed and to be able to detect possible gDNA contamination. The infected cDNA of the harvested time points were pooled into control (0 dpi), early (5 dpi, 10 dpi, and 12 dpi), and late (15 dpi, 23C dpi, and 23L dpi) time points for RT-PCR checks. No amplification of effector and *C. zeina* reference gene primer sets should be seen in maize cDNA. The maize *gst3* reference gene primers (GenBank accession number X06755) (KORSMAN *et al.* 2010) were used as a positive control during maize cDNA amplifications. The PCR cycling conditions for *C. zeina* gDNA, infected cDNA, and maize cDNA amplifications for all primer sets were as follows: denaturation at 94˚C for 5 min, followed by 30 cycles of (94˚C for 30 sec, 58˚C for 45 sec, and 72˚C for 2 min), and a final elongation step at 72˚C for 7 min. For PCR amplification, reaction volumes of 20 µl consisted of 1X NH_4 PCR reaction buffer, 0.2 mM dNTP mix (Bioline, London, UK), 1.5 mM $MgCl_2$, 0.3 µM of each of the primers, 0.025 U of BIOTAQ$_{TM}$ DNA Polymerase (Bioline, London, UK), and made up to 20 µl with sterile distilled water. Template concentrations used in the reactions were 10 ng *C. zeina* gDNA or 10 ng infected cDNA or 10 ng maize cDNA. The quality of the PCR products was verified by standard 2% agarose gel electrophoresis.

3.10 Cloning and sequencing

Products to be cloned were first amplified from *C. zeina* (Mkushi) gDNA through PCR reactions with gene-specific primers and subsequently analysed on an agarose gel. If these amplified products were specific (i.e. single bands on the agarose gel) and of the correct size, the PCR reactions were cleaned by using a Sephadex G-50 clean-up protocol. Sephadex slurry was prepared by adding 45ml distilled water to 3 g Sephadex G-50 powder and microwaving the solution until dissolved. Sephadex cleaning columns were made by spinning the prepared Sephadex slurry (650 µl per eppendorf tube) in a bench-top centrifuge at 2000 rpm (505 g) for three minutes. The prepared column was then added to a new 1.5 ml eppendorf tube and the PCR product was gently pipetted onto the middle of the column and centrifuged at 2000 rpm (505 g) for three minutes. The purified PCR product was in the elute. The Sephadex cleaning columns provided a fast and effective method for purification and recovery of DNA. After the PCR products were purified, the concentrations were determined spectrophotometrically with a Nano-Drop^{TM} 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts).

Cloning of purified PCR products was done by using the InsTAclone PCR Cloning Kit (Thermo Scientific, Waltham, Massachusetts) following the manufacturer's specifications. A map of the cloning vector pTZ57R/T can be found in the (Appendix, Figure S1). Chemically competent cells were prepared from *E. coli* JM109 cells by means of a bacterial

transformation kit included in the InsTAclone PCR cloning kit (Thermo Scientific, Waltham, Massachusetts). Recombinant clones were identified based on blue/white screening which involved the plating out of transformed bacterial colonies on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG). Positively transformed colonies were white in colour, while negatively transformed colonies were dark blue in colour. Five positively transformed colonies were further analysed through colony PCR. The cloning kit makes use of the pTZ57R/T cloning vector and insert DNA can be amplified by using standard M13/pUC primers. The PCR cycling conditions for the M13/pUC primer set were as follows: denaturation at 94˚C for 2 min, followed by 30 cycles of (94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 1 min), and a final elongation step at 72˚C for 7 min. For PCR amplification, reaction volumes of 20 µl consisted of 1X NH_4 PCR reaction buffer, 0.2 mM dNTP mix (Bioline, London, UK), 1.5 mM $MgCl_2$, 0.3 µM of each of the primers, 0.025 U of BIOTAQ$_{TM}$ DNA Polymerase (Bioline, London, UK), and made up to 20 µl with sterile distilled water. Cloned products were sequenced as a further step of validation. Plasmid extractions for sequencing purposes were done by using the GeneJET^{TM} Plasmid Miniprep Kit (Thermo Scientific, Waltham, Massachusetts). Extractions were done according to the manufacturer's specifications. Extracted plasmid samples were analysed with a Nano-Drop^{TM} 1000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts) to determine the concentrations and also analysed by 2% agarose gel electrophoresis to confirm product presence after the extraction protocol. Plasmid samples, containing the *C. zeina* (Mkushi) gDNA inserts, with a concentration of at least 100 ng/µl were used for sequencing reactions. Sequencing reactions were done by Inqaba Biotec (Pretoria, South Africa) with standard M13F and M13R primers. Forward and reverse sequencing reactions were done for each sample. Sequenced products were analysed and aligned by using CLC Bio software (Appendix Figure S2-S6).

3.11 Relative expression analysis of effector and reference genes

Real-time quantitative PCR (RT-qPCR) analysis was done on the Bio-Rad CFX96 Touch^{TM} Real-Time PCR Detection System. The run layout was based on the sample maximization method (HELLEMANS *et al.* 2007). Expression of the *C. zeina* (Mkushi) effector (*Avr4*, *Ecp2*, and *Ecp6*) and reference genes (*GAPDH*, *β-TUB*, *EF-1*, *40S*, *Cyt III*, and *Cyt b*) was determined over eight time points, with each time point consisting of three biological repeats measured in triplicate. Standard samples present on each plate were also done in triplicate with three biological replicates. The standard samples used for standard curve generation purposes consisted of plasmid DNA (Appendix, Figure S1) (InsTAclone PCR cloning kit, Thermo Scientific, Waltham, Massachusetts) containing cloned gDNA *C. zeina* (Mkushi)

gene products specific to each effector or reference gene analysed. For example, standard samples used for the *Avr4* standard curve, consisted of plasmid DNA containing *C. zeina* (Mkushi) *Avr4* gDNA cloned product. Due to limited cDNA, plasmid DNA contained cloned gDNA instead of cDNA for all standard samples. For RT-qPCR amplification, reaction volumes of 10 µl consisted of 5ul Lightcycler® 480 SYBR Green I Master Mix (Roche Diagnostics, Basel, Switzerland), 0.5 µM of each of the primers, 4 µl sterile distilled water, and 1 µl cDNA template or water in the case of non-template controls. The cycling conditions consisted of a pre-incubation step of 95˚C for 10min, followed by 45 cycles of (95˚C for 10 sec, 60˚C for 10 sec, and 72˚C for 15 sec). Fluorescence was detected continuously at the end of each elongation step. Melting curve analysis was done for every run to measure amplification specificity. A standard curve for each effector and reference gene was set up by using seven dilution points containing plasmid DNA (consisting of cloned target DNA). Dilutions used for effector genes were $1x10^{-3}$, $1x10^{-4}$, $1x10^{-5}$, $1x10^{-6}$, $1x10^{-7}$, $1x10^{-8}$, and $1x10^{-9}$ ng/µl. Plasmid dilutions used for reference genes were 1, $1x10^{-1}$, $1x10^{-2}$, $1x10^{-3}$, $1x10^{-4}$, $1x10^{-5}$, and $1x10^{-6}$ ng/µl. Gene expression data was analysed by Bio-Rad CFX Manager™ and qBASE^{PLUS} (Biogazelle, Zwijnaarde, Belgium) software. Effector gene relative expression values was normalized by dividing the amount of input fungal cDNA (extrapolated from the respective standard curves) by the geomean of input reference cDNA of the reference genes *GAPDH* and *Cyt III* (VANDESOMPELE *et al.* 2002).

3.12 Fungal quantification

In planta fungal quantification was determined by using the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System. *C. zeina* quantification was done by the method published by Korsman and colleagues (KORSMAN *et al.* 2010). In short, qPCR reactions consisted of 10ul reaction volumes containing 5 µl Lightcycler® 480 SYBR Green I Master Mix (Roche Diagnostics, Basel, Switzerland), 0.5 µM of each of the primers, 4 µl sterile distilled water, and 1 µl DNA template (10 ng/µl) or water in the case of non-template controls. The cycling conditions consisted of a pre-incubation step of 95˚C for 5 min, followed by 45 cycles of (95˚C for 10 sec, 60˚C for 10 sec, and 72˚C for 5 sec). Fluorescence was detected continuously at the end of each elongation step. Melting curve analysis was performed for every run to measure amplification specificity. Standard curves were set up by using seven dilution points. Dilutions used for the *C. zeina* (Mkushi) *cpr1* gene contained pure *C. zeina* extracted DNA diluted in 10 ng maize (B73) DNA so that the final fungal DNA concentrations were 5, 1, $5x10^{-1}$, $5x10^{-2}$, $5x10^{-3}$, $2.5x10^{-3}$, and $1x10^{-3}$ ng/µl. Pure maize (B73) DNA dilutions used for the *gst3* gene were 20, 15, 12.5, 5, 1, $5x10^{-1}$, and $1x10^{-1}$ ng/µl. Gene quantification data was analysed by Bio-Rad CFX Manager™. The amount of fungal DNA in the samples

of interest could be determined from the *cpr1* standard curve. Quantification values were normalized by dividing the amount of fungal DNA by the amount of maize DNA quantified in infected samples, thereby obtaining quantities ng *C. zeina* per ng maize DNA.

3.13 Statistical analysis

Statistical analysis of the relative expression data as well as the fungal quantification data was done with GraphPad Prism 5.04 software using log-transformed normal distributed relative quantification data of all the relevant biological replicates. One-way ANOVA analysis was followed by Tukey's Multiple Comparison test. The level of statistical significance was measured at $p \leq 0.05$. A Pearson's Correlation test was undertaken to determine if there was a linear correlation between effector expression and fungal quantity at specific time points that included all the relevant biological replicates.

4. RESULTS

4.1 Maize inoculation trial

Inoculation of B73 maize (susceptible) plants was carried out by painting a conidial suspension onto both sides of at least one leaf per maize plant as outlined in the materials and methods section. Control plants were inoculated in the same way, but with a 0.01% Tween 20 solution containing no conidia. In order to obtain high levels of humidity necessary for infection during the initial stages, plants were covered with plastic bag canopies (Figure 1). Maintaining humidity levels above 75% required additional humidifiers in the phytotron (Figure 1) (Appendix, Figure S13). No disease symptoms were observed for more than three weeks after inoculation. Symptoms were only observed from 23 dpi. At 23 dpi, some plants appeared to be at initial stages of infection, whereas others already had mature GLS lesions. The symptoms observed at 23 dpi and onwards including a control maize leaf are summarized in figure 2. Time points harvested were 0 dpi (control), 3 dpi, 5 dpi, 10 dpi, 12 dpi, 15 dpi, 23C dpi (representing chlorotic symptoms), and 23L dpi (representing necrotic symptoms). Three biological replicates were harvested for each time point. Replicate maize leaves harvested for the 23C dpi time point showed similar symptoms as seen in figure 2 (A) and replicate leaf material harvested for the 23L dpi time point showed similar symptoms as indicated in figure 2 (C) and (D). Figure 2 (B) shows typical GLS lesions indicating that the infection in the phytotron was successful. Unfortunately, no conidia could be re-isolated from GLS lesions. Lesions showed the presence of conidiophores as visualized under a light microscope (results not shown), but no conidia could be observed.

