Expression analysis of selected maize primary metabolism genes in response to *Cercospora zeina* inoculation in the glasshouse

By

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Declaration

I, Zimbili Ethel Mlunjwa hereby declare that the dissertation, submitted herewith for the degree Magister Scientiae at the University of Pretoria, contains my own independent work and has not been submitted for a degree at this or any other university.

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Zimbili E. Mlunjwa

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Date

Preface

The research presented in this dissertation is based on experimental work conducted in the Molecular Plant-Pathogen Interactions (MPPI) group at the University of Pretoria, South Africa. The thesis is presented as two separate publishable chapters. Thus, due to the nature of the style, repetition between these chapters and the literature review was unavoidable.

Maize is among one of the most produced crops worldwide and it has served as a source of fodder in developed, industrialised countries such as the United State of America. Additionally, it is a staple food in Sub-Saharan African countries such as Kenya, Zambia and South Africa. South Africa is among the top producers of maize globally, and exports it to countries such as Japan, Malaysia and Iran. However, foliar pathogens, such as the grey leaf spot disease (GLS), affect the production of maize. The causal agents of GLS are sibling species of the *Cercospora* genus, namely, *Cercospora zeae-maydis* (the predominant pathogen in the USA) and *Cercospora zeina* (occurs in the USA and is the only GLS causing pathogen in Africa). The disease poses a threat to food security globally as it decreases the yield, production and quality of maize more than 65% and up to 100% in severe cases of complete blighting and lodging of the crop.

Management strategies such as crop rotation, conventional tillage and application of fungicides are used to control the progression of GLS; however, these strategies have limitations. An alternative and effective prevalent method is the use of host resistance combined with chemical control and cultivation practices to reduce yield losses. Therefore, the knowledge of the pathogen (*C. zeina*) and the interaction with a specific host (maize) will help decrease or prevent the progression of GLS.

The primary glyoxylate pathway metabolism genes have been proposed to be important factors in the pathogenicity of the fungal pathogen. Therefore, the aim of the MSc was to understand the expression patterns of the metabolites in susceptible maize genotypes at different disease progression stages. This would contribute to our current understanding of the importance of the plant, fungal biology, plant-pathogen interactions and plant defence responses with regards to the *C. zeina* pathosystem.

Chapter 1 provides an outline of the current understanding of the importance of the plant, fungal biology, plant-pathogen interaction, plant defence responses and with regards to the *C. zeina* – maize pathosystem to-date. This chapter discusses the importance of maize in South Africa and the effect GLS has on yielding. The chapter further gives an overview of the global distribution of *C. zeina* and *C. zeae-maydis*, the development of GLS, the infection strategies used to result in distinct symptoms and the life cycle. This chapter also includes a review on host resistance and discusses the role of the glyoxylate pathway primary metabolism genes, isocitrate lyase (*ICL*) and malate synthase (*MS*) role in maize during fungal infection. The chapter concludes with a section on the functional characterisation of these primary metabolism genes with regards to susceptible maize genotypes at different disease progression stages.

Chapter 2 of this dissertation discusses the inoculation of grey leaf spot disease susceptible and resistant maize genotypes with regards to *Cercospora zeina*. Two inoculation glasshouse trials were conducted for the susceptible maize genotype, B73. The first glasshouse trial samples were collected at the initial day of inoculation and at a later stage when the lesions were fully mature and coalesced, and the second glasshouse trial samples were collected at different disease progression stages. The causal agent was *C. zeina*, which was as proven by amplifying of the *CTB7* gene.

Chapter 3 discusses the results of the expression patterns of the glyoxylate pathway metabolism genes at different disease progression stages. Copy DNA was synthesized from the un-inoculated and inoculated susceptible maize genotype extracted RNA and a standard PCR confirmed that there was no genomic DNA contamination. The specific *ICL* and *MS* maize genes were amplified using RT-PCR designed primers. These amplified gene products were further sequenced and aligned to confirm that the amplified products are *ICL* and *MS*. The expression level of *MS* was further analysed using semi-quantitative reverse transcriptase polymerase chain reaction (semi-qRTPCR). The expression of two transcripts of *MS* was discovered. The transcripts were differentially expressed at the disease progression stages.

Chapter 4 contains the main concluding remarks and suggestions for improvement in future studies. This chapter consists of general discussion with regards to the research findings and the

prospects of using antifungal compounds in maize against the glyoxylate pathway of the fungi as a method of combating GLS.

Listed below are the research outputs generated from this dissertation to-date:

Mlunjwa, Z.E. & Berger, D.K. 2017. Expression of candidate maize defense genes in response to *Cercospora zeina*. The 5th Annual Genomic Research Institute symposium. November 10. University of Pretoria, South Africa. (Poster presentation).

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Mlunjwa, Z.E. & Berger, D.K. 2018. Expression analysis of selected maize primary metabolism genes in response to *Cercospora zeina* inoculation in the glasshouse. African Centre for Gene Technologies 15th Annual forum. University of Witwatersrand, South Africa. (Oral presentation).

An abstract of the research findings from this dissertation was submitted for the 3rd International Conference on Food Safety and Security in Cape Town, South Africa. The organisers of the conference awarded the author a travel award to attend and present the poster.

Summary

Maize is one of the most important grain crops in Africa especially in Southern and sub-Saharan Africa. This crop contributes not only to food security but also to the economy. However, there are factors that affect the development of maize including diseases, which pose as threats to food security. Grey leaf spot (GLS), a foliar disease of maize, is caused by *Cercospora zeina,* an economically important ascomycete worldwide. It causes over 65% yield losses, thereby being a great threat to food security. This disease was first reported in KwaZulu-Natal province, a GLS hot spot in South Africa in 1988. Strategies such as crop rotation, conventional tillage and fungicides are widely used to manage the progression of GLS. However, these strategies are labour-intensive and expensive for farmers. An alternative cost-effective method is the utilization of host resistance. To better understand the mechanism of host resistance, two primary metabolism genes, isocitrate lyase (*ICL*) and malate synthase (*MS*), involved in the glyoxylate pathway, were previously shown to be highly induced by the fungus in a field RNAseq study. This research was aimed at elucidating expression of these two primary metabolism genes. Glasshouse trials were conducted by inoculating the maize genotypes B73 (susceptible) and B73-QTL (resistant) with *C. zeina.* Genomic DNA was extracted and the fungal *CTB7* gene region amplified to confirm that the infection was due to *C. zeina*. RNA was also extracted, and reverse transcribed to copy DNA (cDNA). The quality of RNA was confirmed by performing a reverse transcriptase PCR (RT-PCR) with banana actin primers as a positive control. Primer pairs flanking an intron in the *ICL* and *MS* genes were successfully designed to specifically amplify their respective cDNA. To further confirm these results, the amplified gene products were sequenced using Sanger sequencing platform and thereafter aligned to respective reference genes obtained from NCBI. Overall, *ICL* and *MS* genes were successfully amplified from the infected maize genotypes. The *ICL* amplification needs further optimisation as the bands were not intense to continue with expression analysis. The expression of the two *MS* transcripts increased at the different progression stages indicated that the glyoxylate pathway plays a role in fungal pathogenicity.

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Secondly, I am grateful for the institutes that continued to fund me during my final year of studies namely, the University of Pretoria for providing me with the Postgraduate Scholarship award, and to Monsanto SA (PTY) Ltd, which enabled me to be a full-time student residing near the institute. Furthermore, I would like to thank the Department of Science and Technology together with the Centre of Excellence for funding me to attend the 3rd International Food Security Conference in 2017.

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Declaring Ephesians 3:20

God will do exceedingly, abundantly above all that I ask or think, because I honour Him, His blessings will chase me down and overtake me

List of Abbreviations

%	percentage
±	approximately (plus or minus)
α	alpha representing the p value (statistical significance)
µg	micrograms
µl	microliters
µM	micromolar
µmol	micromole
®	trademark is officially registered
18 S	18 S ribosomal protein gene
20 S	20 S ribosomal protein gene
ºC	degrees Celsius
™	for unregistered trademark
β	beta
ANOVA	analysis of variance
API	apigenin
ATP	adenosine triphosphate
BA	banana actin
BLAST	Basic Local Alignment Search Tool
BLASTN	search a nucleotide database using a nucleotide query
bp	base pair
CAFF	caffeic acid
cDNA	copy deoxyribonucleic acid
cm	centimeters
CTAB	cetyl trimethylammonium bromide
CTB	cercosporin toxin biosynthetic gene
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotide triphosphate
dpi	days post inoculation or days post infection
dT	deoxythymine
EtBr	ethidium bromide
E-value	expected value
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
g	gravitational force
gDNA	genomic DNA
GFP	green fluorescent protein
GLS	gray/ grey leaf spot

i.e.	in other words,
IAA	isoamyl alcohol
ICL	isocitrate lyase
IITA	International Institute of Tropical Agriculture
K	potassium
Kb	kilobases
Kg	kilograms
$m^{-2}s^{-1}$	meter square per second
mA	milliamps
ml	milliliters
$MgCl_2$	magnesium chloride
mm	millimeters
mM	millimolar
M	molar
mRNA	messenger ribonucleic acid
MS	malate synthase
N	nitrogen
NADP-ME	nicotinamide adenine dinucleotide phosphate-malic enzyme
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
ng	nanograms
NILs	near isogenic lines
P	phosphorus
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PDA	potato dextrose agar
PEP	phosphoenol pyruvate
PRR	pattern recognition receptors
P-value	statistical significance
QTL	quantitative trait loci
R-gene	resistant gene
RNA	ribonucleic acid
RNase	ribonuclease
RNASeq	RNA sequencing
ROS	rosmarinic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase polymerase chain reaction

Semi-qRTPCR	Semi-quantitative reverse transcriptase polymerase chain reaction
SNP	single nucleotide polymorphism
T0	transcript
TCA	tricarboxylic acid
U	enzyme units
UN	United Nations
UTR	untranslated region
V	volts
V6	vegetative stage of maize plant
V8 medium	type of vegetable medium

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Chapter 1

Grey leaf spot disease as a detrimental foliar disease of maize caused by *Cercospora zeina* in Southern Africa

1.1. Introduction

The most widely produced cereal crop plants including; *Zea mays* (maize), *Oryza sativa* (rice) and *Triticum aestivum* (wheat) are essential components of consumer diets throughout the world (Meyer *et al*., 2017). Maize is a crucial food source for industrialized countries such as the United States of America (USA) and developing countries such as Latin America and sub-Saharan Africa (Ward et al., 1999; Hoohafkan and Stewart, 2008). Africa produced over 55 million tonnes of cereal products in 2013 (www.fao.org/worldfoodsituation/csdb/en/). South Africa was among one of the top ten countries that produce maize with a production of approximately 12,365,000 tons (FAOSTAT, 2014). The production of maize is required to increase by 70% to combat food insecurity as the world population is expected to increase up to nine billion by 2050 (Anonymous, 2003).

However, the production of maize is often affected by fungal diseases such as grey leaf spot (GLS) disease that severely limit the quality, yield size and production of the crop. GLS is a detrimental foliar disease of great economic and global importance as it limits the yielding potential and production of maize (Latterell and Rossi, 1983; Ward et al., 1999; Meisel et al., 2009) in humid and warm climates of many countries worldwide such as Brazil, Costa Rica, Mexico, USA, China, Africa and many others, where a genetically distinct *Cercospora* species is prevalent (Ward et al., 1999; Goodwin et al., 2001; Berger et al., 2014). The two structurally similar but genetically distinct species, *Cercospora zeae-maydis* (*C. zeae-maydis*) was first reported in 1925 in the United States of America by Tehon and Daniel (1925) in Illinois and *Cercospora zeina* (*C. zeina*) the focus of the study, was observed initially in a province called KwaZulu-Natal in South Africa for the African perspective, in 1988 (Ward, 1996) belonging to the Dothidiomycetes family (Ward et al., 1999).

This foliar disease is becoming a threat to food security and the decrease in production of maize affects the economy therefore, it is crucial to develop strategies to manage and control this disease (Berger et al., 2014). Methods such as crop rotation and conventional tillage (which are labour intensive) and fungicides, which are too expensive for farmers who have low income resources are widely used (Munkvold et al., 2001; Menkir and Ayodele, 2005). An alternative

widespread method is the utilization of host resistance combined with chemical control and cultivation practices to reduce yield losses (Pratt et al., 1997). Knowledge of the pathogen (*C. zeina*) and the interaction with a specific host (maize) will help decrease or prevent the progression of GLS.

The aim of this review is to provide an outline of our current understanding of the importance of the plant, fungal biology, plant-pathogen interactions and plant defence responses with regards to the *C. zeina* pathosystem. Furthermore, a summary is given of some of the key research that has been conducted on *C. zeina* to date.

1.2. The importance of maize

Maize (*Zea mays* subspecies *mays*) is a cereal grain that belongs to the grass family Poaceae. The crop was first domesticated in Mexico, Central America and over the years it has been grown by other countries in South America and parts of Africa such as Uganda, Kenya and including South Africa for multiple purposes (Doebley, 2004). Maize is produced throughout South Africa under different tropical and temperate climatic environments. Maize germination occurs within six to ten days in warm and moist conditions and takes two weeks or longer in cool conditions. The optimal moisture content of the soil should be approximately 60% and the temperature range being between 20 and 30ºC. Maize grain of approximately 8 million tons are produced on a 3.1-million-hectare land in South Africa and half of this consists of white maize that is used for human consumption and serves as a staple diet. This crop contributes not only to food security, but also to the economy financially (Doebley, 2004).

According to the statistical data released by the United Nations (UN) in 2014, it was predicted that approximately 80% of the population in the world will increase from 7.2 billion to the total population ranging from 9.6 to approximately 12.3 billion in the year 2100. It was additionally estimated that the highest growth will occur in Africa, in the sub-Saharan region specifically (Garland et al., 2014). An increase on the side of the population equally requires an increase in the total good production and supply. It is estimated that 85% of the maize produced in Southern Africa is used for human consumption which the remaining percentage is used for animal feed.

However, there are factors that affect the production of maize such as abiotic and biotic factors and these pose as threats to food security (IITA 2009). Abiotic factors exerted by the non-living chemical and physical parts of the environment such as drought, heat and insufficient soil moisture among others affect the growth of maize. These then affect the production and yield of maize. Additionally, biotic factors exerted by living components (such as pests and microorganisms causing diseases) also affect maize production. Pests such as armyworms, African maize stem borer, grasshoppers and microorganism causing corn smut, corn stunt caused by a virus and common rust, gray/grey leaf spot caused by fungi among other examples.

1.3. *Zea mays-Cercospora* pathosystem

1.3.1. Grey leaf spot disease, endemic and distribution

Gray/grey leaf spot (GLS) disease was described initially in the USA in 1925 in Illinois by Tehon and was a minor disease, until the disease was severe due to minimum tillage and the use of maize monoculture (Pratt et al., 1997). This foliar disease of maize significantly restricted the yielding capacity that existed and expanded over 25 years to different humid and warm geographical regions (Lipps et al., 1998; Ward and Nowell, 1998; Nutter and Jenco, 1992) of many continents, such as, Brazil, Costa Rica, Mexico, the USA, China, Africa and many others, where *Cercospora* is prevalent (Ward et al., 1999; Goodwin et al., 2001). The disease is caused by the closely related *Cercospora* species, *Cercospora zeina* (*C. zeina*) and *Cercospora zeae-maydis (C. zeae-maydis)* that belong to a fungal class called, Dothidiomycetes (Ward et al., 1999; Berger et al., 2014). The *C. zeae-maydis* isolates were found in the enormous areas of the Corn Belt in the USA and Mexico (Ringer and Grybauskas, 1995; Lipps et al., 1998), while the C. *zeina* isolates were found throughout sub-Saharan Africa (Ward, 1996) mainly in Rwanda, Kenya, Uganda, Zambia, Zimbabwe and South Africa (Wang et al., 1998, Dunkle and Levy, 2000; Meisel et al., 2009). In Africa, GLS has become a pandemic (Ward, 1996) and it was first reported in the continent from South Africa, in a province called KwaZulu-Natal in 1988 (Ward et al., 1998) and it has extended to other provinces such as, Mpumalanga, Free State and North-West (Meisel et al., 2009; Muller et al., 2016).

The *Cercospora* species could not be easily distinguished using morphological characteristics, which have always been applied previously in the taxonomy of *Cercospora* (Chupp, 1953). However, these species could be clearly differentiated using the genetic distance and physiological characteristics (Wang et al., 1998; Crous et al., 2006).

1.3.2. Morphology, taxonomy and phylogenetic relationship of the *Cercospora* genus

The *Cercospora* genus sibling species causing GLS of maize were previously classified as Group I for *Cercospora zeae-maydis* (*C. zeae-maydis*) and Group II for *Cercospora zeina* (*C. zeina*) (Wang et al., 1998; Goodwin et al., 2001). The *Cercospora* genus consists of more than 600 foliar pathogens that share many physiological and molecular characteristics despite having a narrow host range (Goodwill et al., 2001; Crous and Braun, 2003). Crous et al. (2006) performed a study to investigate and identify the *Cercospora* species that cause the GLS symptoms in South Africa. The South African and the USA isolates were compared to check for the morphological differences *in vitro*. It was demonstrated that *C. zeina* has a relatively slow growth rate compared to *C. zeae-maydis* (Crous et al., 2006).

A study by Swart et al. (2017) further distinguished the morphological difference amongst the *C. zeina* and *C. zeae-maydis* species by looking at the cercosporin accumulation in culture shown in Figure 1.1. The cercosporin (photosensitizing perylenequinone) is a nonspecific toxin that plays a major role in the pathogenicity of a pathogen (Daub and Ehrenshaft, 2000; Weiland et al., 2010).

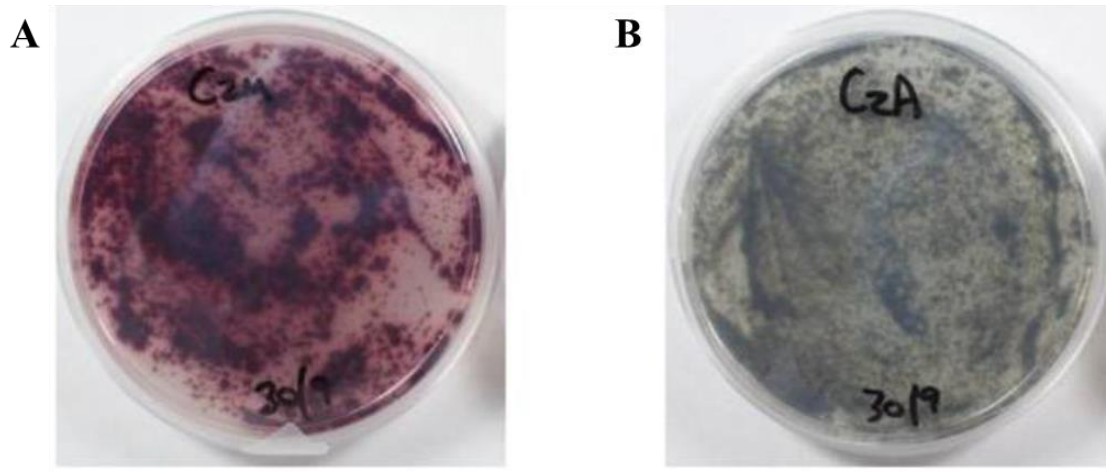

Figure 1.1: The morphological difference between *Cercospora* sibling species, *Cercospora zeina* (A) and *Cercospora zeae-maydis* (B). *C. zeina* species does not produce cercosporin *in vitro* although it carries a complete cercosporin toxin biosynthetic (*CTB*) gene cluster. The different species were cultured on 0.2 potato dextrose agar (PDA) (which allows for the development of a red pigment in a cercosporin-producing pathogens). A *Cercospora zeae-maydis* strain SCOH1-5 produced the cercosporin toxin (Bluhm et al., 2008) and B African *C. zeina* strain CMW25467 (Meisel et al., 2009) did not produce the cercosporin toxin. (Adapted from Swart et al., 2017).

A predicted diagnostic polymerase chain reaction (PCR) further showed the difference between the *C. zeina* and *C. zeae-maydis* species by using the *CTB* gene shown Figure 1.2. The diagnostic PCR shows that *C. zeae-maydis* has the full length *CTB7* gene while the *C. zeina* has a deletion, which allows for the distinction between the two. The deletion in the *CTB7* in *C. zeina* causes a defective version of this gene, however, all other essential machinery needed for synthesizing cercosporin-like molecules are present causing it to produce a structural variant of the cercosporin during maize infection (Korsman et al., 2012; Swart et al., 2017).

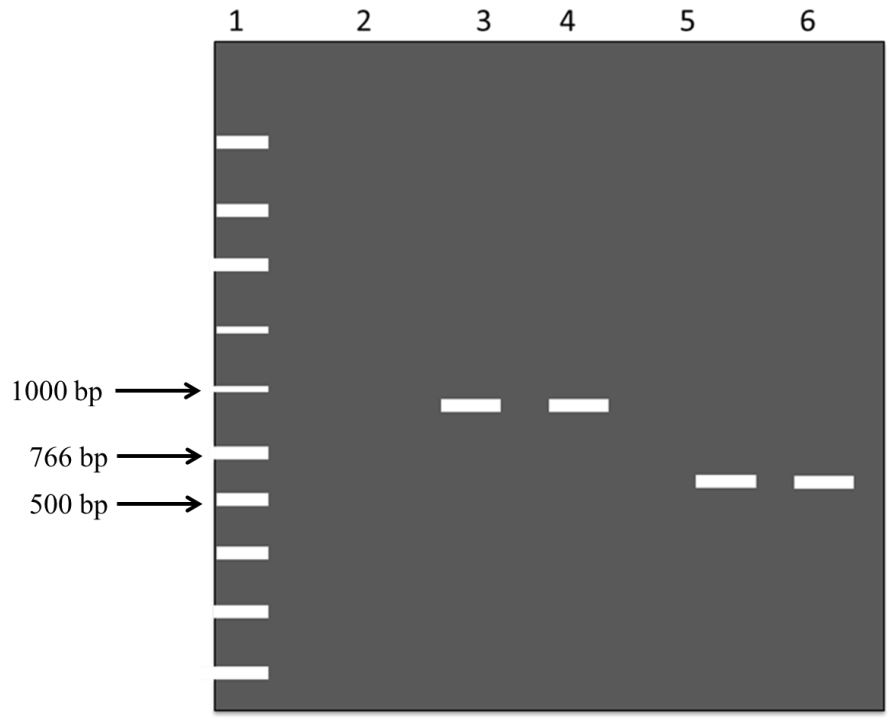

Figure 1.2: A graphic diagram of the predicted diagnostic PCR amplifying, the *CTB7* gene of *C. zeina* and *C. zeae-maydis* isolates. These isolates were amplified using *C. zeina* specific CTB7del_Forward and CTB7del_Reverse primers. The PCR products were separated using an agarose electrophoresis gel that was stained with ethidium bromide. Lane 1 is the NEB Fast-Ruler DNA ladder, lane 2 is the non-template or water control, Lane 3 is the *C. zeae-maydis* positive strain (± 900 bp amplicon), lane 4 is the C. *zeae-maydis* strain (± 900 bp amplicon), lane 5 is the *C. zeina* positive control (smaller ±618 bp amplicon) and lane 6 is the *C. zeina* strain (±618 bp amplicon) (Adapted from Swart et al., 2017).

1.3.3. The infection process of *Cercospora* species

The *Cercospora* species causing maize infection have the same mode of action. The infection strategy of *C. zeae-maydis* has been well described and was found to be like that of other *Cercospora* species (Beckman and Payne, 1982). This is illustrated in Figure 1.3, which shows the infection strategy of *Cercospora zeae-maydis*, *Cochlibolus heterostrophus* and *Setosphaeria turcica*. Firstly, spore germination occurs on the leaf surface followed by infection of maize cells then the production of phytotoxins that are involved in disease development (Wisser et al., 2011). The infection strategy of *C. zeina* species has not yet been formally established, but a study by A.E. Visser (Honours dissertation) partially explained the strategy.

The study used GFP-labelled *C. zeina* isolates and showed that the fungus infects the maize leaf by germination, infection, and colonisation then followed by sporulation which is like the *C. zeae-maydis* infection strategy shown in Figure 1.3 (Beckman and Payne, 1982; Kim et al., 2011; Wisser et al., 2011).

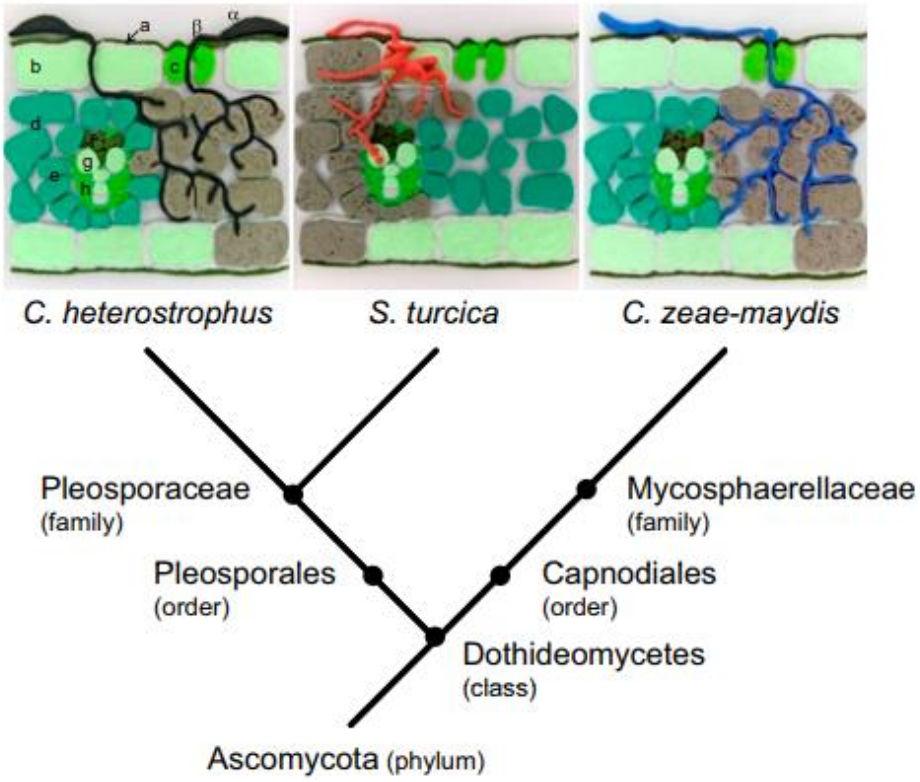

Figure 1.3:Maize leaf infection strategies and taxonomic relationships among the species *Cercospora* species targeting maize. The phylogenetic tree shows *C. heterostrophus*, *S. turcica* and *C. zeae-maydis*. The pictures show maize leaf cross-sections infected by *C. heterostrophus* that causes southern leaf blight, *C. zeae-maydis* that causes grey leaf spot disease and *S. turcica* that causes northern corn are labelled as follows where α conidium (is the black thick structure that is above the cuticle leaf blight. The *C. zeina* species is thought to have the same infection strategy. The *C. zeina* structures of the plant) and β hypha (is represented by the thinner branched blue and red structures inside the maize leaf). Plant structures and tissues were labelled as follows where (a) is the cuticle, (b) is the epidermis, (c) is the stomata which is the point of entry, (d) is the mesophyll region, (e) is the bundle sheathes, (f) is the sclerenchyma, (g) is the phloem and (h) is the xylem. (Adapted from Wisser et al., 2011).

The first step of the GLS disease infection is the dispersal of the fungal spores onto the maize leaves by either rain or wind splash. The spores will attach and then germinate. Once this step is complete and germination tubes demonstrate growth towards the leaf stomata. In the stomata,

differentiation of cells in the hyphae occurs, which leads to the formation of appressorium. After the formation of appressorium, a penetration peg enters the maize cells through the pores of the stomata. The *Cercospora* species colonises mesophyll cells and grows intercellularly for an extended period before switching to a necrotrophic growth phase. In this growth phase, GLS lesions develop and the fungus begins to sporulate and the conidiophore emerges from the stomata and produces conidia, which will give lesions a grey cast and the spores spread once again to repeat the cycle (Beckman and Payne 1982; Kim et al., 2011).

1.3.4. Grey leaf spot disease symptoms

The *Cercospora* species grows throughout the intercellular spaces of the host cells instead of penetrating the cells (Upchurch et al., 1995). The distinct lesions run parallel with leaf veins (Ayers et al., 1984; Stromberg, 1986) and are first observed on the lower leaves of an infected maize with the *Cercospora* species (Ward et al., 1999). Initially, chlorotic spots, tan in colour, appear (approximately 1-3 mm in length) which are irregular or rectangular and have chlorotic borders shown in Figure 1.4A. Mature GLS lesions further expand and result in lesions shown in Figure 1.4B. that further coalesce and blight the entire leaves shown in Figure 1.4C. Blighting of the leaf causes maize to deteriorate and lodging may occur (Stromberg, 1986) as is shown in Figure 1.4D. The latent period of GLS takes longer than other foliar pathogens after infection (14 to 28 days) for the sporulation of mature lesions (Beckman and Payne, 1982; Stromberg, 1986). Sporulating lesions take a greyish cast, which is where the name grey leaf spot comes from (Latterell and Rossi, 1983; Ayers et al., 1984).

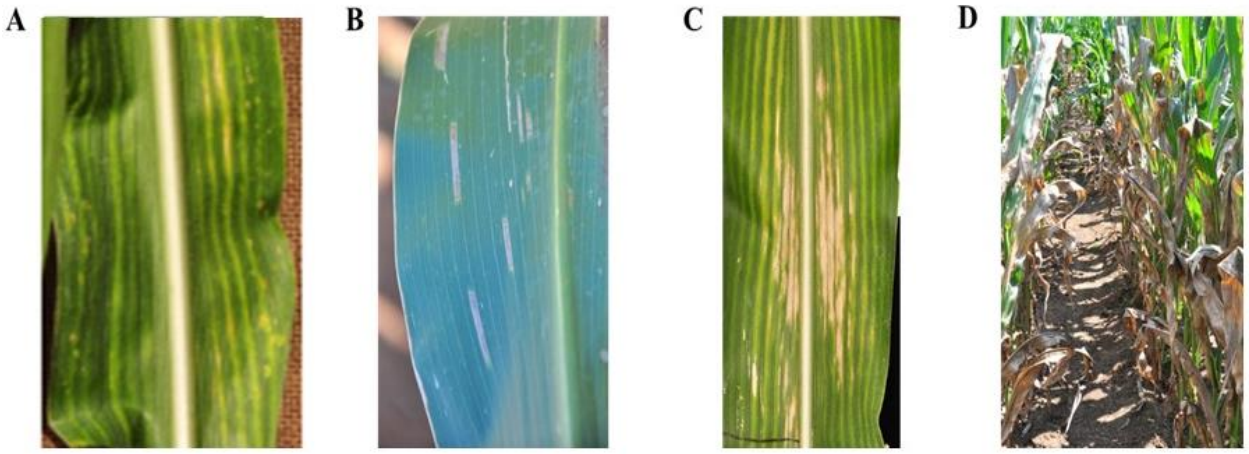

Figure 1.4:The disease progression symptoms of GLS in maize. (A)The immature chlorotic spots. (B) The mature lesions of GLS that are grey and rectangular, occurring parallel to the veins of the leaf. (C) The mature coalesced lesions that cause blighting of the maize leaf. (D) The maize plant becomes predisposed to root rot due to the lodging of GLS. (Photograph credits: Professor Dave Berger).

The sizes, numbers and types of lesions differ greatly among maize genotypes (Ward et al., 1999). Susceptible maize genotypes (inbred and hybrids) commonly display many necrotic lesions (Huff et al., 1988; Lipps and Pratt, 1989) while the moderately resistant maize genotypes exhibit chlorotic and/or lesions that appear fleck (Rupe et al., 1982).This disease is of great economic and global importance as it limits the yield capacity and production of maize, resulting in a loss of as high as 100%, under severe epidemics that cause increased early senescence and rotting of the stalk (Latterell and Rossi, 1983; Meisel et al., 2009). This was illustrated by Ward et al., 1999 where the photosynthetic tissue was reduced and thought to be due to lesions and/or the blighting of entire leaves which results in a decrease in maize yield (Ward et al., 1999). This in turn affects the production of maize as; the inoculum of the fungal pathogen will increase and allow for the fungal spores to be dispersed; and under favourable conditions, it will go through the GLS life cycle therefore, reducing the yielding capacity.

1.3.5. The disease cycle of grey leaf spot

The GLS disease cycle of maize is illustrated in Figure 1.5 (Ward et al., 1999). The maize plant debris is responsible for generating the primary fungal inoculum in the cycle, where it can survive until environmental conditions are favourable (humid and high temperatures). The fungus undergoes germination, colonisation and then produces spores in the maize that was previously infected. The spores are then spread using wind or rain to the lower leaf of newly planted crops.