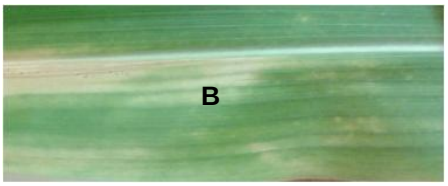
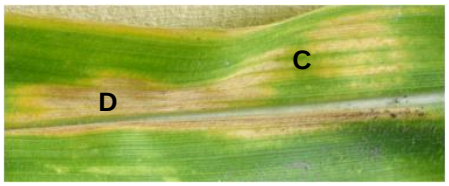
Symptoms observed	Comments
	Control (Healthy leaf)
	**(A)** Chlorotic spots
	**(B)** Grey Leaf Spot (GLS) lesions
	**(C)** Lesions coalescing **(D)** Blighting

Figure 2. GLS disease symptoms developing on maize leaves following inoculation with *C. zeina* conidia. The table indicates the different disease symptoms that were observed. Symptoms were only observed from 23 days post inoculation (dpi) and indicate that some plants were at initial stages of infection, whereas others showed further disease progression. Disease symptoms observed included chlorotic spots (A), GLS lesions (B), lesions coalescing (C), as well as blighting (D). The leaf material harvested for 23C dpi showed similar symptoms as seen in (A), whereas leaf material harvested for 23L dpi had symptoms similar to (C) and (D).

4.2 DNA extractions

Total genomic DNA was extracted from infected maize leaves for three replicates of each of the eight time points harvested during the maize inoculation trial. Total genomic DNA was extracted for fungal quantification purposes through qPCR. Relatively high DNA concentrations were extracted for most time points with gDNA concentrations ranging between 2.8 ng/µl and 190.8 ng/µl. Low DNA concentrations (concentrations below 30 ng/µl) were obtained for 10 dpi (replicate a), 12 dpi (replicate b), 15 dpi (replicate a), and 23 dpi (chlorotic leaf material, replicate b) and very low DNA concentrations (concentrations below

15ng/ul) were obtained for 0 dpi (replicates b and c) and 15 dpi (replicate b). DNA extractions for samples with low concentrations were repeated, but similar results were obtained. The extracted gDNA for all samples were of high quality as 260/280 and 260/230 spectrophotometer ratios were all approximately 1.8 (with the lowest reading being 1.67) and 2.0-2.2 (with the lowest reading being 1.98), respectively, indicating pure samples with no impurities (organic material). Figure 3 indicates the DNA quality of the analysed samples. DNA extracted for all samples were intact and not degraded. No bands were visible for 0 dpi (replicates b and c) and 15 dpi (replicate b) as these time points had very low concentrations of DNA (concentrations below 15 ng/µl). However these very low concentration time points showed good quality DNA and spectrophotometer readings indicated that these time points could be used in further analysis.

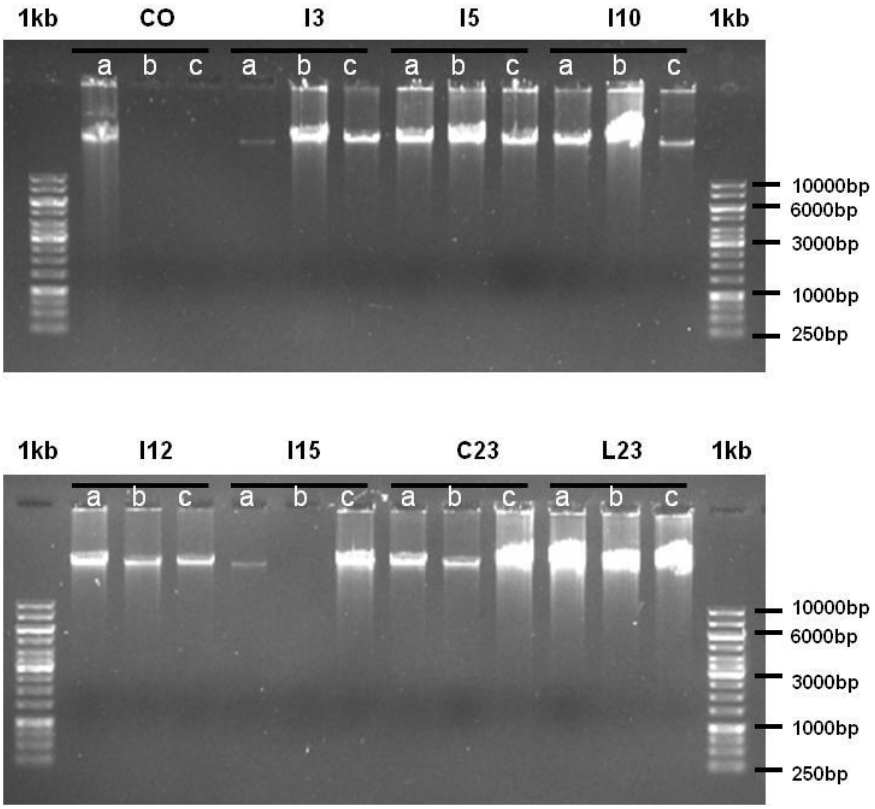


Figure 3. DNA quality of extracted gDNA across all time points harvested during the maize inoculation trial. The figure shows two gel images of DNA extracted from leaf material across all infection time points. Samples were analysed on 0.8% agarose gels. Three biological replicates, represented by (a), (b), and (c), of each time point were analysed. Time points analysed were 0 dpi (C0), 3 dpi (I3), 5 dpi (I5), 10 dpi (I10), 12 dpi (I12), 15 dpi (I15), 23 dpi representing chlorotic leaf material (C23), and 23 dpi representing necrotic leaf material (L23).

4.3 RNA extractions and cDNA synthesis

Total RNA was extracted from three biological replicates of each of the harvested time points during the maize inoculation trial. High concentration RNA was extracted across all samples with concentrations ranging from 206.1 to 858.0 ng/µl. Spectrophotometer results also indicated that high quality RNA with few impurities was extracted across all samples as 260/280 and 260/230 ratios were all very close to 2.0 and 2.0-2.2, respectively. RNA quality was further analysed through gel electrophoresis on a denaturing formaldehyde agarose gel. RNA extracted from all samples showed no degradation as distinct 28S and 18S bands were visible for all samples (Figure 4). Purified RNA was used for cDNA synthesis.

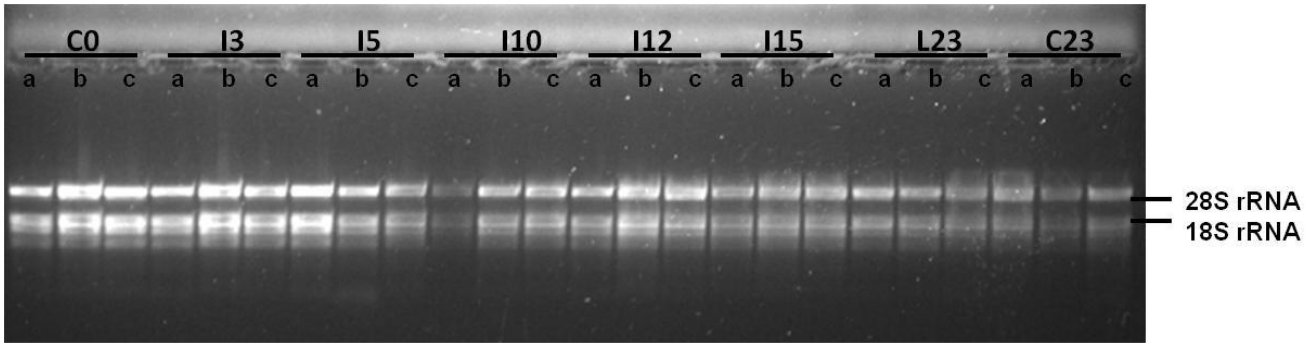


Figure 4. RNA quality across all time points harvested during the maize inoculation trial.
The quality of the extracted total RNA across all samples harvested were analysed by gel electrophoresis. The samples were analysed on a 0.9% formaldehyde gel. All samples showed intact RNA as the 28S rRNA and 18S rRNA bands are clearly visible. Three biological replicates (a), (b), and (c) were included for each sample.

4.4 RT-PCR optimization of effector and reference genes

Annotated gene regions for the six *C. zeina* (Mkushi) putative reference genes are shown in figure 5. The *C. zeina β-TUB* gene consisted of five exons of 25 bp, 24 bp, 55 bp, 55 bp, and 1185 bp as well as four intron regions of 55 bp, 66 bp, 53 bp, and 78 bp (Figure 5D). *C. zeina GAPDH* also has five exons of 12 bp, 50 bp, 64 bp, 688 bp, and 203 bp and four introns of 74 bp, 60 bp, 56 bp, and 80 bp (Figure 5E). The *C. zeina Cyt b* and *Cyt III* genes both consisted of single exons of 248 bp and 258 bp, respectively (Figure 5F and G). Figure 5 (H) shows the annotated *C. zeina 40S* ribosomal protein gene sequence that consisted of four exons of 24 bp, 138 bp, 161 bp, and 364 bp and three introns of 62 bp, 138 bp and 51 bp. The *C. zeina EF-1* gene has five exons of 5 bp, 71 bp, 119 bp, 1059 bp, and 70 bp as well as four intron regions of 73 bp, 254 bp, 55 bp, and 51 bp (Figure 5I). The annotated *C. zeina* (Mkushi) *Avr4*, *Ecp2*, and *Ecp6* effector gene regions as determined in chapter 2 are shown in figure 5 (A), (B), and (C).