The leaves at the bottom of maize are commonly the place of primary infection in the growing maize crop canopy. Then, the secondary inoculum gets generated from the mature GLS lesions and spreads by rain or wind to the upper leaves under favourable environmental conditions. The number of lesions can extremely increase on leaves that are developing higher in the crop canopy (Jenco and Nutter, 1992). When favourable conditions last for long periods (hot and humid), the developing lesions may be coalesced and result in extreme blighting, leading to the necrosis of the tissue of the leaf (Stromberg, 1986; Ward, 1996). This will result in a damage caused by light during the initial season of the developed maize crop. The pathogen is then introduced to the field. However, major losses happen at the beginning if (i) environmental conditions are highly favourable for disease development (humid and warm temperatures) and (ii) sufficient crop residues remain that are infected and spread by wind or rain to crops that were previously without infection. The inoculum from crop remains that are infected can be a serious risk factor to the production of maize in the next season (Ward et al., 1999).

Production loss of maize is a result of blighting caused by lesions in the leaves. Blighting leads to premature death of the leaves, which restricts the ability to capture radiation energy and resulting in a reduction in the photosynthetic potential of the leaves, which in turn decreases the translocation of photosynthate for the maize kernels (Ward et al., 1999). This occurs mostly in the upper eight or nine leaves and results in fewer kernels filled per ear (Allison and Watson, 1966). The disease is becoming a threat to food security and the decrease in production of maize affects the economy. Therefore, it is crucial to develop strategies to manage and control this disease.

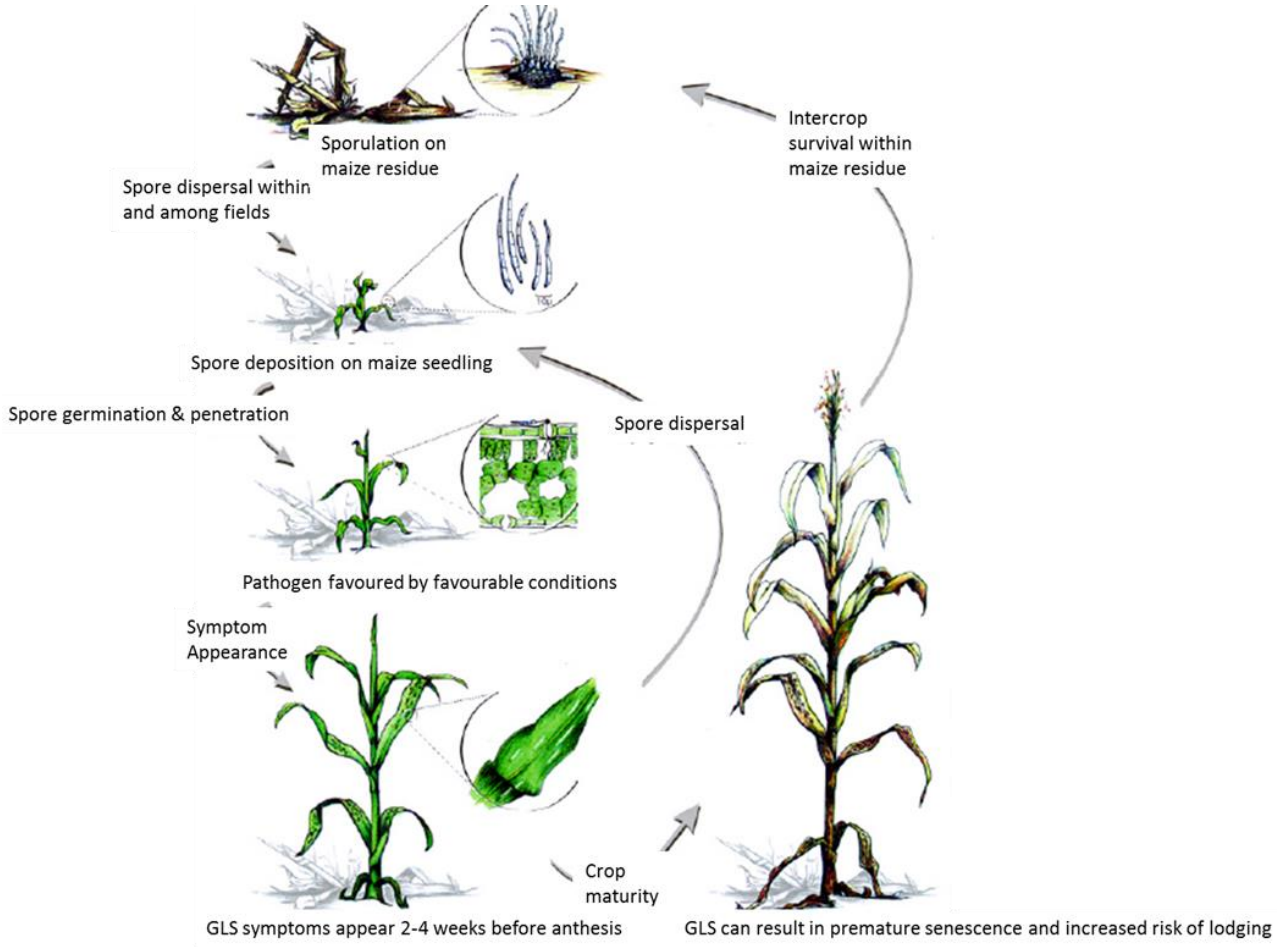

Figure 1.5:The life cycle of GLS disease in maize. The infected maize plant debris serves as the primary inoculum for the development of GLS under favourable conditions. The pathogen undergoes sporulation and the spores get dispersed with the aid of wind or rain to new un-infected maize plants. The fungus penetrates the maize leaf tissue after germination, giving rise to GLS lesions. Mature lesions produce a secondary inoculum, which is dispersed to the upper leaves or other plants which are in the neighbouring or same fields. The fungus survives on the plant debris and the cycle continues (Ward et al., 1999).

1.4. Host resistance

The maize plant can protect itself from the development of diseases using specific gene expression pathways that inhibit the entrance or progression of GLS. The maize can use two specific mechanisms, namely the qualitative host resistance and the quantitative host resistance to induce host resistance. Qualitative host resistance is a race specific host resistant mechanism, regulated by several genes that have a major phenotypic effect. This mechanism is effective and specific to a pathogen isolate it targets, thus making the host specific to the pathogen it recognises. Recognition-proteins and the effector triggered immunity leads to a hypersensitive response once the pathogen has invaded the plant cell (Burdon et al., 1994). Quantitative host resistance is a non-race specific resistance, which involves the expression of numerous genes that have few phenotypic effects and a host is said to be non-specific to the pathogen isolate it targets but recognises a wide range of pathogens, such as targeting the conserved pathogen associated molecular patterns (PAMPs) in pathogens. The PAMP triggered immunity involves recognition of fungi outside the cell by the pattern recognition receptors (PRR) which will trigger a cascade of pathways, causing an expression of the genes that have a small phenotypic effect and result in defence response. As the fungi evolve and develop new effectors, the qualitative resistance is overcome and becomes less effective. Therefore, making the quantitative method a more durable and the preferable method to develop maize hybrids that can be partially resistant to GLS (Carson and Vandyke, 1994).

The resistance of GLS is inherited by the maize quantitatively, by genes that act primarily in a protective manner, where the efficiency of *C. zeina* inoculum is reduced by using the non-race specific genes of maize, which interpose a barrier between the maize (Donahue et al., 1991) and *C. zeina* pathogen (Cromley et al., 2002). This leads to a prolonged latent stage of the pathogen, and reduced sporulation capacity of the C. *zeina*. Additionally, the number of lesions and infection rates will be reduced (Beckman and Payne, 1982; Carson and Goodman, 2006).

1.4.1. The infection trial for candidate maize defence genes

A maize field trial comparing the susceptible B73 infected and un-infected maize genotype was done by the Molecular Plant-Pathogen Interaction Group, at the University of Pretoria led by Professor Dave Berger in 2013. RNA sequencing (RNASeq) was generated for genes that are up-regulated (2368) and down regulated (2079) during *C. zeina* infection and the progression is shown in Figure 1.6. Among the up-regulated genes, two interesting glyoxylate pathway genes that are commonly expressed during seed germination to produce sugar for energy were expressed in the leaf namely, the isocitrate lyase (56-fold change) and the malate synthase (28-fold change) shown in Table 1.1 (Christie et al., 2017).

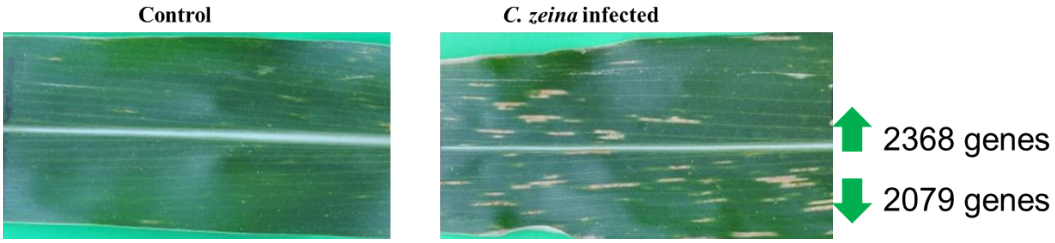

Figure 1.6: Susceptible, B73, maize leaf samples collected from the field trial performed at KwaZulu-Natal in 2013. The maize was inoculated with the *C. zeina* inoculum and control sample was not infected. RNA sequencing was done on the collected B73 maize leaf samples. An increased regulation of 2368 genes was observed while 2079 genes were down-regulated during the fungal infection and disease progression (Christie et al., 2017).

Table 1.1: The RNA sequencing analysis performed on susceptible maize genotype, B73 that were planted in the field in 2013. The RNA-Seq data showed an increase in the regulation of glyoxylate pathway genes. Isocitrate lyase had a 56-fold increase and malate synthase 28-fold increase when un-infected and infected B73 susceptible maize genotypes were compared.

B73 v2 gene name	Fold change	P value (FDR)		UniProt annotation
GRMZM2G056369	56	1.6E-09		Isocitrate lyase
GRMZM2G102183	28	2.4E-11		Malate synthase, glyoxysomal

1.4.2. The role of primary metabolites involved in the glyoxylate pathway in plants

The glyoxylate pathway is a metabolic process that integrates two carbons into the tricarboxylic acid cycle (TCA) of a cell and translocate them by means of gluconeogenesis to produce glucose (Wang et al., 2003) with the use of the major key enzymes namely, isocitrate lyase (ICL) and malate synthase (MS) which are encoded by the *ICL* and *MS* genes. These two enzymes are in separate membrane-bound organelles called the glyoxysomes shown in Figure 1.7A. The key enzymes situated in the glyoxysomes are not always present in all plant tissues but are mostly, during germination when plants develop from the lipid-rich seed (Eastmond et al., 2000). This process helps the plants to acquire glucose before they can be able to make glucose through photosynthesis (Kornberg and Beevers, 1957).

The ICL breaks down isocitrate to form succinate and glyoxylate. The acetyl CoA, together with glyoxylate becomes condensed to produce malate with the aid of malate synthase. The malate becomes oxidized to form oxaloacetate which can be changed to produce phosphoenolpyruvate (PEP) by the PEP carboxykinase, which serve as a precursor of glucose in the glucogenesis pathway to yield energy (Cozzone, 1998) as is shown in Figure 1.7B). Thus, these enzymes play a huge role in seed germination. However, these enzymes have been reported also to play a huge role in the establishment of bacterial and fungal pathogens (they use this energy to progress in the plant host) (Kondrashov et al., 2006; Dunns et al., 2009).

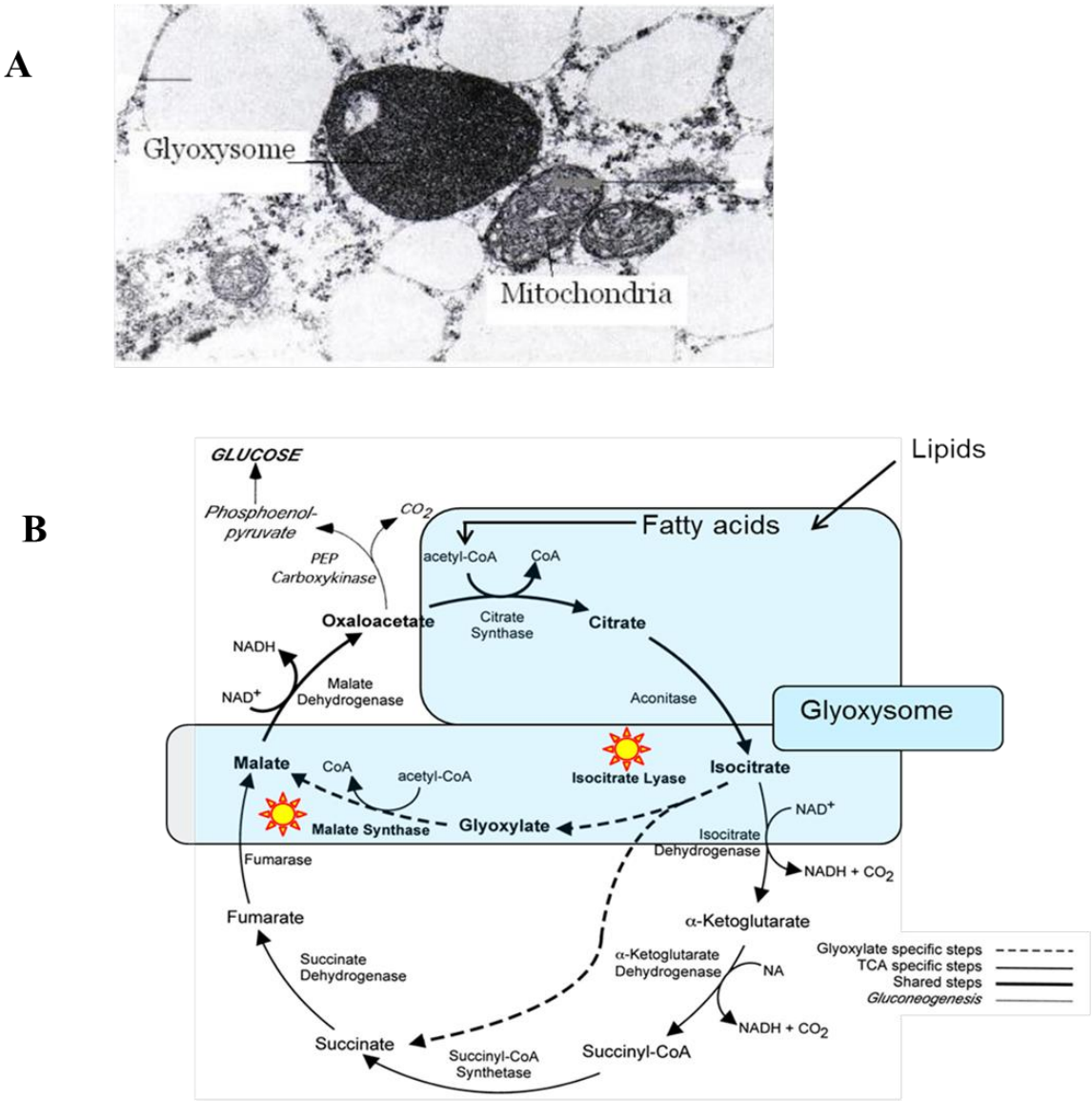

Figure 1.7:The glyoxylate pathway showing the role of the key enzymes, isocitrate lyase (*ICL*) and malate synthase (*MS*). (A) The micrograph shows glyoxysome that is membrane-bound which houses the glyoxylate pathway key enzymes (adapted from Lehninger, online slides). (B) This indicates the bypassing of the TCA cycle. The isocitrate lyase is used to break down citrate to produce glyoxylate. Acetyl CoA, together with glyoxylate, becomes condensed to form malate using malate synthase. The malate becomes oxidized to form oxaloacetate which can be changed to form phosphoenolpyruvate (PEP) by the PEP carboxykinase, which serves as a precursor of glucose. (Adapted from Eukaryotic Cell, 2002).