Gene-specific primers were designed to the annotated gene regions of *C. zeina Avr4*, *Ecp2*, and *Ecp6* effector genes as well as to the *C. zeina β-TUB*, *GAPDH*, *EF-1*, *Cyt III*, *Cyt b*, and *40S* putative reference genes. Primer regions for each gene are indicated in figure 5. Table 1 gives a summary of the designed primers including their primer sequences, T_m, and expected amplicon lengths. Primers designed to the *GAPDH* and *EF-1* genes, were designed over the intron/exon boundaries. Therefore, the GAPDH and EF-1 primers could be used to check for gDNA contamination in infected cDNA samples. The specificity of the primers was tested through conventional PCR amplification prior to RT-qPCR.

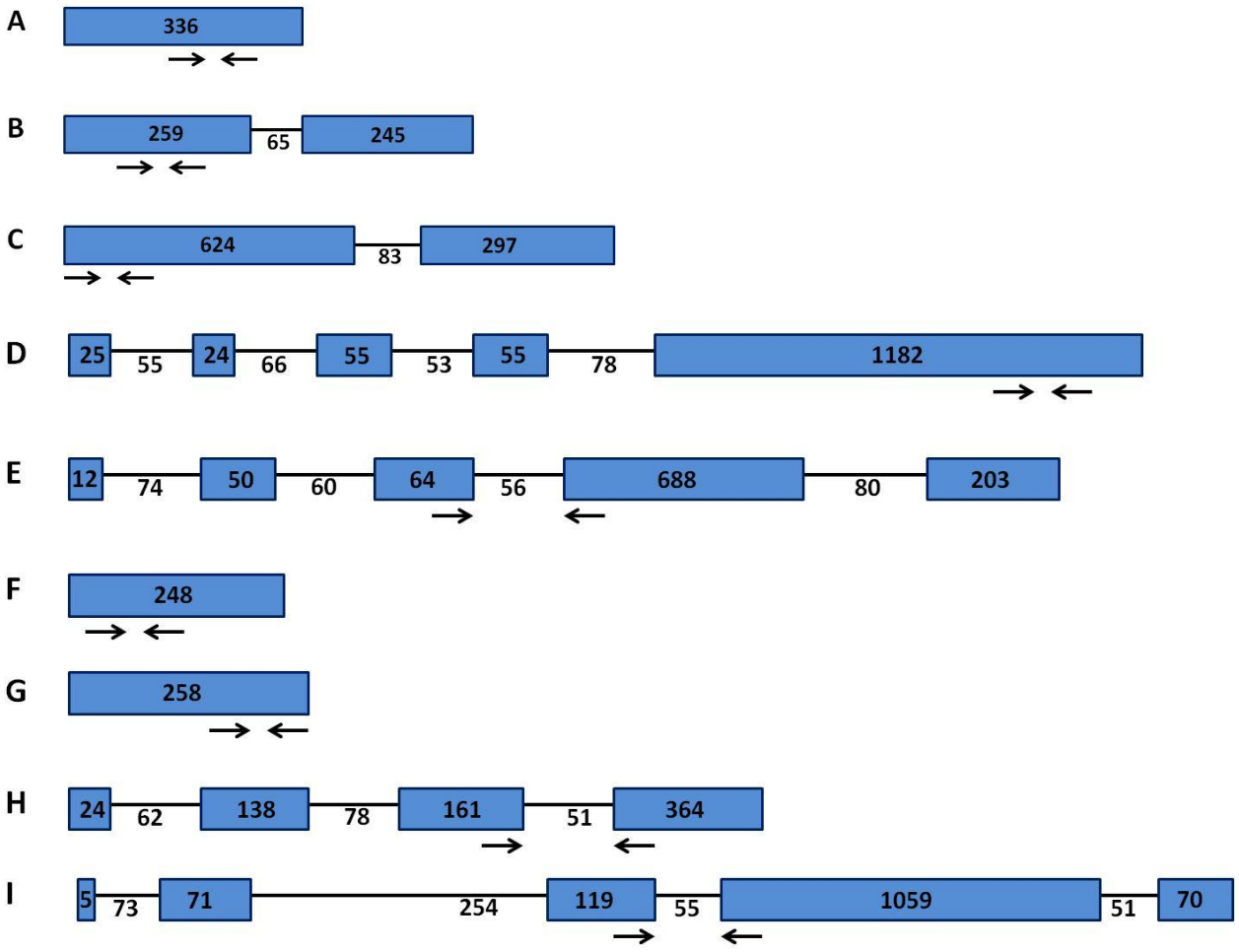


Figure 5. Annotations and primer regions for effector and reference genes.
The figure shows the gene structures of *C. zeina* (Mkushi) *Avr4* (A), *Ecp2* (B), and *Ecp6* (C) effector genes as well as *C. zeina* (Mkushi) *β-TUB* (D), *GAPDH* (E), *Cyt b* (F), *Cyt III* (G), *40S* (H), and *EF-1* (I) reference genes. Exons are indicated as blue blocks and introns as black lines. The sizes of the introns and exons are indicated in the figures. Primer sequences designed to the gene regions are indicated as black arrows. Figures are not drawn on scale.

Primer specificity was tested using *C. zeina* (Mkushi) gDNA to determine if single products of the expected sizes were amplified. The quality of the PCR products was verified through gel electrophoresis using a GeneRuler^{TM} 50 bp DNA Ladder (Thermo Scientific, Waltham, Massachusetts) to determine product sizes (Figure 6). The expected product sizes (Table 1) of approximately 148 bp, 137 bp, and 143 bp were obtained for the *Avr4*, *Ecp2*, and *Ecp6* amplifications, respectively. The expected product sizes (Table 1) of approximately 93 bp, 161 bp, 154 bp, 102 bp, 108 bp, and 111 bp were obtained for the reference genes *β-TUB*, *GAPDH*, *EF-1*, *40S*, *Cyt III*, and *Cyt b*. The PCR controls showed no amplification as expected and no primer dimers were present. Therefore the primers showed gene-specific amplification in *C. zeina* (Mkushi).

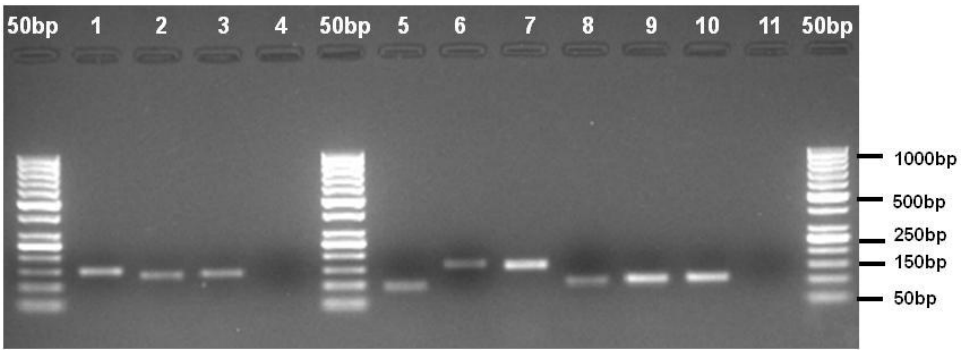


Figure 6. *C. zeina* (Mkushi) gDNA amplification. The figure shows a 2% agarose gel containing the amplification products of the effector genes and reference genes form *C. zeina* (Mkushi) gDNA. (1) *Avr4*, (2) *Ecp2*, (3) *Ecp6*, (4) PCR control, (5) *β-TUB*, (6) *GAPDH*, (7) *EF-1*, (8) *40S*, (9) *Cyt III*, (10) *Cyt b*, and (11) PCR control. The GeneRuler^{TM} 50 bp DNA Ladder (Thermo Scientific, Waltham, Massachusetts) was used to determine the product sizes. The expected product gDNA sizes of all the amplified products (lanes 1-3 and 5-10) were obtained: 148 bp, 137 bp, 143 bp, 93 bp, 161 bp, 154 bp, 102 bp, 108 bp, and 111 bp, respectively. The PCR controls contained no template and were amplified with *Avr4* and *β-TUB* primers respectively.

cDNA across all time points was pooled into control (0 dpi), early (3 dpi, 5 dpi, and 10 dpi), and late (15 dpi, 23C dpi, and 23L dpi) time points. The control time point (0 dpi) consisted of B73 maize cDNA. All primers were tested on the early and late cDNA time points to test if the genes were expressed and if gDNA contamination was present. No amplification could be observed for any of the primers from the cDNA pools (results not shown). As the fungal cDNA is diluted in the maize cDNA, conventional PCR might not have been sensitive enough to detect fungal gene expression.

Primers were also checked against the maize cDNA (control time point) to ensure that primers do not amplify maize genes. The maize *gst3* gene primers were used as a positive control for maize cDNA amplification. Negative controls contained no template. The quality of the PCR products was verified through gel electrophoresis using a GeneRuler^{TM} 50 bp DNA Ladder (Thermo Scientific, Waltham, Massachusetts) and GeneRuler^{TM} 100 bp DNA Ladder (Thermo Scientific, Waltham, Massachusetts) to determine product sizes. As expected, no amplification was seen for the *C. zeina* effector and reference genes. The amplification product seen for *gst3* was of the expected size of approximately 106 bp (Figure 7). The non-template controls showed no amplification and no primer dimers were present. Therefore it could be expected that the designed *C. zeina* effector and reference gene primers are specific and would not amplify maize genes.

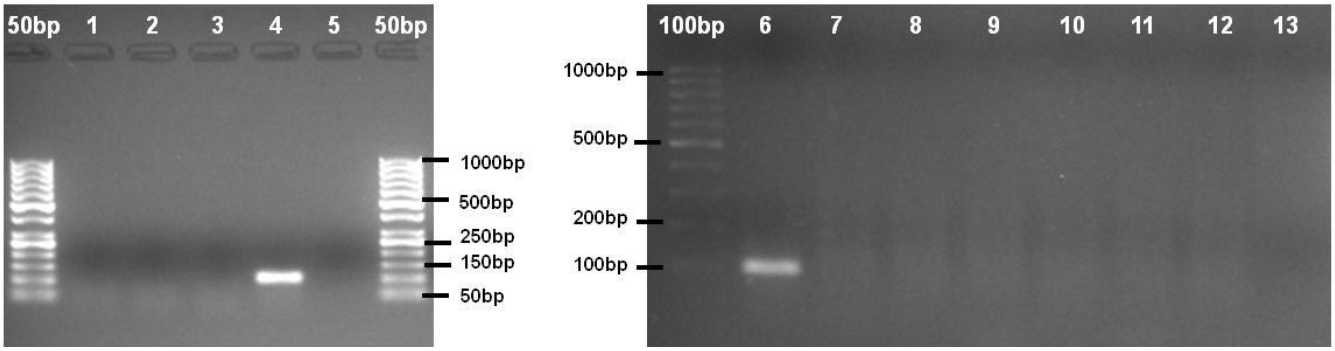


Figure 7. Maize cDNA amplification. cDNA isolated from the 0 dpi (contains only maize cDNA) was used for amplification of effector genes as well as the reference genes. (1) *Avr4*, (2) *Ecp2*, (3) *Ecp6*, (4) *gst3*, (5) control, (6) *gst3*, (7) *β-TUB*, (8) *GAPDH*, (9) *EF-1*, (10) *40S*, (11) *Cyt III*, (12) *Cyt b*, and (13) control. The maize *gst3* gene was used as a positive control for maize cDNA amplification. Amplification products were analysed on a 2% agarose gel. Controls contained no template and were amplified with *Avr4* and *β-TUB* primers respectively. GeneRuler^{TM} 50 bp DNA Ladder (Thermo Scientific, Waltham, Massachusetts) and GeneRuler^{TM} 100 bp DNA Ladder (Thermo Scientific, Waltham, Massachusetts) was used to determine product sizes. No *C. zeina* genes showed amplification from B73 maize cDNA.

4.5 Relative expression analysis of *C. zeina* effector and reference genes

RT-qPCR analysis of the three *C. zeina* effector genes (*Avr4*, *Ecp2*, and *Ecp6*) and six putative *C. zeina* reference genes (*GAPDH*, *β-TUB*, *EF-1*, *40S*, *Cyt III*, and *Cyt b*) were performed across eight infection time points that consisted of three biological replicates with three technical replicates each. Gene expression data was analysed by Bio-Rad CFX Manager^{TM} and qBASE^{PLUS} (Biogazelle, Zwijnaarde, Belgium) software. The Ct (threshold cycle) values of RT-qPCR replicates used for analysis differed with less than 0.5 cycles. For

expression normalisation, the expression results of the six putative *C. zeina* reference genes (*GAPDH*, *β-TUB*, *EF-1*, *40S*, *Cyt III*, and *Cyt b*) were analysed further in qBASE^{PLUS} software with geNorm to determine the expression stability of each putative reference gene across all the time points. Good reference genes will show stable expression across all time points analysed (VANDESOMPELE *et al.* 2002). Reference gene expression stability was determined by geNorm indicators, namely pair wise variation (CV) and gene stability (M) indicators (HELLEMANS *et al.* 2007; VANDESOMPELE *et al.* 2002). Reference genes should have CV-values less than 0.2 and M-values less than 0.5 to be suitable for normalisation of expression data (HELLEMANS *et al.* 2007; VANDESOMPELE *et al.* 2002). Only *GAPDH* and *Cyt-III* matched the above mentioned criteria for the specific experimental conditions. The *GAPDH* gene had an M-value of 0.371 and a CV-value of 0.121 (Table 2). The *Cyt III* gene also had an M-value of 0.371 and a CV value of 0.117 (Table). Therefore, *GAPDH* and *Cyt III* were stably expressed across all time points analysed in this study and were good reference genes for normalisation of expression of *C. zeina Avr4*, *Ecp2*, and *Ecp6*. The respective M and CV stability values for the poor *C. zeina* reference genes (*β-TUB*, *EF-1*, *40S*, and *Cyt b*) were not shown.

Reference target	M	CV
GAPDH	0.371	0.121
Cyt III	0.371	0.117
Average	0.371	0.119

Table 2. *C. zeina* reference gene stability analysis following maize infection.
The table summarises the expression stability values of the *C. zeina* reference genes *GAPDH* and *Cyt III*. M-values less than 0.5 and CV-values less than 0.2 indicate reference genes suitable for normalisation of expression data.

Melting-curve analysis of the RT-qPCR products showed single peaks indicating single homogenous cDNA products and the absence of primer dimers (Figure 8). Melting curves for the samples of interest (cDNA) were shown in green, whereas the melting curves for standard plasmid DNA samples (containing gDNA and not cDNA as described in the relevant materials and methods section) were indicated in blue. The specific melting temperatures of the *C. zeina Avr4*, *Ecp2*, *Ecp6*, *GAPDH*, and *Cyt III* amplicons were 85˚C, 86.5˚C, 85˚C, 84.5˚C, and 75˚C (Figure 8). The melting curves for the cDNA and plasmid DNA samples for each gene analysed formed the same peak as primers would amplify identical amplicons irrespective of cDNA or plasmid DNA (containing gDNA gene copies)

amplification. One exception was the *C. zeina GAPDH* gene that showed two different amplicon peaks for cDNA (84.5˚C) and plasmid DNA (86˚C) (Figure 8). These different peaks were due to the *GAPDH* primers that were designed to span an intron (Figure 5E); therefore amplification from plasmid DNA (containing a cloned gDNA *GAPDH* gene copy) would have produced a larger amplicon with a different melting temperature than amplification from cDNA. This result also shows that there was no gDNA amplification in the cDNA samples.

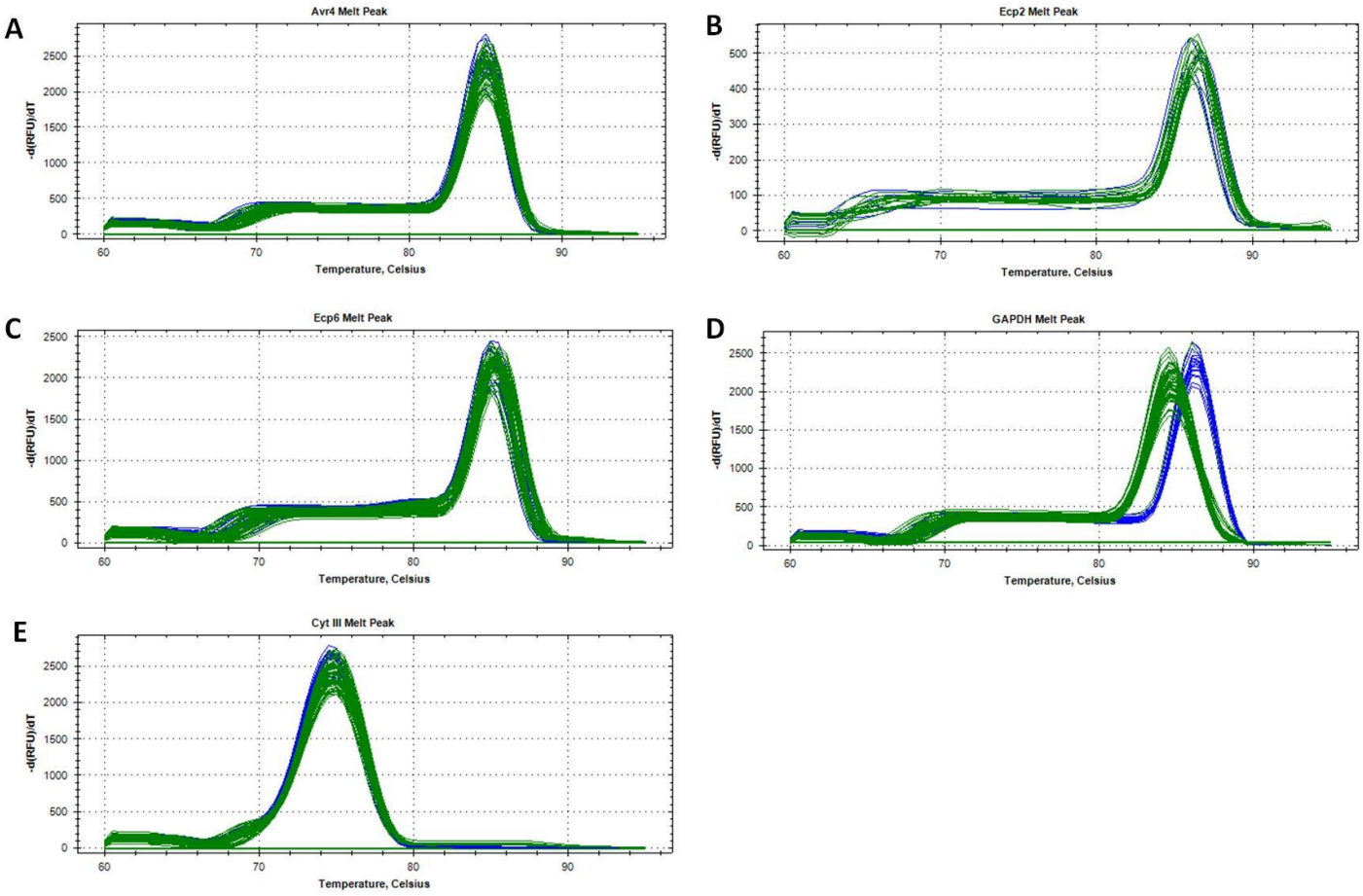


Figure 8. Melting curves from RT-qPCR analysis of *C. zeina* effector and reference genes.
The melting curves of *C. zeina* (Mkushi) effectors (*Avr4*, *Ecp2*, and *Ecp6*) and stable *C. zeina* (Mkushi) reference genes (*GAPDH* and *Cyt III*) were plotted as the negative rate of change in relative fluorescence units (RFU) as the temperature (T) changes. The melting curve analysis revealed single peaks for all the amplicons analysed indicating single and specific amplification products with no primer dimers. Samples of interest (cDNA) were indicated in green and standard samples (plasmid DNA containing cloned gDNA copies of the respective genes) were indicated in blue. The melting temperatures of *C. zeina Avr4*, *Ecp2*, *Ecp6*, *GAPDH*, and *Cyt III* were 85˚C, 86.5˚C, 85˚C, 84.5˚C, and 75˚C. The melting temperature of the standard samples of *GAPDH* (plasmid DNA containing a cloned gDNA copy of *GAPDH*) was 86˚C, which differed from the cDNA samples, as the *GAPDH* primers were designed to span an intron sequence and therefore produced a bigger fragment from the standard plasmid samples.

Selected RT-qPCR products were run on a 2% agarose gel to confirm the amplification specificity and the absence of primer dimers (Figure 9). The expected cDNA product sizes of 148 bp, 137 bp, 143 bp, 103 bp, and 108 bp were obtained for Avr4, Ecp2, Ecp6, GAPDH, and Cyt III confirming amplification specificity. Amplification of the *GAPDH* RT-qPCR product also confirmed that the cDNA was free of DNA contamination as only a 103 bp fragment amplified as opposed to a 161 bp gDNA fragment. The amplified RT-qPCR products from standard samples were sequenced as further confirmation of amplification specificity. The sequenced products all aligned to the respective predicted gene regions (Figures S2-S6, Appendix), confirming that the correct genes were amplified during RT-qPCR analysis.