1.4.2.1. The contribution of isocitrate lyase to fungal pathogenicity

The ICL enzyme plays a role in the first step of the glyoxylate pathway, where it breaks down isocitrate to glyoxylate. Then acetyl CoA together with glyoxylate will be changed to pyruvate. Thereafter, converts pyruvate to glucose or carbohydrates through, glucogenesis. In a recent study done by Wang et al., 2003, the expression of *ICL* has been observed as a requirement for the virulence of *Magnaporthe grisea*, which causes rice blast, the greatest disease of rice (Rauyaree et al., 2001). In the study, it has been shown that the isocitrate lyase (*ICL1)* gene is elevated (Howard and Valent, 1996; Talbot and Foster, 2001). This indicated that the glyoxylate pathway can be used as an intervention for diverse fungal infections (Ward et al., 2003). Another study, illustrating the function of the *ICL* enzyme in fungal pathogenicity of *Leptosphaeria maculans*, which causes blackleg disease of crucifers such as cauliflower and canola was performed. The study showed that the *ICL1* was expressed in the process of infection in the *Brassica napus* cotyledons. The suppression of the *ICL1* locus resulted in the rates of germination in pycnidiospores being reduced. This limited the fungal growth and the fungal pathogenicity on cotyledons. The reduction of pathogenicity of the mutant pathogen was thought to be due to the failure of utilising carbon produced by the crop from the glyoxylate pathway (Idnurm & Howlett, 2002).

Asakura et al., (2006) further confirmed the role of *ICL* in *C. lagenarium* pathogenicity. The fungal pathogen results in anthracnose of numerous plants, which are of great agricultural importance such as watermelon and bitter-melon, among others. In this study, an *ICL1* mutant of the fungi failed to develop on fatty acid or acetate, which was also seen in other fungal and prokaryotic *ICL* mutants.

1.4.2.2. Inhibition of fungal isocitrate lyase in fungi for plant resistance

A cellular localization experiment by Asakura et al. (2006) showed that the encoded *ICL* gene could be merged with green fluorescent protein (GFP) in for *C. lagenarium*. The expression of the merged *ICL1-GFP* was observed in the hyphae, conidia, peroxisomes and appressoria of the fungal pathogen. This modified fungus could damage the lipid bodies and germinate to develop appressoria, same as the wild-type strain (*ICL1*) (Asakura et al., 2006). Additionally, some *ICL1*-deficient conidia were inoculated onto cucumber cotyledons and leaves. This resulted in a decrease in the number of lesions on the cotyledons and leaves even though the fungi remained pathogenic.

Aggressive experiments were also performed whereby spores were inoculated into wound spots of the host plant and penetration assays on cotyledons of cucumber plants. This showed that, the mutant did not penetrate the hyphae thus, proving that the *ICL* gene is necessary at the early stages of infection stages for *C. lagenarium*. Overall, these studies showed that the *ICL* gene is important for fungal pathogenicity and, inhibiting *ICL*, leads to less disease progression in the plant (Asakura et al., 2006).

1.4.2.3. Malate synthase and pathogenicity in fungi

The malate synthase condenses glyoxylate and acetyl CoA to make malate. This will be further converted to form phosphoenolpyruvate which will yield energy (glucose formation). A study by Solomon et al. (2004) used a fungal pathogen that is necrotrophic, *Stagonospora nodorum*, which is responsible for causing glume blotch and leaf disease in economically significant cereals like wheat. The fungus requires internal, stored carbon sources at the beginning of infection and it was suggested in the study that the lipids are metabolised through the glyoxylate pathway that yield glucose and support fungal development. This was shown by an increase in expression of the *MS1* gene in the fungus in wheat with spores that had not germinated and decreased drastically later after germination (Solomon et al., 2004).

1.4.2.4. Inhibition of fungal malate synthase for plant resistance

Unexpectedly, patterns that are opposite were observed when the activity of *MS* was measured. The activity could not be detected in spores that had not yet been germinated but the activity was found to be highly significant in spores that have germinated. The *MLS1* null mutant spores were used to inoculate the seedlings and leaves of wheat and these spores could not induce lesions that were necrotic on either tissue. Therefore, this proved that the gene is crucial for virulence on wheat, which causes a decrease in spores that can germinate and a reduction in the hyphal length in *MSL1*-deficient mutants/null *MSL1* mutants (Solomon et al., 2004). Therefore, targeting the fungal malate synthase and inhibiting it, decreased disease progression, and hence having a plant that has a reduced *ICL* production during infection, would contribute towards reducing fungal infection.

1.5. Conclusion

The grey leaf spot disease was initially considered as a minor disease in Illinois in the United States by Tehon and Daniels in 1925 until it resulted in significant yield losses (Latterell and Rossi, 1983). The *C. zeae-maydis* isolates were found in USA and Mexico (Ringer and Grybauskas, 1995 and Lipps et al., 1998), while the C. *zeina* isolates were found throughout sub-Saharan Africa (Ward, 1996) and South Africa particularly (Wang et al., 1998; Dunkle and Levy, 2000; Okori et al., 2003; Meisel et al., 2009). The fungal spores spread throughout South Africa to other provinces including Mpumalanga, Free State and North-West (Ward et al., 1999; Meisel et al., 2009). Epidemics of these *Cercospora* species may occur if the new races evolve and this will change the way the host interacts with the pathogen to induce host resistance. The interaction amongst the pathogen and host is very dynamic. Since the pathogens and the hosts go through continuous adaptations where the host develops new host immunity and the pathogen develops strategies to evade host detection. The combination of quantitative resistance and qualitative resistance is considered to have the best results in managing GLS (Carson & Van dyke, 1994).

The glyoxylate pathway genes *ICL* and *MS* play an important role in the host-pathogen system as seen in Table 1.1, where the expression of these genes increased during fungal infection. Understanding the respective roles of either in defence mechanisms in the plant or pathogenicity in the fungus can help characterise the progression of GLS and manage it at different stages of infection. The role of isocitrate lyase and malate synthase in maize in response to *C. zeina* infection is unknown. The hypotheses for this study were therefore, that the isocitrate lyase expression is increased at the different disease infection stages to help with fungal development. However, with the contradicting studies on malate synthase, where the hypothesis was that the malate synthase gene is differentially expressed at the different disease progression stages in maize. Thus, this specific study was aimed at investigating the function of these primary metabolism genes with regards to a susceptible maize genotype at different disease progression stages.

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Chapter 2

Inoculation of grey leaf spot disease on susceptible and resistant maize genotypes with *Cercospora zeina*

2.1. Abstract

Background: *Cercospora zeina* (*C. zeina*), which caused GLS is an economically important disease worldwide. The ascomycete grows well and causes GLS under humid and warm conditions, which are the optimal conditions to grow maize. GLS causes over 65% yield, quality and productivity losses, thereby representing a great threat to food security. This disease was first reported in KwaZulu-Natal province, a GLS hot spot in South Africa, in 1988. The visual scores on the susceptible and resistant maize genotypes after inoculation with *C. zeina* were investigated in this study. **Methods:** Two separate glasshouse inoculation trials were conducted in different growing seasons where the maize genotypes; B73, a GLS susceptible and B73-QTL, a GLS resistant were inoculated with a prepared *C. zeina* conidial suspension. Genomic DNA was extracted from the un-inoculated and inoculated maize genotypes and the fungal cercosporin toxin biosynthesis7 (*CTB7)* gene region was amplified to confirm that the infection was due to *C. zeina* and not any other fungal or *Cercospora* species. **Results and Discussion:** In glasshouse trial 1 (2016), the maize infection at two-time points (0 and 32 days' post inoculation) showed that the susceptible maize genotype B73 (2016) had more lesions on average compared to the B73-QTL containing maize genotypes. B73-QTL (83) had more lesions on average compared to the B73-QTL (82) genotype. However, the B73-QTL (82) did not have significant resistance compared to the B73-QTL (83) or B73 (2016) genotypes. In glasshouse trial 2 (2017), the maize scored at different disease progression stages (un-inoculated, chlorotic spots, several lesions and coalesced lesions) showed that the B73-QTL (2017) containing maize genotype had less lesions compared to the B73 maize genotypes. However, the B73-QTL did not have significance resistance compared to the B73 at the different disease stages. Overall, the trial showed that the top portion of the leaf samples that were more exposed to the sun and humidity for the susceptible maize genotypes, showed more disease progressions compared to the resistant maize genotypes. In line with this the inoculations were successful as the *CTB7* gene was amplified in the inoculated samples and was not present in the un-inoculated samples as expected. In conclusion, the susceptible B73 and B73-QTL maize genotype did not show any significance. This difference could be due to the small sample size therefore; the B73 maize genotype was used for future work to check the expression of the candidate maize defence genes that were shown to be highly regulated in inoculated B73 genotypes from RNA sequencing data.

2.2. Introduction

The Group II *Cercospora* species, *Cercospora zeina* (*C. zeina*), causing grey leaf spot (GLS) disease, poses as a threat to food security in Southern Africa (Dunkle and Levy, 2000). The disease spread from the initial place it was identified, KwaZulu-Natal in South Africa, to neighbouring provinces (Wang et al., 1998; Meisel et al., 2009; Berger et al., 2014; Muller et al., 2016). The *C. zeina* fungus overwinters in the field in a form of mycelia on the infected host maize debris, which will germinate and cause infection in the next season of plantation (Korsman et al., 2012; Ntuli, 2016). The progression of GLS is due to increasing temperatures and the presence of high relative humidity early in spring (Ward et al, 1999). The spores get dispersed by rain droplets and the wind to the lower leaves of healthy susceptible maize seedlings (Ward et al., 1999 and Berger et al., 2014). The sporulation of C. zeina was observed on maize leaves at 25-30˚C at 100% relative humidity (Paul and Munkvold 2005). Following sporulation, the conidial spores germinate at temperatures ranging between 23˚C and 30˚C with extended periods of high relative humidity which are greater than 90% (Bluhm et al., 2008).

The initial infection of maize leaves occurs from the formation of germ tubes from the conidial spore that attach across the leaf surface and penetrate the stomata of the leaf. (Dhami et al., 2015). When penetration has occurred, the conidiophore emerges from the stomata, where the first disease symptoms begin to show such as chlorotic spots, which turn into tan lesions, which at a later stage or in severe cases of disease development, the lesions usually coalesce to form extensive necrosis and blighting (Beckman and Payne, 1982; Ringer and Grybauskas, 1995; Ward et al., 1999). The tan spots that form at the beginning are between 1-3 mm in length and rectangular surrounded by a chlorotic border which can be seen if observed under transmitted light microscopy (Mathur and Kongsdal, 2003; Meisel et al., 2009). The mature lesions slowly running parallel and within the leaf veins, ranging from 5-70 mm long and 2-4 mm wide (Ward et al., 1999).

The necrosis and blighting of the susceptible leaf is followed by stalk deterioration (Stromberg, 2009). GLS has a longer dormant period than other foliar diseases where the lesions can take 14 to 28 days before they sporulate. Different maize genotypes show different lesion sizes, number

and type due to their difference in resistance, and the most susceptible genotypes or inbreed show necrotic lesions while moderately susceptible maize genotypes show chlorotic or tan or grey lesions. Therefore, management strategies such as the use of fungicides and genetic resistance help to control or prevent the disease (Ward et al., 1999). The use of resistant cultivars gives the most reduced labour and cost-effective tool to combat the pathogen (Ward et al., 1999). In addition, a combined use of the resistant cultivars which have favourable genetic traits together and with fungicides can result in a high efficiency method to combat GLS (Zhang et al., 2012).

The favourable genetic traits can be found in nature either qualitatively or quantitatively. The qualitative traits are Mendelian traits that can, in absent or present form either exhibit full protection or none, which is controlled by a single resistant gene, R-gene (Poland et al., 2009). The quantitative trait however, does not follow Mendelian genetics but exhibits a continuous variation resulting in quantitative disease resistance that is controlled by different genes which can be pleotropic and epistatic (St Clair, 2010). The resistance of the QTL containing maize genotypes results in partial prevention or reduction of the disease in susceptible maize genotypes (Zhang et al., 2012).

In this study, a glasshouse trial was performed using a GLS susceptible maize genotype, B73 and the quantitative resistant (partially resistant) maize genotype B73-QTL in a homozygous state (Wentzel, 2015). The maize genotypes were inoculated with *C. zeina* to quantify the resistance of the QTL to the fungal pathogen compared to that of the B73 susceptible genotype. This study could be used to select for maize plant genotypes and breeding for maize genotypes that could be used in regions that are affected by the foliar diseases. Collection were done at two-time points for the first trial and at different disease progression stages for the second trial. The B73 maize leaf samples were used as negative controls of the population.

Hypothesis

- Positive control plants containing QTL (B73-QTLs) would have greater disease resistance and thus lower levels of *C. zeina* infection than those of the negative control plants (B73).

Null hypothesis

- The plants containing the QTL would not have any reduction in disease symptoms when compared the B73 maize genotype which lacks the QTL.

2.3. Materials and Methods

2.3.1. Description of the maize genotypes

The plant material contained a QTL for GLS resistance in bin 10.06/10.07 on maize chromosome 10 reported in Berger et al. (2014). The source of the QTL was the maize inbred CML444, which was identified by genetic mapping in a RIL (recombinant inbred line developed using single seed descents from the filial 2 generation) population from CML444 X SC Malawi (Berger et al., 2014). The QTL was introduced into the public inbred maize genotype B73 (Schnable et al., 2009) to produce the B73-QTL genotype (i.e. near-isogenic lines (NILs) with and without the QTL) as a collaborative result between PANNAR SEED (Pty) Ltd and the University of Pretoria (unpublished). The breeding started with a cross between the parents CML444 and B73, followed by backcross breeding for several generations and then selfing for several generations. Marker assisted breeding was used during the backcross breeding to select for the QTL from CML444 (using SSR markers) and to select against the CML444 in the rest of the genome (using SNP markers). Proprietary markers were used for this work which was carried out by the service of the Forest Molecular Genetics, a DNA fingerprinting team at the University of Pretoria. Due to the availability of seed, B73-QTL plants at different generations were used for each glasshouse trial, but they were all fixed in homozygous state for the GLS resistance QTL in bin 10.06/10.07, and therefore could be considered equivalent.

2.3.2. Glasshouse trial 1: Maize samples for inoculation at two-time points

The NILs for Glasshouse trial 1 (2016):

- B73 (2016) (breeder code: AW2239)
- Two sources of the B73-QTL genotype, namely 82 (breeder code: MGN78483-1) which was in the BC3S6 generation (i.e. three cycles of backcrossing plus six cycles of selfing), and 83 (MGN78484-1) which was BC2S7 generation. These were named for this MSc dissertation as B73-QTL (82) and B73-QTL (83), respectively.
- The leaf sample (leaf B and leaf C) from B73, B73-QTL (82) and B73-QTL (83) were inoculated with *C. zeina* and were scored (16th May 2016) by Claudio De Nuzzo a Masters student from the University of Pretoria, Molecular and Plant Interactions Group (MPPI), before they were harvested after 32 days' post inoculation (32 dpi) (De Nuzzo, 2016).

2.3.4. Glasshouse trial 2: Maize samples for inoculation at different progression stages

The NILs for this trial were:

- B73 (2017) (breeder code LGT 60393)
- B73-QTL (2017) (LGT 61419), named B73-QTL (2017) in this MSc dissertation. The backcross and self-generations of this genotype are not known, however the presence of the GLS QTL on chromosome 10 in homozygous state had been confirmed using the four-marker panel by the FMG marker service at the University of Pretoria in 2012 (data not shown).
- The leaf sample (leaf B and leaf C) B73 and B73-QTL (2017) maize genotypes that were inoculated with *C. zeina* were scored (19th May 2017) before they were harvested after 31 days' post inoculation (31 dpi) at different disease progression stages.

2.3.5. Growth of maize genotypes for glasshouse trials

The maize glasshouse trial was run at the Experimental farm at the University of Pretoria in Phytotron A. Two types of maize were grown in the glasshouse trial, B73 which lacks the QTL and the QTL containing maize genotypes. Two seeds were planted per pot until they had germinated where the weaker and smaller one of the two was discarded leaving one plant per

pot. The plants continued to grow in pots containing a coir/sand mix (1:1) in a greenhouse set at 21 ± 5˚C and 60% relative humidity. The plants were on an automatic watering system with one dripper being situated in each pot. The plants were watered for 15 minutes twice daily, which is equal to 500 ml of water per watering session. The plants were fertilized with Wonder leaf and lawn 7N:1P:3K by placing 5 ml into a hole with the dripper in the pot. Hydroponic 3 Kg/5000litre and Solucal 2 Kg/5000 litre (Solu-Cal, USA, 2013) was added to the JoJo tank to apply nutrients to the pots through the watering system twice daily. The misters were initially set to be active four times a day for 10 minutes however this was changed after inoculation to twice a day for 30 minutes due to changes in temperature. To check if the plants had adapted to the growth conditions of the glasshouse trial, the growth of the plants was monitored for a period of 3 weeks shown in Figure 2.2. Leaves of plants at the V6 stage were inoculated with *C. zeina* as previously described (Korsman et al., 2012). The glasshouse trial temperature and humidity were monitored using the HOBO®Pro v2 external and relative humidity data logger that was checked at the end of each week.