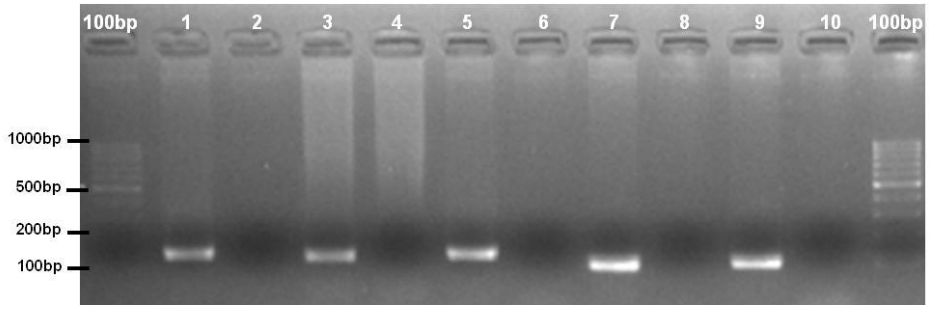


Figure 9. Amplification specificity of *C. zeina* effector and reference RT-qPCR products. Selected RT-qPCR products were analysed on a 2% agarose gel with a 100 bp DNA ladder (Thermo Scientific, Waltham, Massachusetts). Lanes: (1) *Avr4*, (2) control, (3) *Ecp2*, (4) control, (5) *Ecp6*, (6) control, (7) *GAPDH*, (8) control, (9) *Cyt III*, (10) control. The expected cDNA product sizes of approximately 148 bp, 137 bp, 143 bp, 103 bp, and 108 bp were obtained for *Avr4*, *Ecp2*, *Ecp6*, *GAPDH*, and *Cyt III*, respectively. Products are also present as single bands indicating amplification specificity. The 103 bp amplicon obtained for *GAPDH* confirms the absence of gDNA contamination. The qPCR non-template controls (NTC) were clear and showed no primer dimers.

Standard curves were generated from plasmid DNA containing a gDNA insert of each respective gene to determine the amplification efficiency of the reactions (Figure 10). Amplification efficiencies were determined by the slope of the standard curve as well as the calculated R^2 value (correlation coefficient). Amplification efficiencies between 0.90 and 1.10, which correspond to slopes between -3.58 to -3.10, indicate good reaction efficiency (TAYLOR *et al.* 2010). R^2 values closer to 1.00 are associated with a good correlation (how well the data fits the curve) (TAYLOR *et al.* 2010). The standard curve for *Avr4* showed an efficiency of 0.97 and an R^2 value of 0.99. *Ecp2* had a low efficiency value of 0.64 and an R^2 value of 0.99. The amplification efficiency of *Ecp6* was 1.11 with an R^2 value of 0.99. *GAPDH* had an amplification efficiency of 0.9 and an R^2 value of 0.99. *Cyt III* had an amplification efficiency of 0.86 and an R^2 value of 0.99. Standard error of the average

threshold cycle obtained at each dilution point for the effector genes as well as the reference genes were low, i.e. <0.03 Ct values (Figure 10), indicating good consistency in dilution replicates.

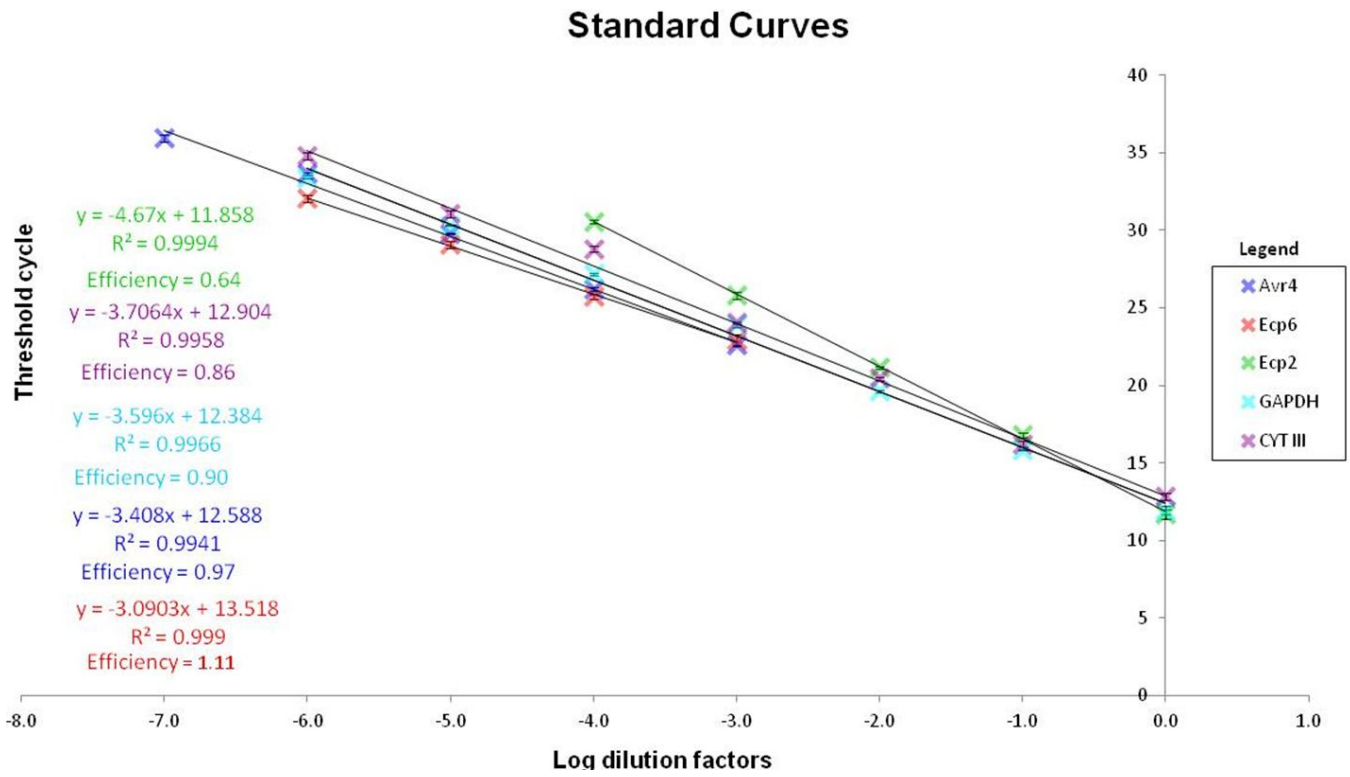


Figure 10. Standard curves generated for *C. zeina* effector and reference genes.
Plasmid DNA containing gDNA copies of respective genes analysed was used for the standard curves. The standard curves for *Avr4, Ecp2*, and *Ecp6* as well for the reference genes *GAPDH* and *Cyt III* were generated by plotting the threshold cycle (Ct) values against the log10 concentration. A linear trend line was drawn for the data points of each gene. The equation of the regression curve, the correlation coefficient (R^2), as well as the efficiency of each curve is indicated on the graph. Standard error of the average threshold cycle obtained at each dilution point is indicated by the error bars. Error bars represent the standard error of the mean (SEM).

The normalised relative expression of *Avr4* during infection is shown in figure 11A. *Avr4* showed relatively constant expression levels across all time points during expression and the control time point (0 dpi) showed no expression. The normalized expression levels observed all showed a statistically significant fold increase compared to the control time point (0 dpi) as indicated by the (*) in the figure. Normalized *Avr4* expression levels were low and ranged from approximately 0.003 to 0.004. Therefore the normalized *Avr4* expression levels were approximately 0.003 to 0.004 fold higher than the expression levels of the *GAPDH* and *Cyt III* reference genes.

The normalised relative expression levels of *Ecp2* (Figure 11B) were low and showed relatively constant expression during infection. No statistically significant increases were seen in expression levels between time points, except between the control time point (0 dpi) and 3 dpi, 12dpi, 15 dpi 23C dpi, and 23L dpi. No error bars or statistical calculations could be made for 5 dpi and 10 dpi time points, as the data for these time points are represented by one biological replicate only as indicated by the ($^{\#}$) in the figure. The data for the 5 dpi and 10 dpi time points were included to show that there was expression in these time points. Normalized *Ecp2* expression levels ranged from approximately 0.004 to 0.005. This means that the normalized *Ecp2* expression levels were approximately 0.004 to 0.005 fold higher than the expression levels of the *GAPDH* and *Cyt III* reference genes.

Similar results were obtained for *Ecp6*, where *Ecp6* relative normalised expression levels (Figure 11C) remained constant across infection time points. All time points showed a statistically significant increase in relative expression levels when compared to the control time point (0 dpi). Normalized *Ecp6* expression levels were higher when compared to the normalized *Avr4* and *Ecp2* expression and ranged from approximately 0.020 to 0.045 relative to the housekeeping genes.

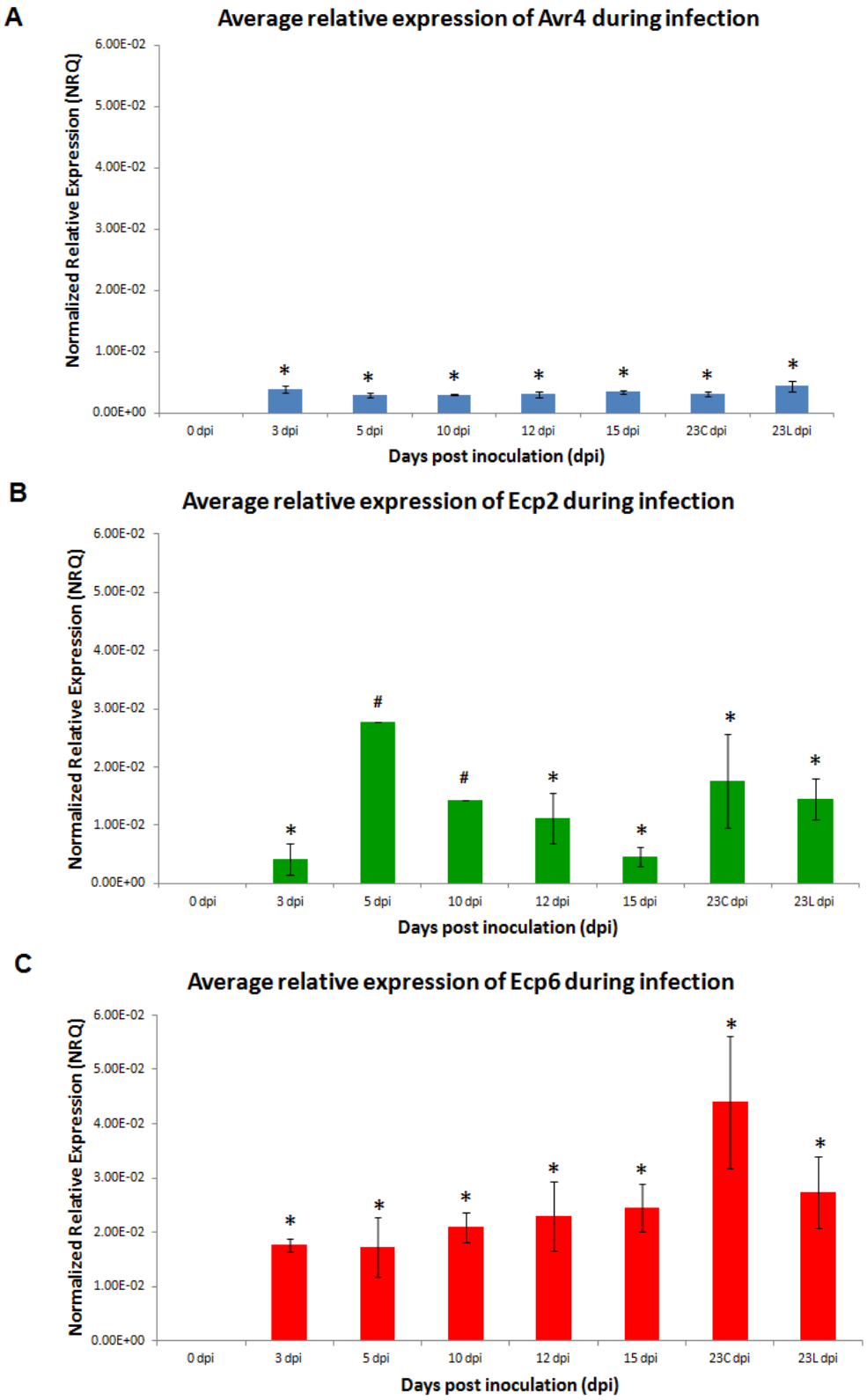


Figure 11. Normalised RT-qPCR expression profiles of *Avr4*, *Ecp2*, and *Ecp6*.
The average relative expression was calculated for each of the *C. zeina* effector genes during infection. The infection time points included 0 days post inoculation (dpi) as the control, 3 dpi, 5 dpi, 10 dpi, 12 dpi, 15 dpi, 23C dpi (representing chlorotic symptoms), and 23L dpi (representing necrotic symptoms). Effector gene relative expression values were normalized by dividing the amount of input fungal cDNA (extrapolated from the respective standard curves) by the geomean of input reference cDNA of the reference genes *GAPDH* and *Cyt III.* Standard error of the average relative expression is indicated by the error bars in the figures. Statistical analysis of the relative expression data was done by one-way ANOVA analysis followed by a Tukey's Multiple Comparison tests. ***** Indicates a statistical significant change in mean expression value when compared to 0 dpi ($p \leq 0.05$). # Indicates data from a single biological replicate.

4.6 Fungal quantification through qPCR

Melting-curve analysis of qPCR products for *in planta* fungal quantification showed that the amplification of both *C. zeina* (Mkushi) *cpr1* and the B73 maize *gst3* gene were specific with no primer dimers (Figure 12). The specific melting temperatures were 84.5˚C for the *C. zeina cpr1* gene and 81˚C for the maize *gst3* gene. The *C. zeina* (Mkushi) *cpr1* melt-curve also shows specific amplification from *C. zeina* (Mkushi) (KORSMAN *et al.* 2010), which is further confirmation of Koch's postulates.

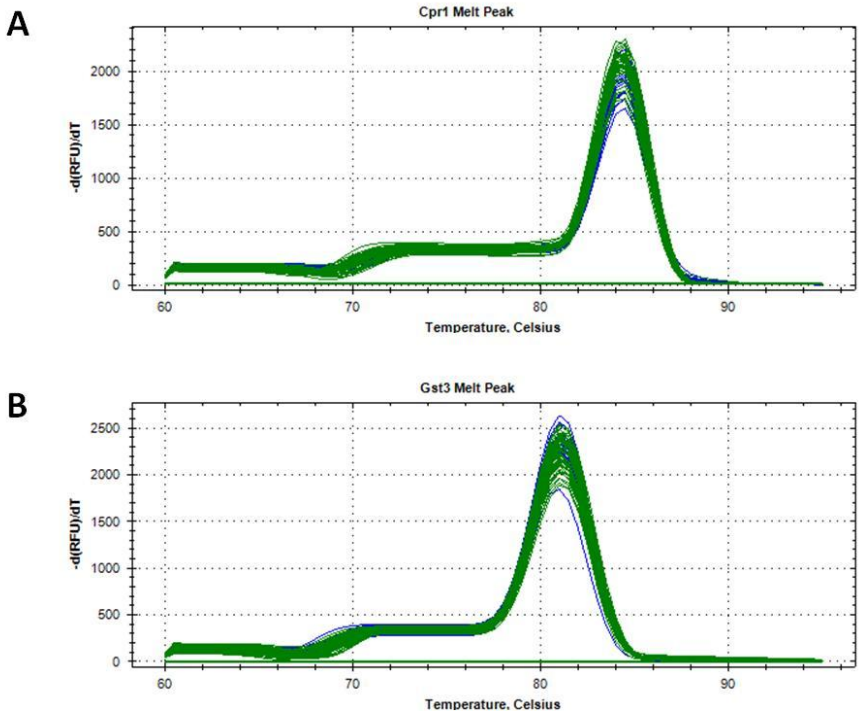


Figure 12. Melting curves from qPCR analysis for *C. zeina* (Mkushi) quantification *in planta*. The melting curves for the *C. zeina* (Mkushi) *cpr1* gene (A) and the B73 maize *gst3* gene (B) were plotted as the negative rate of change in relative fluorescence units (RFU) as the temperature (T) changes. The melting curve analysis revealed single peaks for all the amplicons analysed indicating single and specific amplification products with no primer dimers. Amplification of samples was indicated in green and standard samples (see materials and methods section) used for the standard curve were indicated in blue. The specific melting temperatures for *C. zeina cpr1* and the maize *gst3* gene were 84.5˚C and 81˚C.

Selected qPCR products were run on a 2% agarose gel to confirm the amplification specificity and the absence of primer dimers (Figure 13). The expected product sizes of approximately 106 bp and 164 bp were obtained for *C. zeina* (Mkushi) *gst3* and B73 maize *cpr1*, respectively.

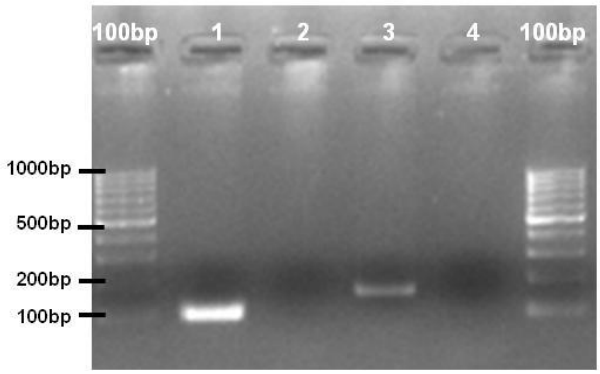


Figure 13. Amplification specificity of qPCR products used for fungal quantification.
Selected qPCR products were analysed on a 2% agarose gel with a 100 bp DNA ladder (Thermo Scientific, Waltham, Massachusetts). Lanes: (1) gst3, (2) control, (3) Cpr1, (4) control. The expected product sizes of approximately 106 bp and 164 bp were obtained for *C. zeina* (Mkushi) *gst3* and B73 maize *cpr1*, respectively. Products are also present as single bands indicating amplification specificity. The qPCR non-template controls (NTC) were clear and showed no primer dimers.

The standard curves obtained for *C. zeina* (Mkushi) *cpr1* and for the B73 maize *gst3* genes are shown in figure 14 (A) and (B). Amplification efficiency of the qPCR reactions were based on the slope of each standard curve with values close to two indicating a good efficiency. The correlation coefficient was indicated by the R^2 -value with values closer to 0.99 indicating a good correlation. The standard curve for the *C. zeina cpr1* gene had a good efficiency of 0.91 and an R^2- value of 0.99. The *gst3* standard curve also had a good efficiency of 0.89 and an R^2- value of 0.97. Standard error of the average threshold cycle obtained at each dilution point for the *cpr1* and *gst3* standard curves were low, i.e. <0.02 Ct values (Figure 14) indicating good consistency in dilution replicates.