Fungal material

The *C. zeina* Mkushi strain (CMW 25467) grown on autoclaved V8 medium (made up of 200 ml V8 juice, 20 g of bacteriological agar, 3.49 g of calcium carbonate and distilled water brought up to 1000 ml) was used to inoculate the B73 and B73-QTL containing maize genotypes (Meisel et al., 2009; Korsman et al., 2012). The conidial spores were sub-cultured after a week when the plates looked like those in Figure 2.3.

2.3.6. Inoculation preparation and plant infection

The conidia were scraped out with a hockey stick spreader which was suspended in 0.01% Tween 20 solution for 2016 and 0.05% Tween 20 in 2017. The *C. zeina* suspension was aspirated into a 50-ml falcon tube. Dilutions of the suspension were made to get the concentration (1:5). The conidial suspension concentration was measured using a Neubauer haemocytometer (BLAUBRAND®, Germany, 2016) which contains four distinct counting chambers, and whereby the conidia were counted for each counting chamber and the average of

the four taken. The conidia were diluted to obtain a $3x10^5$ conidia /ml for 2016 and $1.1x10^6$ conidia/ml for 2017 spore suspension. The spore suspension was applied to the top and bottom sides of each marked off leaf section on all three leaves, Leaf A at the bottom, Leaf B in the middle and Leaf C at the top (V6 stage) using a small brush as is shown in Figure 2.1 below. The leaves of plants that were labelled un-inoculated were painted with the 0.01% or 0.05% Tween 20 while the leaves of plants that were labelled inoculated painted with the fungal suspension. The inoculated 0 dpi leaves for B73 and B73-QTL plants were collected. The temperature was adjusted to 25˚C and water supply was reduced to 2 times per day in the morning at 8:00 am and midday at 1:00 pm.

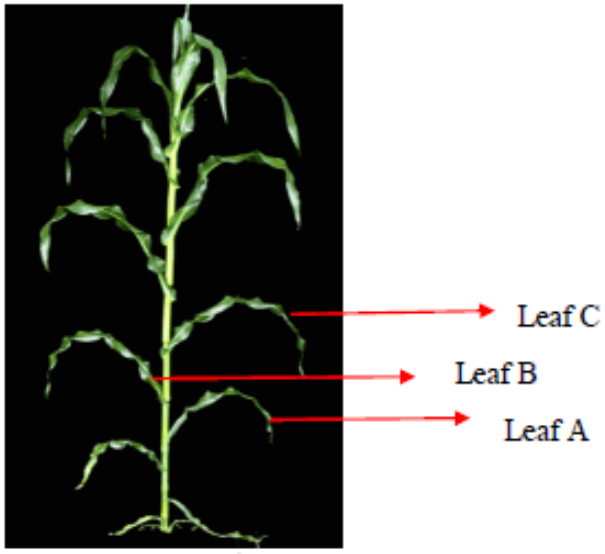


Figure 2.1: Illustration of the inoculated leaf A at bottom, leaf B in the middle and leaf C from a V15 growth stage maize plant (Ritchie et al., 1992).

2.3.7. Visual scoring of the disease

A self-developed scale was used to scale the disease progression of each leaf sample that was harvested during the point of harvesting. The scale was from 1 to 5, where level 1 indicate that there was no disease progression to level 5 that indicated fully mature coalesced lesions. The scale used can be found in Figure 2.11.

2.3.8. Confirmation of inoculation by amplification of the *CTB7* gene

The harvested leaf material was used to extract genomic DNA (gDNA) using an improved cetyl trimethylammonium bromide (CTAB) technique previously described (Meisel et al., 2009; Korsman et al., 2012). A diagnostic PCR reaction containing 10 µM of both the forward and reverse primers, 4.0 µl of double distilled water and Kapa2G Robust HotStart ReadyMix (1U KAPA2G Robust HotStart DNA Polymerase, 0.2 mM each dNTP, 2 mM MgCl2). The forward primer (5'-AAG AGT GCT TGT GAA TGG-3') and reverse primer (5'-GAT GCG GGT GAA GTA GAA A-3') amplifying the CTB7 gene was performed to confirm that the causal agent for the grey leaf spot symptoms is *C. zeina* not by other foliar pathogens (Swart et al., 2014). After PCR, the product was run on a 1.2% agarose gel for 1 hour at 100 V to visualize the PCR product and prove Koch's postulates.

2.3.9. Statistical analysis of glasshouse data

2.3.9.1. Glasshouse trial 1: Maize samples for inoculation at two-time points (2016)

The GLS disease score data obtained from the un-inoculated (0 dpi) and inoculated maize genotypes (32 dpi) was analysed using a One-way ANOVA test from GraphPad Prism 5.0 tool to determine whether the differences in data obtained were significant or not. A t-test using unequal variances was used due to differences in the amount of plants in each maize genotype. The B73-QTL (82) and B73-QTL (83) maize genotypes (Leaf B and C) were compared as well to determine if there was any statistical significance in the data obtained. The B73 (2016) maize genotype data was compared with both the B73-QTL (82) and B73-QTL (83) maize genotypes data for both disease scores. There was no statistical significance if ($\alpha \geq 0.05$).

2.3.9.2. Glasshouse trial 2: Maize samples for inoculation at different progression stages (2017)

The GLS disease score data obtained from the un-inoculated (0 dpi) and inoculated maize (Leaf A and B) collected at different disease progression genotypes (31 dpi) were also analysed using a One-way ANOVA test from GraphPad Prism 5.0 tool. Statistical analysis was done using the Tukey's Multiple Comparison test on B73 (2017) and B73-QTL (2017) (SU, S1, S2, S4 and S5) maize genotypes to determine if there was any statistical significance in the data obtained. There was no statistical significance if ($\alpha \geq 0.05$).

2.4. Results

2.4.1. Growth stage of maize genotypes

The maize plants were developed from the vegetative (V) to the reproductive (R) stage, which was determined by counting the amount of leaves that contained a fully formed leaf collar (Nielsen, 2014). The leaf collars were light in colour and located at the base of the leaf blade, where contact was made with the stem of the plant (Nielsen, 2014). Figure 2.2 shows the growth for all the maize genotypes grown in the glasshouse over a period of three weeks, showing slightly stunted growth stage over the three-week period for the plants that contain the QTL for the first glasshouse trial. The plants were then inoculated at the V6 stage of development.

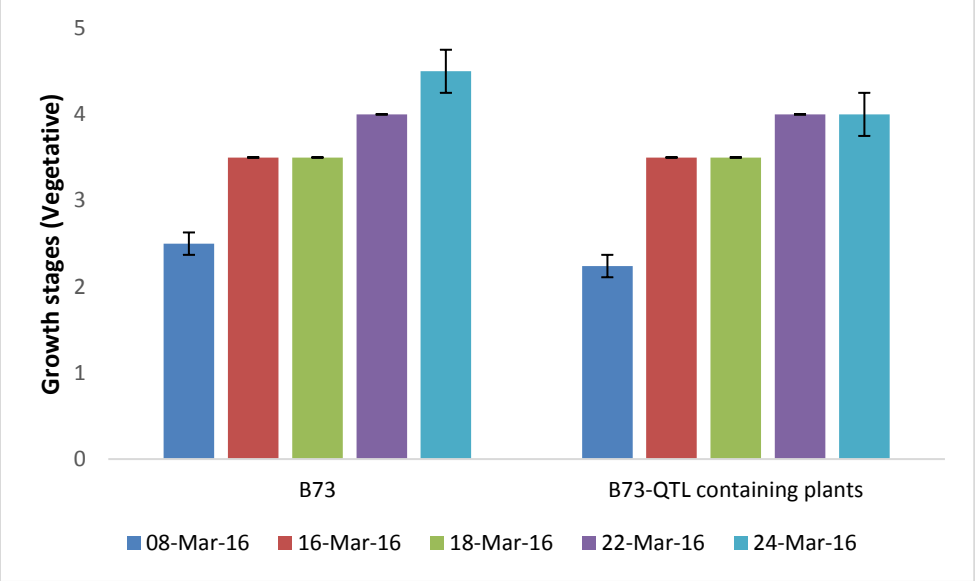


Figure 2.2: Growth stage obtained over three weeks of planting for the B73 and QTL containing [B73-QTL (82) and B73-QTL (83)] maize genotypes.

2.4.1.1. Glasshouse trial inoculation

The *C. zeina* Mkhushi strain cultures, CMW 254627, grew very well in the prepared medium as is shown in Figure 2.3 and the concentration of the conidial suspension diluted to $3x10^5$ conidia /ml for 2016 and $1.1x10^6$ conidia /ml for 2017 glasshouse trial.

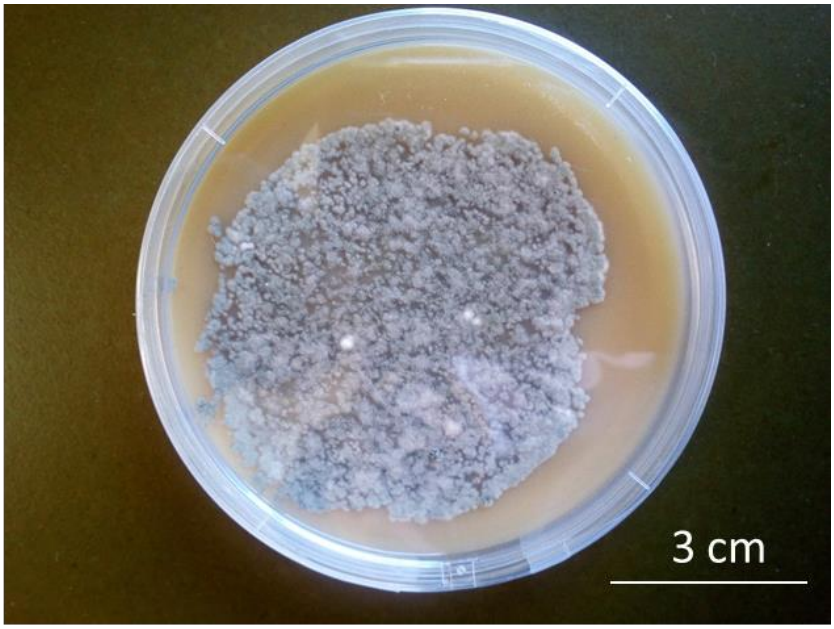


Figure 2.3: *Cercospora zeina* cultured in the dark for maize inoculation and DNA isolation. Wild-type strain CMW25467, *C. zeina*, cultured on V8 agar medium at 25ºC in constant darkness to stimulate conidiation (conidial spore concentration of $1.1x10^6$/ml for 2017 and $3x10^5$ /ml for 2016 glasshouse trial). Scale bar is 1 cm:3 cm. (Photographs taken by Professor Dave Berger).

2.4.2. Glasshouse trial harvesting

The mature GLS lesions running parallel to the leaf veins were visible at 32 days' post inoculation (dpi) for the first glasshouse trail and were harvested for the B73 (2016), B73-QTL (82) and B73-QTL (83) genotypes shown in Figure 2.4.

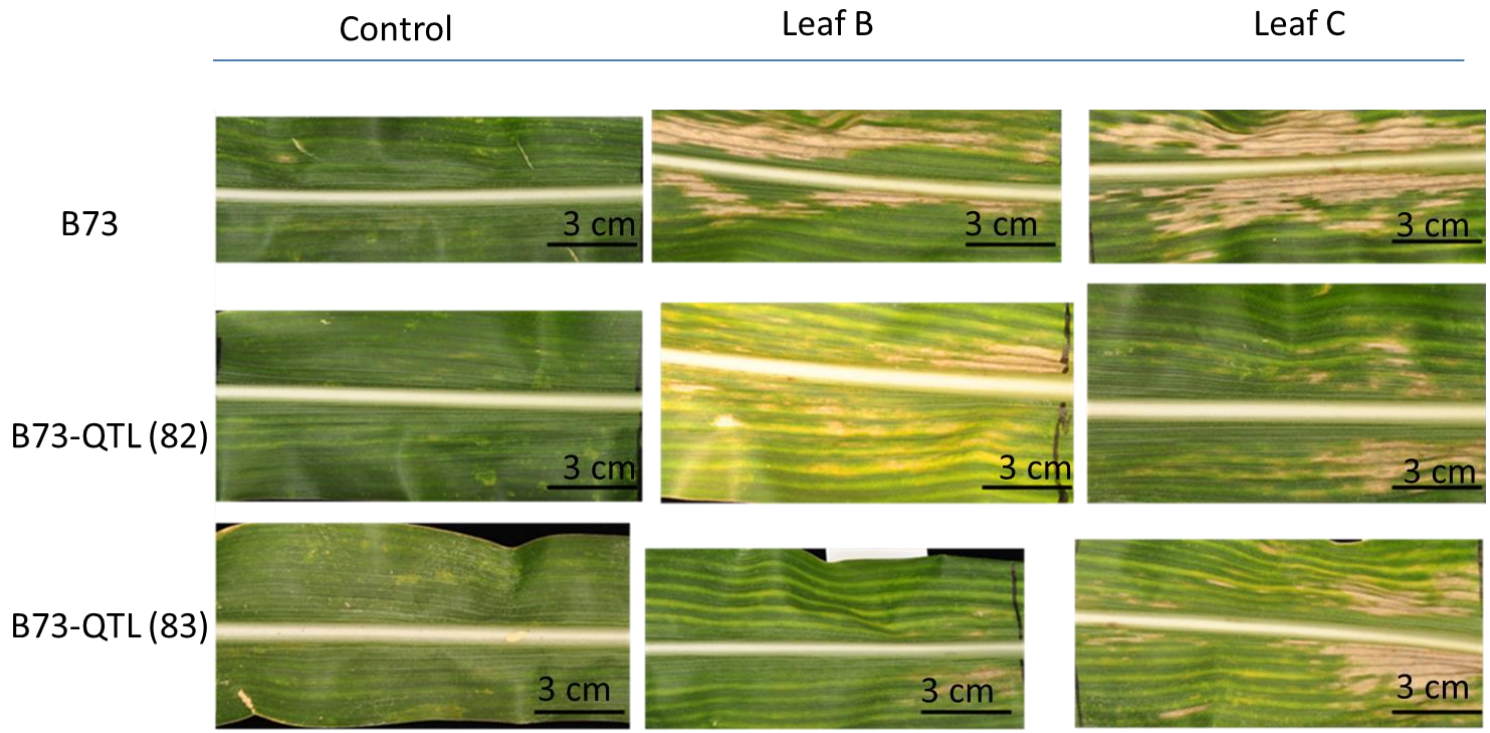


Figure 2.4: Maize genotype B73(2016), B73-QTL (82) and B73-QTL (83) developed GLS symptoms after glasshouse trial inoculation (2016) with *C. zeina* CMW 25467. The GLS susceptible B73 and resistant B73-QTL containing maize genotypes, B73-QTL (82) and B73-QTL (83) were inoculated with the conidial suspension and the leaf samples were collected in triplicates for leaf B and C at 32 dpi. The control mock inoculations did not show any symptoms as expected. Scale bars is 1 cm:3 cm. Photographs taken by Professor Dave Berger. The leaf samples showing different stages of disease progression.

Some GLS mature lesions running parallel to the leaf veins were visible at 31 days' post inoculation (dpi) for the second glasshouse trail shown in Figure 2.5 were collected for both the B73(2017) and B73-QTL (2017) maize genotypes.