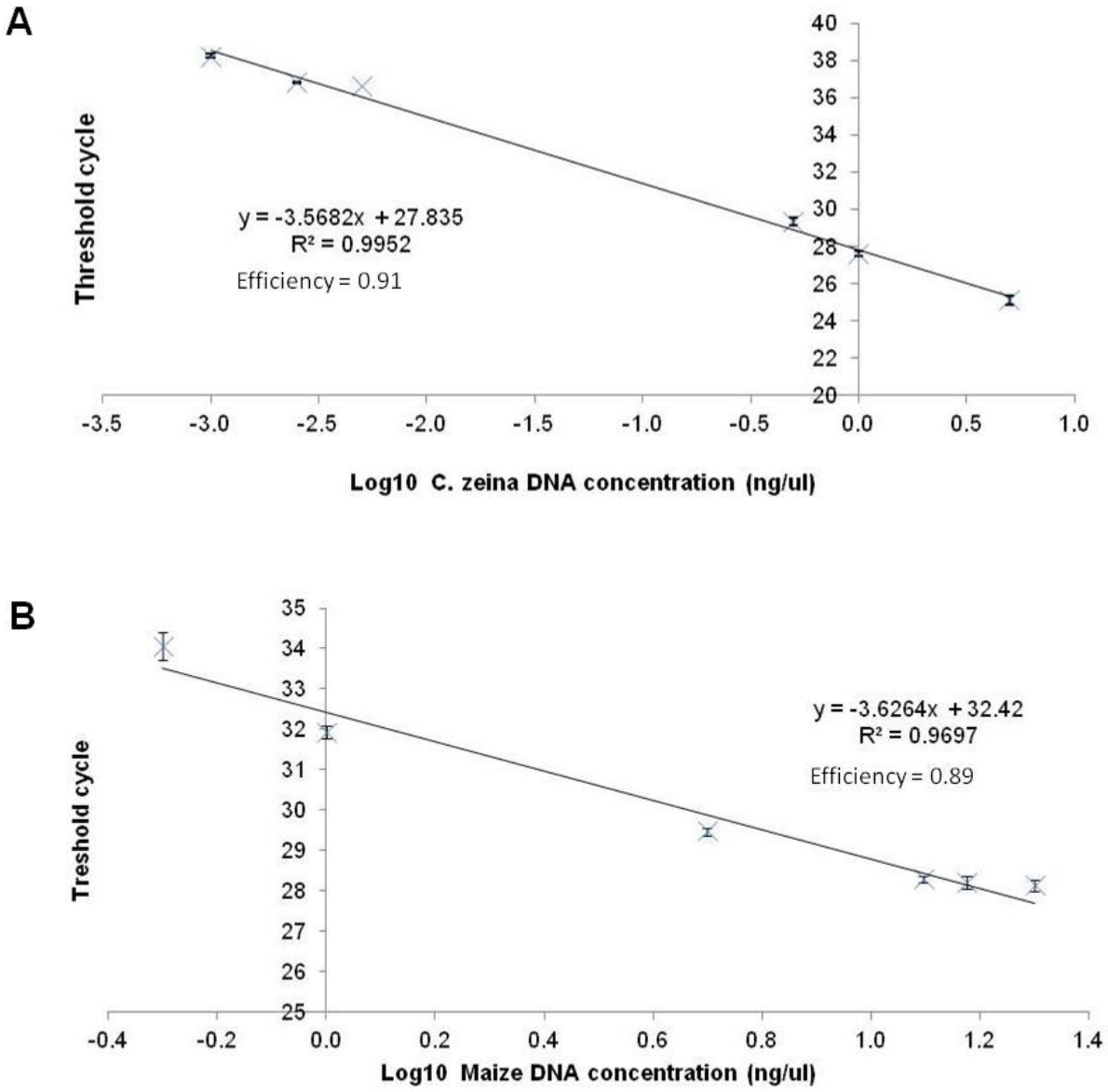


Figure 14. Standard curves generated for *C. zeina* (Mkushi) *cpr1* and B73 maize *gst3* for *in planta* fungal quantification. The figure indicates the standard curves obtained for *C. zeina* DNA concentration through the *cpr1* gene (A) and for maize DNA concentration through the *gst3* gene (B). Standard curves were obtained by plotting the threshold cycle (Ct) values against the log10 concentration of either *C. zeina* (Mkushi) DNA (A) or B73 maize DNA (B). A linear trend line was drawn for the data points of each gene. The equation of the regression curve, the correlation coefficient (R^2), as well as the efficiency of each curve was indicated on the graph. Standard error of the average threshold cycle obtained at each dilution point is indicated by the error bars. Error bars represent the standard error of the mean (SEM).

The quantity of *C. zeina* (Mkushi) present in each biological replicate was extrapolated from the *cpr1* standard curve and normalized to the amount of maize DNA present in the same samples as extrapolated from the *gst3* standard curve (KORSMAN *et al.* 2010). Normalized fungal quantities were pooled for each time point and are indicated in figure 15. Fungal quantities were determined in terms of ng *C. zeina* DNA per ng of maize DNA.

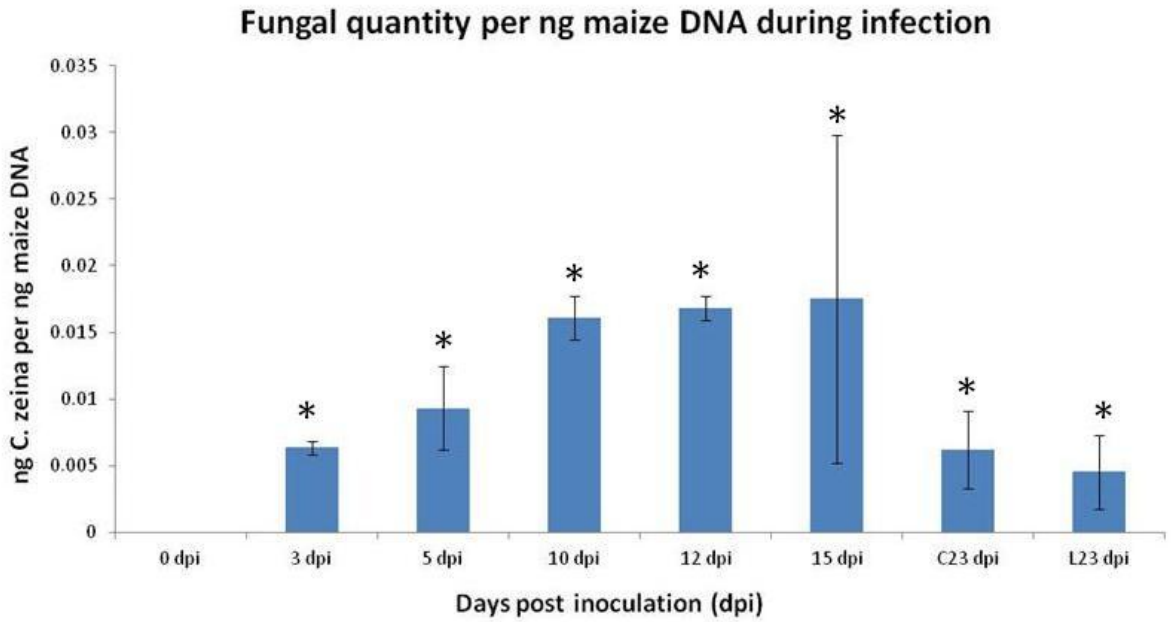


Figure 15. *C. zeina* (Mkushi) fungal quantity at each time point following maize infection.
The average amount of *C. zeina* (Mkushi) present at specific time points during infection in terms of ng fungal DNA per ng maize DNA as determined through a qPCR method for fungal quantification (KORSMAN *et al.* 2010). The time points included 0 days post inoculation (dpi) as the control, 3 dpi, 5 dpi, 10 dpi, 12 dpi, 15 dpi, 23C dpi (representing chlorotic symptoms), and 23L dpi (representing necrotic symptoms). Standard error of the average relative expression is indicated by the error bars in the figures. Bars on the graphs represent the standard error of the mean (SEM). Statistical analysis of the fungal quantity data was done by one-way ANOVA analysis followed by a Tukey's Multiple Comparison tests. *Indicates a statistical significant change in mean quantification value when compared to 0 dpi ($p \leq 0.05$).

All time points showed a statistically significant increase in fungal material when compared to the control time point (0 dpi). The 23C dpi time point represents chlorotic symptoms that are associated with initial stages of infection. The amount of fungal material obtained at the 23C dpi time point corresponds to the amount of fungal material obtained at 3-5 dpi. The 23L dpi time point represents necrotic leaf symptoms that are associated with late stages of infection. It was expected that higher fungal quantities should be associated with this time point. However, low fungal quantities corresponding to earlier time points were obtained.

4.7 Relationship between gene expression and fungal quantity

A Pearson's product-moment correlation coefficient was computed to assess the relationship between fungal quantity and effector gene expression at specific time points during infection (PEARSON 1895). The Pearson's correlation statistical test measures if there is a linear relationship or correlation between two variables, giving it a value between negative one and positive one, where negative one means a total negative correlation, zero means no correlation exists, and positive one means a total positive correlation (STATISTICS 2008). Overall, there was a significantly moderate, positive correlation (p=0.02) (Table 3) between fungal quantity and *Avr4* gene expression at all the time points assessed. Increases in fungal quantity were correlated with increases in *Avr4* gene expression. A similar result was observed for *Ecp6* gene expression and fungal quantity, where fungal quantity showed a weak, positive correlation to *Ecp6* gene expression (p=0.03) (Table 3). No statistical significant correlation was found between *Ecp2* gene expression and fungal quantity (p=0.59) (Table 3). A correlation was considered statistically significant when p≤0.05.

Table 3. Pearson's correlation test results.

Effector gene	R- value[a]	P- value[b]	**Notes** (STATISTICS 2008)
Avr4	0.42	0.02	Positive correlation, moderate relationship
Ecp2	0.12	0.59	Correlation not statistically significant
Ecp6	0.21	0.03	Positive correlation, weak relationship

[a] Pearson's correlation value
[b] Statistical significance with p≤0.05 indicating a statistical significant correlation

5. DISCUSSION

This study has proven that *C. zeina* infection trials can be successfully achieved in a phytotron. Two suitable *C. zeina* reference genes namely *GAPDH* and *Cyt III* have also been identified for accurate normalisation of *in planta* fungal gene expression. These reference genes were used to determine the relative *in planta* expression profiles of *C. zeina Avr4*, *Ecp2*, and *Ecp6*. All three effector genes showed constant expression during early and late time points of infection. The relative expression of the three effector genes was also correlated to the fungal quantities present at each time point analysed. *Avr4* and *Ecp6* expression showed a positive, weak correlation to fungal quantity. Therefore, as fungal quantity increases (or infection progresses), *Avr4* and *Ecp6* relative expression also increases. There was no significant correlation between *Ecp2* expression and fungal quantity. Since there was only a statistical difference found between the control time point (0 dpi) and the other time points for all the assays, the correlation was not so meaningful.

Inoculation trials with *C. zeina* in our laboratory have always been done in the glasshouse or the field. Even though glasshouse and field inoculation trials have been very successful, these trials are limited to maize growing seasons. The University of Pretoria also has phytotron facilities available for plant growth and fungal infections. Phytotrons provide an environment for maize inoculation trials where the temperature and humidity can be controlled. Therefore, inoculations in the phytotron can be done all year round and under specific controlled conditions. The inoculation trial in the phytotron undertaken in this study was done to determine if infection is possible in the phytotron as a basis for future trials, especially if genetically modified *C. zeina* strains are used, as these cannot be released into the environment. Infection was established as proven by the characteristic GLS disease symptoms obtained during the phytotron inoculation trial. Even though infection was achieved, a variety of symptoms indicating different disease stages were only seen after three weeks. A possible reason why symptoms were not observed earlier could be because of humidity levels that were not constant during the inoculation trial in the phytotron. Humidity plays a very important role during *C. zeina* infection, especially during the initial stages (BECKMAN and PAYNE 1982). During the first few days of the inoculation trial, the maize plants were covered in black plastic bag canopies to increase the humidity levels. The plastic bag canopies as well as extra humidifiers were needed to obtain humidity levels above 75%. Without the extra humidifiers, humidity levels fluctuated between 60-100% (Appendix, Figure S13). Re-isolation of conidia, by using a light microscope, was also not possible as no conidia were found attached to conidiophores from GLS lesions. Fans necessary for temperature control that is present in the phytotron might be a possible explanation as the draft caused by the fans might have blown the conidia away. In future,

maize leaves could be protected from the strong drafts by making a division between the fans and the maize plants. Another option could also be to leave the plants in the phytotron for longer before attempting to re-isolate conidia. It might also be worthwhile to extend darkness periods for conditions optimal for conidiation (BLUHM *et al.* 2008).