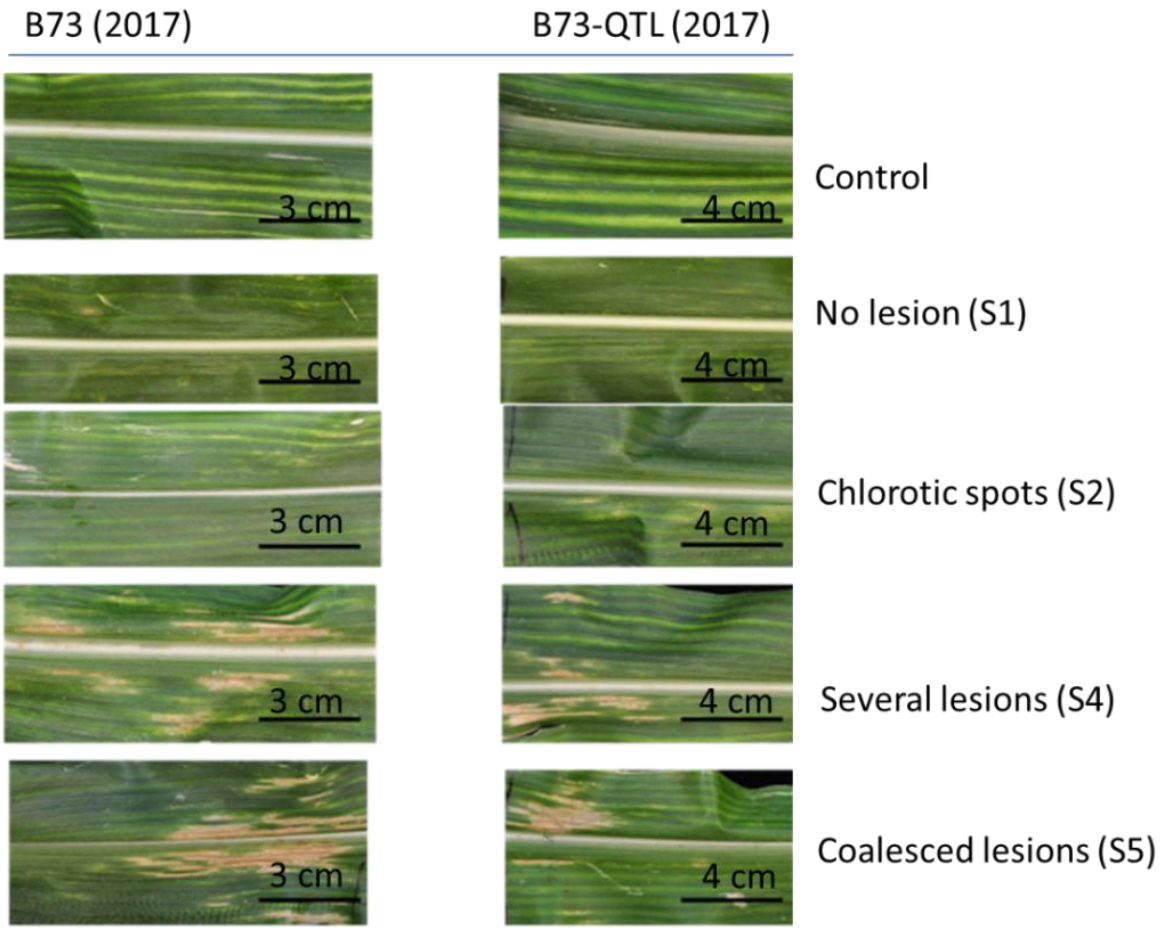
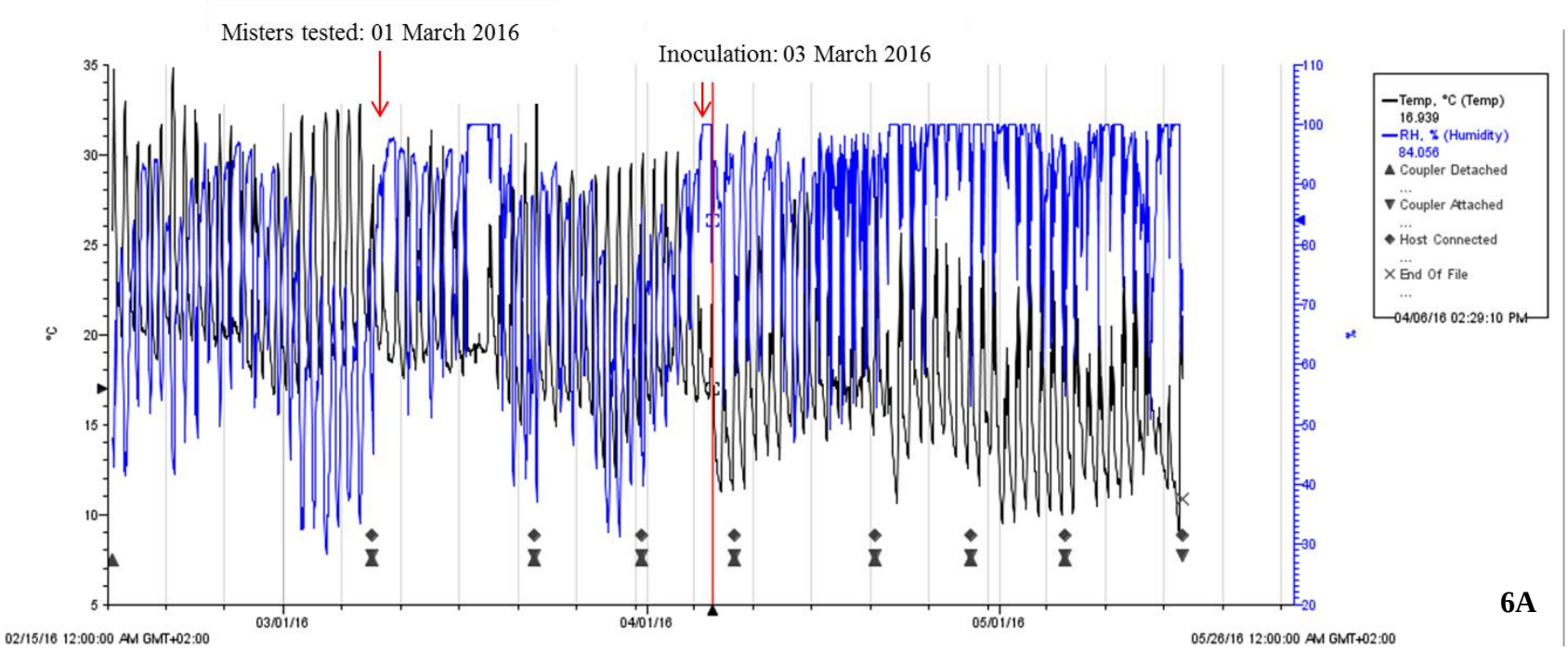


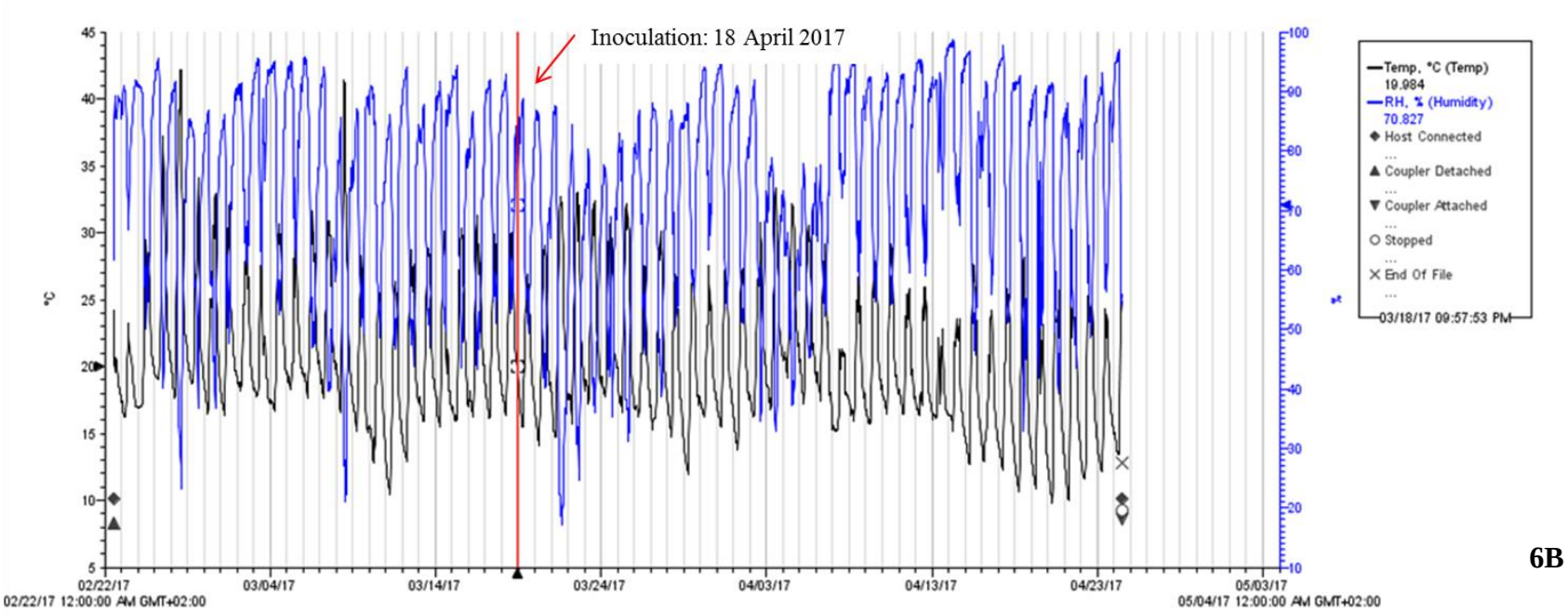
Figure 2.5: Maize genotype B73 and B73-QTL developed GLS symptoms after glasshouse trial inoculation with *C. zeina* CMW 2546 (year 2017). The GLS susceptible B73 (scale bar 1 cm:3 cm) and resistant B73-QTL (scale bar 1 cm:4 cm) containing maize genotypes were inoculated with the conidial suspension and the leaf samples collected in triplicates for leaf samples showing the GLS symptoms at different stages (31 dpi), namely S1 for no lesions, S2 for chlorotic spots, S4 for several mature lesions and S5 for coalesced lesions. The control mock inoculations did not show any symptoms as expected. (Photographs taken by Professor Dave Berger).

The GLS symptoms developed in the upper leaf samples B (middle leaf) and C (upper leaf) instead of the lower leaf samples (leaf A), which is the opposite of what occurs in the field regarding the progression of GLS that usually integrates from the lower leaf showing greater disease symptoms (shown in Figure 2.7 and Table 2.4). The reason for this contradiction in both glasshouse trails is possibly due to the exposure of the upper leaves to sun (heat) and moisture (humidity) during the spraying by the misters from the glasshouse trial, creating a more favourable optimal condition for the disease to progress. The optimal growth conditions were monitored by measuring the temperature and humidity.

The humidity and the temperature in the glasshouse trial are shown in Figure 2.6 where the misters were not active in the first section with lower humidity readings and the second section showed the region where the misters were tested to determine if they were functional, which showed an increase in humidity which was expected. Finally, the third section showed the point of inoculation and switching on of the misters. The final section was of great importance as this was the step, where the misters kept the humidity around 90-100%, which was essential for a successful inoculation. The temperature was approximately higher than 26°C (optimal growing condition for *C. zeina*) and dropped to less than 15°C during the night hence, there was prolonged disease symptom progression.



6A



6B

Figure 2.6: Temperature and humidity levels obtained during the glasshouse trial inoculation for 2016 (6A) and 2017 (6B). The conditions in the phytotron were recorded with a HOBO® Pro v2 external temperature humidity data logger. The temperature (ºC) is indicated in the black lines while the relative humidity (%) is indicated in the blue line.

2.4.3. Grey leaf spot disease score for the B73 and B73-QTL maize genotypes

2.4.3.1. Glasshouse trial 1 disease scores

A self-determined scale from 1 to 5 in Figure 2.11 was used to score the disease progression of each leaf that was harvested (Table 2.1) where 1 was for no disease, 2 was for chlorotic spots, 3 was for single lesions, 4 was for several lesions and 5 was for coalesced lesions. The disease scores in this table were used for statistical analysis shown in Table 2.2 for Tukey's test and Table 2. 3 for the Dunn's test. The average disease scores for B73 (2016), B73-QTL (82) and B73-QTL (83) are illustrated in Figure 2.7.

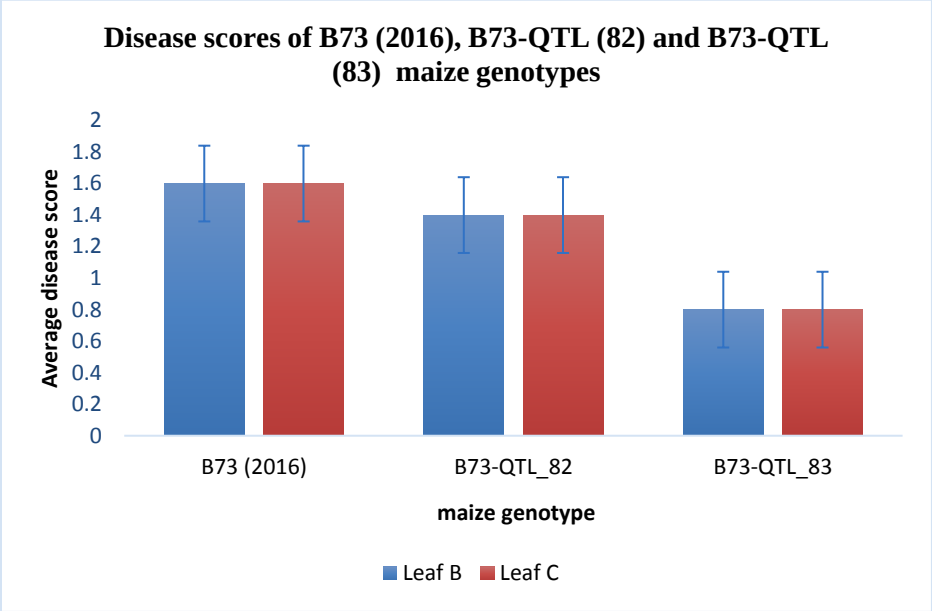


Figure 2.7: Disease scores of the B73 and B73-QTL (82) and B73-QTL (83) containing maize genotypes. Disease scores for harvested leaves B and C. There was no statistical significance of the disease progression between the different maize genotypes and the leaf in each genotype.

2.4.3.2. Glasshouse trial 2 disease scores

The disease scores in Table 2.4 were used for statistical analysis for the Tukey's test shown in Table 2.5 and Table 2.6 for the Dunn's test. The average disease scores for B73 (2017 B73-QTL (2017) illustrated in Figure 2.8.

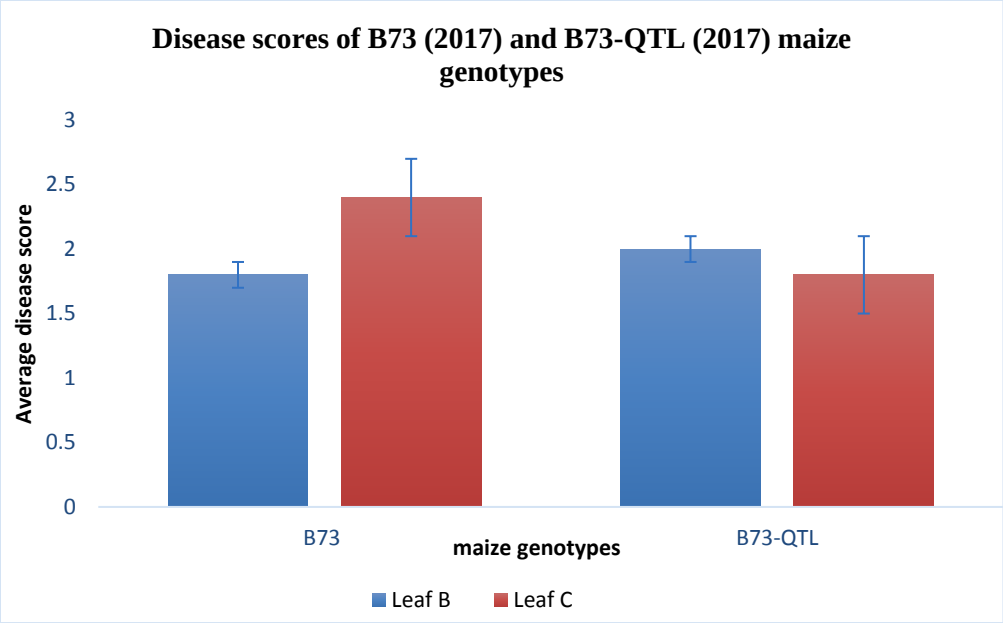


Figure 2.8: Disease score of the B73 (2017) and B73-QTL (2017) containing maize genotypes. Disease scores for harvested Leaf B and C. There was no statistical significant difference of the disease progression between the different maize genotypes and the leaf in each genotype.

2.4.4. Confirmation of infection by *C. zeina*

DNA was extracted from the maize genotypes from the first and second glasshouse trials. The DNA quality check of the extracted gDNA indicates clear bands in the lanes (shown in Figure 2.9). The gDNA was used to amplify the *CTB7* gene to prove the Koch's postulates. Figure 2.10 below shows the amplification of bands in lanes 4, 7, 8, 9, 12 and 13 where lanes 2, 3, 6 and 11 are the negative controls which do not have amplification as expected. The bands seen in lanes in the below gel show the *CTB7* genes which are approximately 620 bp in size; this serves as a confirmation that it was *C. zeina* Mkushi that caused the disease symptoms in the glasshouse trial.

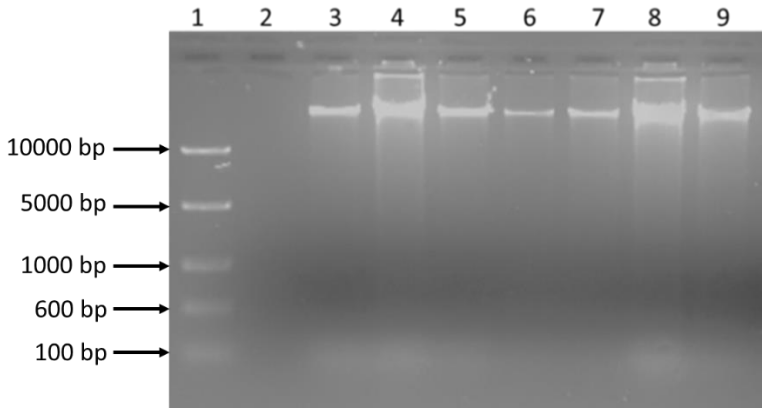


Figure 2.9: DNA extracted from the harvested maize leaves in the glasshouse. Lane 1: Quick-load 2-log (1.0-10 kb) DNA ladder, lane 3: 2016 B73 un-inoculated sample, lane 4: 2016 B73 inoculated sample, lane 5: 2016 B73-QTL (82) inoculated sample, lane 6: 2016 B73-QTL (83) inoculated sample, lane 7: 2017 B73 un-inoculated sample, lane 8: 2017 B73 inoculated sample and lane 9: 2017 B73-QTL inoculated sample. Lane 3-9 showed genomic at the same level larger than the 10 kb band of the molecular marker as expected.

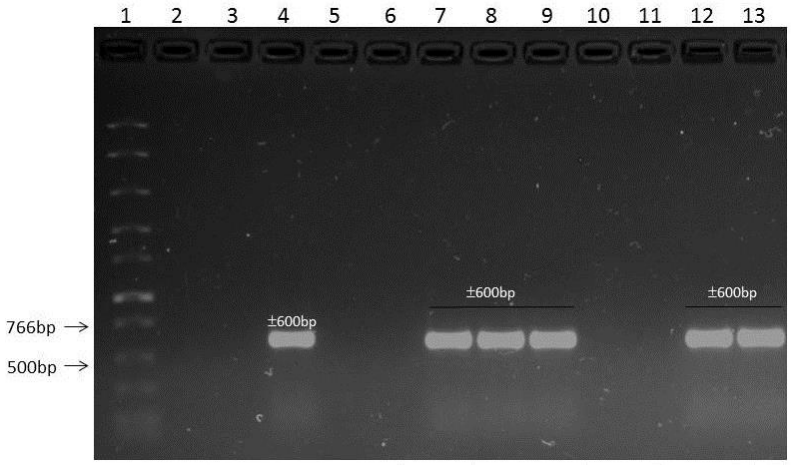


Figure 2.10: *CTB7* gene amplification by PCR to prove Koch's postulates. Lane 1: NEB FAST Ruler 50-10000 bp molecular marker, lane 2: the non-template control, lane 3: negative control with double distilled water as template, lane 4: *C. zeina* Mkushi positive control, lane 6: 2016 B73 un-inoculated, lane 7: 2016 B73 inoculated, lane 8: 2016 B73-QTL (82) inoculated, lane 9: 2016 B73-QTL (83) inoculated, lane 11: 2017 B73 un-inoculated, lane 12: 2017 B73 inoculated, lane 13: 2017 B73-QTL inoculated, lane 6 and lane 13: nothing loaded.

2.5. Discussion

Growth of the maize genotypes in the glasshouse trial

The average growth stage data in Figure 2.2 showed that maize genotypes containing the full QTL had a slightly lower average growth stage in the period of growth but it was not statistically different. This lower growth rate can be due to the additional genetic load of the full QTL, causing a slight change in the way the plants grow. Per Bhatia and Munkvold (2002) even the best partially resistant hybrids that are available can suffer significant yield loss, thus affecting the growth rate of the resistant genotypes (Gorman et al., 1997). But, this change did not have a detrimental negative effect on the survival of the plants. Thus, all the plants were inoculated at the same time at the same growth stage, V6.

Inoculation studies and monitoring of glasshouse trial

The symptoms of GLS started to show at the 19 dpi and later at 21 dpi mature tan lesions appeared (Korsman et al., 2012). However, even though the inoculations were successful, both glasshouse trials were delayed by one week (31 dpi for 2017 and 32 dpi for 2016). This delay was due to the decreased temperatures, which were present during the time of infection and slightly after infection, these colder temperatures (winter time) caused a delay in germination of the conidia and in turn a delay in the onset of the disease (Ward et al., 1999). Humidity of greater than 90% and temperatures greater than 30˚C have a crucial role in helping the conidia germinate (Ringer and Grybauskas, 1995), a slight change in these affects the development of the disease (Ringer and Grybauskas, 1995; Ward et al., 1999; Paul and Munkvold, 2005; Bluhm et al., 2008).

Glasshouse trial maize leaf harvest

The infections in the field show that the lower leaves developed more disease symptoms than the upper leaves (Beckman and Payne, 1982; Paul and Munkvold, 2005). However, the upper leaf samples B (middle) and C (at the top) had greater disease symptoms present than the lower leaf

sample (A), shown in Figure 2.7 and Figure 2.8. This is opposite of a natural occurring infection, which initially develops from the lower leaves, which are more affected by the grey leaf spot disease (Beckman and Payne, 1982; Ward et al., 1999). *Cercospora* species can survive the winter on infected corn residues in the soil surface. When the temperature rises mostly in spring, the fungus on corn residue produces spores that are splashed by the rain or blown by the wind to the young leaves, and then the spores are blown to the upper leaves for secondary infection (Ward, et al., 1993; Ward et al., 1999). The opposite unfamiliar symptoms can be caused by several different factors, which should do with the placement of plants in the glasshouse and their access to sunlight (Beckman and Payne, 1982). Beckman and Payne (1982) reported that optimal sunlight (UV radiation) and high temperatures increase the growth of the *Cercospora* species (*C. zeae-maydis*) *in vitro* (on V8 media) and *in planta* (in a glasshouse trial) however, constant light or darkness (with low temperatures) decreased the development of the GLS. The sunlight was not evenly distributed throughout the glasshouse and the leaf samples that were more exposed to the sunlight had more disease present on them than those which lacked sufficient sunlight exposure. The sunlight would mainly enter the glasshouse from above, which would limit the exposure of lower leaves to the sun and decrease the temperature which may explain why lower leaves had lower levels of disease present on them than that of the upper leaves (Paul and Munkvold, 2005).

The lesions of the susceptible (B73) and resistant [B73-QTL (82) and B73-QTL (83)] maize genotypes (when comparing the disease scores relatively) that occurred in the first glasshouse trial did not differ in size as is shown in Figure 2.4. The lesions (shown in Figure 2.5) from the second glasshouse trial in B73 (2017) and B73-QTL (2017) genotypes were relatively the same size at the disease development stage. The only thing that differed was the number of lesions present per leaf sample from the different maize genotypes, where the maize genotypes containing the QTL seemed to have less lesions present however, still the same size as lesions present on the B73 maize genotypes which lacks the QTL. These results agree with results obtained by Zhang et al. (2012), where they witnessed the same lesion sizes but different numbers in their quantitative resistant maize genotypes therefore, they concluded that GLS disease resistance occurred mainly in fungal invasions and not during progression.

Results from the graphs in Figure 2.7 and Figure 2.8 shows that the plants that contained the QTL had slightly less disease present on them compared to the maize genotype without the QTL, B73. According to the results obtained, it is evident that a general trend is present which indicates that the QTL provides control of grey leaf spot disease in maize caused by *C. zeina*. But, the disease progression for both the glasshouse trials was not statistically significant, meaning the resistant genotypes did not have a huge difference regarding the disease progression when compared to the susceptible maize genotype. The susceptible B73, maize genotype also contains resistance alleles that are present at some loci in the genome however, these alleles are normally weak and cannot overcome the disease (Zhang et al, 2012). Therefore, the quantitative disease resistance can be effective in disease control in two ways, the first would be that once the pathogen is detected, counter pathogen attack mechanisms can occur and secondly, after initial infection has occurred, pathogen progression may be blocked (Zhang et al., 2012).

Glasshouse trial statistical analysis.

The statistical analysis data [(Table 2.2 and Table 2. 3) for 2016 glasshouse trial and (Table 2.5 and Table 2.6) for 2017 glasshouse trial)] and the graphs (Figure 2.7 and Figure 2.8) show that none of the comparisons between any of the maize genotypes exhibited a p value smaller than 0.05, which means that the data is non-significant as more than 5% of the data obtained is due to chance. This result is opposite of what Korsman et al. (2009) reported. This is possibly due to the small sample size whereby, the B73-QTL (82) maize genotype contained only seven plants. While the B73-QTL (83) maize genotype had four plants and the B73 maize genotype contained eight plants. The solution is to use of a larger population size in the future for greater comparisons which may result in significant statistical data.

DNA extractions and confirmation of successful inoculation

The genomic DNA was extracted successfully which is shown by the gel electrophoresis in Figure 2.9, where all the bands were greater than 10 kilo bases (kb) in length. The DNA that was successfully extracted was required for further molecular quantification of a *C. zeina* specific gene, *CTB7,* in the maize genotypes used in the glasshouse trial.

Proving Koch's Postulate

Koch's postulates are an important mechanism that is used to prove that a pathogen is the causal agent of a specific symptom that is visualised (Agrios, 2005). Koch's postulate, which confirms that the *C. zeina* is the causal agent of GLS, can be proven in four specific criteria, which need to be met namely, (i) The suspected causal agent must be present within all the diseased plants examined, (ii) The suspected causal agent must be isolated from the diseased plants and be grown in a pure culture, (iii) The pure culture must be used to inoculate healthy uninfected plants which then need to mimic the disease or symptoms, (iv) The same pathogen or causal agent needs to be isolated from the experimental population as was isolated from the initial population. A study by Meisel et al., 2009 proved Koch's postulates because *C. zeina* was identified as the causal agent for GLS in several countries Southern Africa in countries such as South Africa, Zambia and Zimbabwe. In the study, maize leaf samples that showed GLS disease symptoms and were harvested from the previously mentioned countries were used to recover single conidial cultures. The cultural, morphological and PCR diagnostic based tests that used *C. zeina* and *C zeae-maydis* specific primers proved that the causal agent of GLS in the Southern African countries namely, Zimbabwe, South Africa and Zambia was *C. zeina*.

In this study the maize leaf samples that showed GLS symptoms were harvested and used for single spore isolation (Swart et al., 2017).The single spores were then sub-cultured into a V8 media. The resultant conidial suspension was used for the 2016 and 2017 glasshouse trials to inoculate B73 and B73-QTL containing maize genotypes. The infected maize genotypes showed similar GLS disease symptoms and morphology. A PCR-based test was performed using *CTB7* primers that were specific for *C. zeina* (600 bp) as is shown in Figure 2.10 (Korsman et al., 2012). This molecular diagnostic technique was important to prove that the fungi that caused the GLS symptoms were caused only by *C. zeina.* The positive control would have had a bigger PCR product if *C. zeae-maydis* was the causal agent of GLS. However, there would have been contamination if the negative control had a band which represents the amplification of the *C. zeina* specific gene, *CTB7*. The bigger band in the positive control would mean that the *C. zeae-maydis* was the causal agent and if it had a band in the negative lanes it would have meant that there was contamination of the negative control (the spores spread throughout the glasshouse).

2.6. Conclusion

From the growth stage data obtained, a slight effect was witnessed in plants containing the full QTL, the maize lines exhibited a slightly lower average growth stage over a period of three weeks, which may indicate that the QTL may result in slower growth of the maize. The maize genotypes that contained the full QTL in their maize genome showed more disease resistance to *C. zeina* seen from the visual scores of the harvested leaves from both the glasshouse trials. The glasshouse trials showed a higher level of disease progression or presence in the B73 maize genotype compared to the B73-QTL maize genotypes from both glasshouse trials. However, the statistical analysis showed no significant difference.

The visual score data obtained from the glasshouse showed that it is not statistically significant to make a scientific conclusion that the QTL containing genotypes confer disease resistance after statistical analysis was performed. Therefore, the presence of the QTL may aid in controlling grey leaf spot disease caused by *C. zeina*. However, further tests with larger populations need to be done. The QTL will play a crucial role in breeding programs as well as understanding in the function QTLs play in disease control. Koch's postulates were proven for the study as the *CTB7* gene was amplified from genomic DNA extracted from the infected maize samples.

A large sample size can be used in the future study to get more clarity on the resistance of QTL containing maize lines compared to the B73 inbred genotypes. In the future, further research can be done to quantify the amount of the *C. zeina* pathogen in the infected maize, using quantitative PCR and identifying and determining candidate genes associated with the resistant maize genotypes that play an important role in disease resistance.

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2.8. Appendix

Disease scores

The self-determined scores below were used to scale the level of disease progression from both the glasshouse trials with the help of de Nuzzo, 2016. The disease score data for the 2016 (Figure 2.7 and Table 2.1) and 2017 (Figure 2.8 and
Table 2.4) respectively.

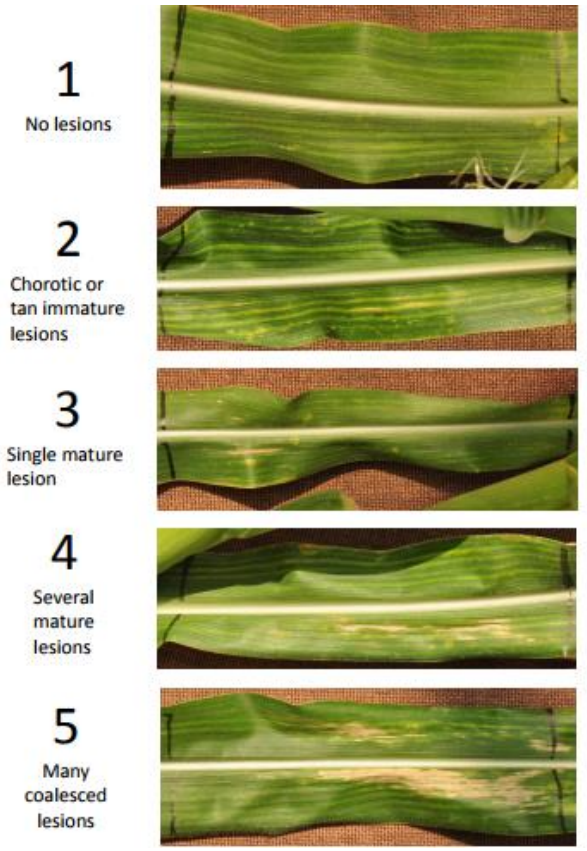

Figure 2.11: The self-determined disease scores for grey leaf spot disease (De Nuzzo, 2016).

Table 2.1: Disease scores for the B73 (2016) and B73-QTL (82) and B73-QTL (83) maize genotypes inoculated with *C. zeina* for 2016.

Plant name	Breeder code for seed source	Leaf B	Leaf C
(82)_1	MGN78483-1	2 (B)	4
(82)_2		2 (B)	3
(82)_3		3	1
(82)_4		4	4
(82)_5		5	5
(82)_6		3	3
(82)_7		1 (P)	5
(82)_9#			
(82)_10#			
(82)_11#			
(83)_1	MGN78484-1	3	4
(83)_2*			
(83)_3		1	1
(83)_4		3	3
(83)_5*			
(83)_6*			
(83)_7		3 (P)	1 (B)
(83)_9#			
(83)_10#			
(83)_11#			
B73_1	AW2239	3	3
B73_2		5	5
B73_3		5 (P)	5
B73_4		5	5
B73_5		2	2 (P)
B73_6		2	3
B73_7		3	4
B73_8		3	3
B73_9#			
B73_10#			
B73_11#			

*Indicated plants which died or did not germinate.
#Indicate 0 dpi plants.
BBlotches.
I Insect damage.
P Plant damages.

Table 2.2: The Tukey's multiple comparison tests between the B73 (2016) and B73-QTL (82) B73-QTL (83) samples for leaves B and C.