It was a challenge to extract *C. zeina* DNA and RNA from a maize environment as the infected maize leaves used for the extraction contain fungal material that is effectively already diluted by the maize material. RNA extractions from filamentous fungi are also difficult as filamentous fungi contain rigid cell walls and excrete carbohydrates during submerged growth, which interferes with the extractions (SANCHEZ-RODRIGUEZ *et al.* 2008). However, DNA extractions yielded pure and relatively high DNA concentrations for most maize/fungal samples. Overall, DNA extractions were successful and produced high quality gDNA. RNA extractions also yielded intact and high quality RNA samples. The maximum amount of RNA template was used for cDNA synthesis, in an attempt to obtain higher amounts of fungal cDNA, as low amounts of cDNA complicate detection of genes expressed at lower levels. Therefore the highest amount of fungal cDNA possible was needed for optimal detection of fungal gene expression. During the optimization steps, conventional PCR reactions were not sensitive enough to detect fungal cDNA amplification, which was a further indication of low fungal cDNA concentrations as a result of dilution by maize leaf material. RT-qPCR reactions were sensitive enough to detect effector gene expression, except *C. zeina Ecp2* expression. Double the amount of template cDNA concentration had to be added to the RT-qPCR reactions before *Ecp2* expression could be detected. The importance of high concentrations of total fungal RNA extractions was highlighted by this result. Higher quality RNA would provide higher concentrations of cDNA to be able to detect very low effector gene expression. Excess carbohydrates produced by *C. zeina* during infection that are not effectively removed during the extractions, might be a reason why RNA extractions from some samples were less effective. In an *in planta* study done on efficient RNA extractions from the filamentous fungus *M. graminicola*, a nucleic isolation protocol including sodium dodecyl sulphate (SDS)-based buffers was developed to avoid excess carbohydrates during extractions (SANCHEZ-RODRIGUEZ *et al.* 2008). This protocol delivered higher concentrations of extracted fungal RNA and showed similar results when tested on *C. fulvum*. In future, this protocol could be tested on *C. zeina* to determine if higher amounts of RNA could be extracted for subsequent cDNA synthesis.

RT-qPCR is an established technique for gene expression analysis. Relative expression of genes analysed are normalised by the expression of suitable reference genes (HUGGETT *et al.* 2005). Reference genes need to be stably expressed across all the experimental

conditions analysed for accurate normalization. No reference genes have been identified for *C. zeina* and limited information on reference genes in *Cercospora* species is available. A single reference gene, namely *β-tubulin*, was identified for normalisation of *C. zeae-maydis* gene expression during maize infection (BLUHM *et al.* 2008). A recent study validated a few reference genes for *in planta* expression analysis from the biotrophic fungus *Hemileia vastatrix* (VIEIRA *et al.* 2011). The study identified *β-tubulin (β-TUB)*, g*lyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, e*longation factor (EF-1)*, *40S ribosomal protein (40S)*, c*ytochrome c oxidase subunit III (Cyt III)*, c*ytochrome b (Cyt b)* as suitable reference genes. It was also shown that *glyceraldehyde-3-phosphate dehydrogenase*, *40S ribosomal protein*, and *cytochrome c oxidase subunit III* were more suitable for *in planta* expression normalisation (VIEIRA *et al.* 2011). This study identified and validated *C. zeina* (Mkushi) *GAPDH* and *Cyt III* reference genes for *C. zeina in planta* expression normalisation.

Relative expression profiles of the *C. zeina* effector genes revealed that *Avr4*, *Ecp2* and *Ecp6* are expressed during infection. The three effector genes showed constant expression at the time points analysed. *Avr4*, *Ecp2*, and *Ecp6* therefore play a role during pathogenesis. Transcripts were below the detection threshold for the control time point (0 dpi) samples, indicating that no background amplification of maize genes or endophytes were present. Limited information is available on *in planta* relative expression levels of *Avr4*, *Ecp2*, and *Ecp6*. In *C. fulvum* and *M. fijiensis*, *Avr4* has been shown to protect fungal hyphae against hydrolysis by plant chitinases (STERGIOPOULOS *et al.* 2010; VAN DEN BURG *et al.* 2006). According to this information it is expected that *C. zeina Avr4* should have a constant expression levels during infection as it plays a defensive role for the fungus against plant chitinases. This corroborates the finding of steady transcript levels of *Avr4* in *C. fulvum* (JOOSTEN et al. 1997). *Ecp6* also plays an important defensive role during infection as shown in *C. fulvum* where *Ecp6* binds to chitin oligomers to evade possible detection by its host (DE JONGE and THOMMA 2009). It could therefore also be expected that *C. zeina Ecp6* should show constant expression levels during infection. A study of the *in planta* expression levels of two *M. graminicola Ecp6* genes (*Mg3LysM* and *Mg1LysM*) showed that the expression levels of these two genes were strongly upregulated, especially during symptomless leaf infection stages (MARSHALL *et al.* 2011). *C. zeina Ecp6* relative expression levels can be compared to what was found in *M. graminicola* as the expression levels showed an increase when compared to 0 dpi.

Ecp2 on the other hand has been proposed to be involved in the necrotrophic stages of infection (STERGIOPOULOS *et al.* 2010). It might therefore be expected that *C. zeina Ecp2* is expressed during later stages of infection, which is in contrast to the constant expression

levels observed in this study. It has been reported that *C. fulvum Ecp2* is expressed at low levels at *in vitro* conditions and at high levels at *in planta* conditions (VAN DEN ACKERVEKEN *et al.* 1993). In contrast, *Ecp2* is expressed at very low levels during *C. zeina in planta* infection as expression could only be detected when double the amount of cDNA was added to the RT-qPCR reaction. The input ng of fungal material extrapolated from the *Ecp2* regression curve was halved to compensate for the higher concentration cDNA used in the RT-qPCR reactions. However, the *C. zeina Ecp2* standard curve generated from plasmid DNA template showed a low amplification efficiency of 64%. Low RT-qPCR efficiencies are usually due to Taq polymerase inhibitors, non-optimal annealing temperatures, old/inactive Taq polymerase, poorly designed primers, or amplicons with secondary structures (TAYLOR *et al.* 2010). The *C. zeina Ecp2* primers were designed according to specific parameters and the amplicon was also analysed for possible secondary structures. *C. zeina Ecp2* primers showed specific amplification when analysed in an RT-PCR reaction, showing that the primers were specific and that the annealing temperature was optimal. The GC content of the *Ecp2* primers were 52.6% (forward primer) and 50% (reverse primer). The Taq polymerase used was also of high quality and worked perfectly in the *Avr4*, *Ecp6*, *GAPDH*, and *Cyt III* RT-qPCR reactions. The plasmid DNA used for the generation of the *C. zeina Ecp2* standard curve was purified and analysed spectrophotometrically as well as by gel electrophoresis to ensure that no polymerase inhibitors were present. A possible solution to the low amplification efficiency would have been to use cDNA instead of cloned *Ecp2* product for the standard curve if more cDNA product was available, but unfortunately cDNA was limited. Even though results were obtained for the relative expression levels of *Ecp2*, it was not ideal as the PCR efficiency was very low. The low PCR efficiency might also explain why the relative *Ecp2* expression levels could not be detected at 1X cDNA concentration. Future experiments would involve a re-design of the *Ecp2* RT-qPCR primers in an attempt to obtain a higher PCR efficiency to be able to publish the *Ecp2* relative expression data.

The fungal effector expression was correlated to the fungal load present at each time point. Overall there was a weak, positive correlation between fungal quantity and effector gene expression. Increases in fungal quantity were correlated to increases in *Avr4* and *Ecp6* gene expression. The *Ecp2* gene expression showed no significant correlation with an increase in fungal quantity. Fungal quantity increased across the time points when compared to the 0 dpi, indicating successful *C. zeina* infection, even though no symptoms were observed before 23 dpi. Fungal quantity at 23C dpi (representing chlorotic symptoms) was similar to fungal quantities observed at early time points. Chlorotic symptoms are associated with initial stages of infection and therefore it was expected that the 23C dpi time point should have similar amounts of *C. zeina* present as observed at 10 dpi. However, the 23L dpi time

point showed similar fungal quantity as observed at early time points. It was expected that the 23L dpi (associated with necrotic leaf material) time point would have high fungal quantity as necrotic symptoms are associated with late stages of infection. A possible reason why the necrotic disease time point harvested (23L dpi) contained low amounts of fungal material according to qPCR analysis, could be due to secondary compounds that are released in dying leaf material that possibly interfere with the qPCR reaction causing an underestimation of fungal load at this time point. Importantly, the expression seen for all the genes up until 15 dpi was associated with no symptom development. Ideally more time points are needed during the chlorotic (23C dpi) and necrotic (23L dpi) symptom development stages.

This study was also not able to show Koch's postulates as no conidia could be re-isolated from infected maize leaf material. However, melting curve analysis and subsequent cloning and sequencing of cDNA products of the *C. zeina* genes analysed in this study, showed *C. zeina* specific amplification, indicating that *C. zeina* had infected maize leaves. In addition, more reference genes should also be identified that are suitable for *in planta* gene expression analysis. According to the MIQE guidelines (BUSTIN *et al.* 2009) for publication of relative expression studies, at least three reference genes are needed for normalisation of gene expression.

6. CONCLUSION

This study proved that successful *C. zeina* inoculation can be achieved in a phytotron. Successful inoculation was based on the typical GLS lesions observed, the fungal quantification data, and the relative gene expression data. The infection established in the phytotron enables future infection studies that are not limited to maize growing seasons and could be done at any time during the year. It was also shown that the *C. zeina* effectors *Avr4*, *Ecp2*, and *Ecp6* are expressed during infection, which gives more insight into the molecular mechanisms of infection and opens up new research opportunities to possibly obtain specific virulence targets for disease management purposes. All three effector genes investigated, showed relatively constant expression during infection. This study also identified two suitable *in planta* reference genes, namely *GAPDH* and *Cyt III*, for relative quantification of gene expression in *C. zeina*.

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