SUMMARY			
Table Analysed	Pre-hoc (normalised data)		
One-way analysis of variance			
Value of P	**0.31**		
Summary of p value	**ns**		
Are means significantly different? (P < 0.05)	**No**		
Number of groups	6		
F	1.25		
R square	0.16		
ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	10.49	5	2.10
Residual (within columns)	53.82	32	1.68
Total	64.32	37	

Tukey's Multiple Comparison Test

	Mean Difference	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Leaf B-B73 vs Leaf C-B73	-0.25	0.55	No	ns	-2.22 to 1.72
Leaf B-B73 vs Leaf B-B73-QTL (82)	0.64	1.35	No	ns	-1.39 to 2.68
Leaf B-B73 vs Leaf C-B73-QTL (82)	-0.07	0.15	No	ns	-2.11 to 1.96
Leaf B-B73 vs Leaf B-B73-QT L (83)	1.00	1.78	No	ns	-1.41 to 3.41
Leaf B-B73 vs Leaf C-B73-QTL (83)	1.25	2.23	No	ns	-1.16 to 3.66
Leaf C-B73 vs Leaf B-B73-QTL (82)	0.89	1.88	No	ns	-1.14 to 2.93

Leaf C-B73 vs Leaf C-B73-QTL (82)	0.18	0.38	No	ns	-1.86 to 2.21
Leaf C-B73 vs Leaf B-B73-QTL (83)	1.25	2.23	No	ns	-1.16 to 3.66
Leaf C-B73 vs Leaf C-B73-QTL (83)	1.50	2.67	No	ns	-0.91 to 3.91
Leaf B-B73-QTL (82) vs Leaf C-B73-QTL (82)	-0.71	1.46	No	ns	-2.82 to 1.39
Leaf B-B73-QTL (82) vs Leaf B-B73-QTL (83)	0.36	0.62	No	ns	-2.11 to 2.82
Leaf B-B73-QTL (82) vs Leaf C-B73-QTL (83)	0.61	1.06	No	ns	-1.86 to 3.07
Leaf C-B73-QTL (82) vs Leaf B-B73-QTL (83)	1.07	1.86	No	ns	-1.39 to 3.54
Leaf C-B73-QTL (82) vs Leaf C-B73-QTL (83)	1.32	2.30	No	ns	-1.14 to 3.77
Leaf B-B73-QTL (83) vs Leaf C-B73-QTL (83)	0.25	0.39	No	ns	-2.53 to 3.03

Table 2. 3: One-way ANOVA Dunn's between the B73 (2016) and B73-QTL (82) B73-QTL (83) samples for leaves B and C.

SUMMARY	
Table Analysed	Normalised distribution
Test for Kruskal-Wallis	
Value of P	0.36
Approximate or exact value of p	Gaussian Approximation
Summary of p value	**Non-significant**
Are the median values	**No**

significantly different? (P < 0.05)	
The number of groups	6
Statistical value of Kruskal-Wallis	5.46

Dunn's Multiple Comparison Test

	Rank sum difference	**Significant? P < 0.05?**	**Summary**
Leaf B-B73 vs Leaf C-B73	-2.38	No	ns
Leaf B-B73 vs Leaf B-B73-QTL (82)	4.88	No	ns
Leaf B-B73 vs Leaf C-B73-QTL (82)	-1.55	No	ns
Leaf B-B73 vs Leaf B-B73-QTL (83)	7.44	No	ns
Leaf B-B73 vs Leaf C-B73-QTL (83)	8.69	No	ns
Leaf C-B73 vs Leaf B-B73-QTL (82)	7.26	No	ns
Leaf C-B73 vs Leaf C-B73-QTL (82)	0.83	No	ns
Leaf C-B73 vs Leaf B-B73-QTL (83)	9.81	No	ns
Leaf C-B73 vs Leaf C-B73-QTL (83)	11.06	No	ns
Leaf B-B73-QTL (82) vs Leaf C-B73-QTL (82)	-6.43	No	ns
Leaf B-B73-QTL (82) vs Leaf B-B73-QTL (83)	2.55	No	ns
Leaf B-B73-QTL (82) vs Leaf C-B73-QTL (83)	3.80	No	ns
Leaf C-B73-QTL (82) vs Leaf B-B73-QTL (83)	8.98	No	ns
Leaf C-B73-QTL (82) vs Leaf C-B73-QTL (83)	10.23	No	ns

Leaf B-B73-QTL-83 vs Leaf C-B73-QTL (83)	1.25	No	ns

ns is non-significant, P-value indicating strong evidence against null hypothesis, F is known as F-distribution, compares models in statistics that were fitted to a data set to find the model that best fits the sampled population, R square measures how close the data is to the fitted regression line, ANOVA stands for analysis of variance, Residual is the difference between the predicted value and the detected value of the dependent variable, Mean diff measures statistical dispersion between means, q is a p-value that has been adjusted for the proportion of false positives you can expect to get from a test , CI of diff is Confidence Interval on the difference between Means and vs. stands for versus (against).

Table 2.4: Disease scores for B73 (2017) and B73-QTL (2017) maize genotypes inoculated with *C. zeina* for 2017.

Plant name	New correct label	description	Colour of plant label in glasshouse	Leaf A (bottom)	Leaf B (middle)	Leaf C (Top)
B73-1	B73 Plant: LGT 60393-1	T0: Inoc	Green	Collected 22 February 2017		
B73-2	B73 Plant: LGT 60393-2	T0: Inoc	Green	Collected 22 February 2017		
B73-3	B73 Plant: LGT 60393-3	T0: Inoc	Green	Collected 22 February 2017		
B73-1	B73 Plant: LGT 60393-1	T1: Uninoc	Blue			
B73-2	B73 Plant: LGT 60393-2	T1: Uninoc	Blue			
B73-3	B73 Plant: LGT 60393-3	T1: Uninoc	Blue			
B73-1	B73 Plant: LGT 60393-1	T1: Inoc	Blue	1	3	5
B73-2	B73 Plant: LGT 60393-2	T1: Inoc	Blue	1	1	3
B73-3	B73 Plant: LGT 60393-3	T1: Inoc	Blue	2 or 3	3	1
B73-1	B73 Plant: LGT 60393-1	T2: Uninoc	White			
B73-2	B73 Plant: LGT 60393-2	T2: Uninoc	White			
B73-3	B73 Plant: LGT 60393-3	T2: Uninoc	White			
B73-1	B73 Plant: LGT 60393-1	T2: Inoc	White	3	1	1
B73-2	B73 Plant: LGT 60393-2	T2: Inoc	White	t1	2 or 3	2
B73-3	B73 Plant: LGT 60393-3	T2: Inoc	White	1	4	5
B73-1	B73 Plant: LGT 60393-1	T3: Uninoc	Grey			

B73-2	B73 Plant: LGT 60393-2	T3: Uninoc	Grey			
B73-3	B73 Plant: LGT 60393-3	T3: Uninoc	Grey			
B73-1	B73 Plant: LGT 60393-1	T3: Inoc	Grey	2	2 or 3	2
B73-2	B73 Plant: LGT 60393-2	T3: Inoc	Grey	1	4	4
B73-3	B73 Plant: LGT 60393-3	T3: Inoc	Grey	3	2	5
B73-QTL-1	B73-QTL plant: LGT 61419-1	T0: Inoc	Green	Collected 22 February 2017		
B73-QTL-2	B73-QTL plant: LGT 61419-2	T0: Inoc	Green	Collected 22 February 2017		
B73-QTL-3	B73-QTL plant: LGT 61419-3	T0: Inoc	Green	Collected 22 February 2017		
B73-QTL-1	B73-QTL plant: LGT 61419-1	T1: Uninoc	Blue			
B73-QTL-2	B73-QTL plant: LGT 61419-2	T1: Uninoc	Blue			
B73-QTL-3	B73-QTL plant: LGT 61419-3	T1: Uninoc	Blue			
B73-QTL-1	B73-QTL plant: LGT 61419-1	T1: Inoc	Blue	2	5	1 or 2
B73-QTL-2	B73-QTL plant: LGT 61419-2	T1: Inoc	Blue	2 or 3	4	2 or 3
B73-QTL-3	B73-QTL plant: LGT 61419-3	T1: Inoc	Blue	3	1	1
B73-QTL-1	B73-QTL plant: LGT 61419-1	T2: Uninoc	White			
B73-QTL-2	B73-QTL plant: LGT 61419-2	T2: Uninoc	White			
B73-QTL-3	B73-QTL plant: LGT 61419-3	T2: Uninoc	White			
B73-QTL-1	B73-QTL plant: LGT 61419-1	T2: Inoc	White	1	3	2
B73-QTL-2	B73-QTL plant: LGT 61419-2	T2: Inoc	White	4	2	1
B73-QTL-3	B73-QTL plant: LGT 61419-3	T2: Inoc	White	1	1	5

B73-QTL-1	B73-QTL plant: LGT 61419-1	T3: Uninoc	Grey			
B73-QTL-2	B73-QTL plant: LGT 61419-2	T3: Uninoc	Grey			
B73-QTL-3	B73-QTL plant: LGT 61419-3	T3: Uninoc	Grey			
B73-QTL-1	B73-QTL plant: LGT 61419-1	T3: Inoc	Grey	1: dry plant		
B73-QTL-2	B73-QTL plant: LGT 61419-2	T3: Inoc	Grey	1	1	1
B73-QTL-3	B73-QTL plant: LGT 61419-3	T3 :Inoc	Grey	1	4	2

Table 2.5: ANOVA Tukey's multiple comparison tests between the B73 (2017) and B73-QTL (2017) samples for leaves B and C.

SUMMARY			
Table Analysed	Pre-hoc (normalised data)		
One-way analysis of variance			
P value	**0.46**		
P value summary	**ns**		
Are means signif. Different? (P < 0.05)	**No**		
Number of groups	4.00		
F	0.89		
ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	5.64	3	1.88
Residual (within columns)	67.33	32	2.10
Total	72.97	35	

Tukey's Multiple Comparison Test

	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
leaf b-B73 vs leaf c-B73	-0.5556	1.149	No	ns	-2.41 to 1.30
leaf b-B73 vs leaf b-B73-QTL	0.1111	0.2298	No	ns	-1.74 to 1.97
leaf b-B73 vs leaf C-B73-QTL	0.5556	1.149	No	ns	-1.30 to 2.41
leaf c-B73 vs leaf b-B73-QTL	0.6667	1.379	No	ns	-1.19 to 2.52
leaf c-B73 vs leaf C-B73-QTL	1.111	2.298	No	ns	-0.74 to 2.97
leaf b-B73-QTL vs leaf C-B73-QTL	0.4444	0.9192	No	ns	-1.41 to 2.30

Table 2.6: One-way ANOVA Dunn's multiple comparison tests between the B73 (2017) and B73-QTL (2017) samples for leaves B and C.

SUMMARY		
Table Analysed	Normalised data	
Kruskal-Wallis test		
P value		0.46
Exact or approximate P value?	Gaussian Approximation	
P value summary	ns	
Do the medians vary signif? (P < 0.05)	No	
Number of groups		4
Kruskal-Wallis statistic		2.56

Dunn's Multiple Comparison Test

	Difference in rank sum	**Significant? P < 0.05?**	**Summary**
leaf b-B73 vs leaf c-B73	-2.89	No	ns
leaf b-B73 vs leaf b-B73-QTL	1.83	No	ns
leaf b-B73 vs leaf C-B73-QTL	4.61	No	ns
leaf c-B73 vs leaf b-B73-QTL	4.72	No	ns
leaf c-B73 vs leaf C-B73-QTL	7.50	No	ns
leaf b-B73-QTL vs leaf C-B73-QTL	2.78	No	ns

ns is non-significant, P-value indicating strong evidence against null hypothesis, F is known as F-distribution, compares models in statistics that were fitted to a data set to find the model that best fits the sampled population, R square measures how close the data is to the fitted regression line, ANOVA stands for analysis of variance, Residual is the difference between the predicted value and the detected value of the dependent variable, Mean diff measures statistical dispersion between means, q is a p-value that has been adjusted for the proportion of false positives you can expect to get from a test , CI of diff is Confidence Interval on the difference between Means and vs. stands for versus (against).

DNA extraction from harvested samples

The DNA was pure as the A260/280 values were greater than 1.9 for the extracted DNA. The DNA was used to amplify the *CTB7* gene to confirm that the causal agent was indeed *C. zeina* for the GLS symptoms in the glasshouse trials.

Table 2.7: DNA extraction concentration and purity check using Nanodrop spectrophotometry for B73 (2016), B73-QTL (82) and B73-QTL (83).

#	Sample ID	Nucleic acid (ng/µl)	A260 (Abs)	A280 (Abs)	260/280	260/230
1	*B73_10 uninoculated-2016	3.5	0.007	0.036	1.93	0.59
2	B73 uninoculated-2017	393.5	7.869	4.106	1.92	2.00
3	B73_3 inoculated-2016	375.6	7.513	3.902	1.93	2.04
4	B73 inoculated-2017	97.7	1.955	1.027	1.90	1.70
6	82_3 inoculated2016	301.4	6.028	3.126	1.93	1.91
7	83_3 inoculated-2016	369.6	7.393	3.837	1.93	2.10
8	B73-QTL inociulated-2017	1563.9	31.279	15.102	2.07	2.16

^{A260} Absorbance at 260 nm.
^{A280} Absorbance at 280 nm.
$^{260/280}$ Ratio of absorbance between 260 nm and 280nm
$^{260/230}$Ratio of absorbance between 260 nm and 230 nm
* Was not further used for analysis as it had low concentration

Chapter 3

Expression analysis of selected maize primary metabolism genes in response to *Cercospora zeina* inoculation in the glasshouse

3.1. Abstract

Background: *Cercospora zeina* causes grey leaf spot (GLS), a foliar infection of maize in Southern Africa. The disease is of great threat to the economy, agricultural sector and food security. Strategies such as crop rotation, conventional tillage and fungicides are widely used to manage the progression of GLS. However, these strategies are labour-intensive and expensive for farmers. An alternative, cost-effective method is the utilization of host resistance. To better understand the plant-pathogen interactions during GLS progression, two candidate maize defence genes, malate synthase (*MS*) and isocitrate lyase (*ICL*). The genes are involved in the glyoxylate pathway, were previously shown to be highly induced when comparing un-infected and infected maize in a field RNA sequencing study. This research was aimed at elucidating expression of these two candidate genes.

Methods: Glasshouse trials were conducted by inoculating the susceptible B73 maize genotypes with *C. zeina*. Maize samples harvested at two-time points (0 and day 32 days' post inoculation) and at different disease progression stages (un-inoculated, chlorotic spots, several lesions and coalesced lesions) were used to extract RNA and reverse transcribed to copy DNA (cDNA). The quality of RNA was confirmed by performing reverse transcriptase PCR (RT-PCR) with banana actin (*BA*) primers as a positive control. Primer pairs flanking an intron in the *ICL* and *MS* genes were successfully designed to specifically amplify their respective cDNA. To further confirm these results, the amplified gene products were sequenced using the Sanger sequencing platform and thereafter aligned to respective reference genes obtained from NCBI. The expression levels of *MS* were quantified using the semi-quantitative RTPCR (semi-qRTPCR) technique at different stages of the disease progression.

Results and Discussion: Good quality RNA was successfully extracted and converted to pure cDNA. The cDNA of the candidate maize defence genes, *ICL* and *MS* was successfully amplified. However, the intensity of the *ICL* was relatively low and could not be quantified thus, it needs further optimisation. On the other hand, the expression levels at the different disease stages for the *MS* were quantified successfully using semi-qRTPCR, where the *BA* gene was used to normalise gene expression.

Conclusion: Overall, *MS* gene was successfully amplified from the infected maize genotypes and different transcripts were expressed at the different time points. However, the expression at the different disease progression stages was not statistically significant.

3.2. Introduction

The *Cercospora* species are responsible for causing foliar disease of maize, grey leaf spot disease (Ward et al., 1999; Meisel et al., 2009) in numerous countries (Korsman et al., 2012). The foliar disease causal agents require ways of germination and development without the aid of external nutrients, on the surface of the leaf. The *Cercospora* species enter by breaking the tough cuticles of the plant, attack primary tissues, and to resist the complex physical and chemical mechanisms created by plants for defence. These species carry out morphogenetic programmes, responsible for the development of infection structures in the leaf, such as, the glyoxylate pathway (Wang et al., 2003). The pathway provides a way of joining the carbon and lipid metabolism (Weber et al., 2001), which may be essential to permitting the germination and development of invasion structures to continue before the invasion of the host (Wang et al., 2003). The glyoxylate cycle makes ways for the integration of the carbon (2) compounds into the tricarboxylic acid cycle (TCA cycle). The compounds are further carried through the gluconeogenesis cycle to generate glucose in a cell (Wang et al., 2003) with the use of two key enzymes, malate synthase and isocitrate lyase.

Isocitrate lyase role in fungal pathogenicity

Isocitrate lyase (*ICL*) plays a role in the initial step of the glyoxylate pathway, where it promotes the production of glyoxylate using isocitrate and allows acetyl CoA to be transformed to pyruvate. Thereafter, converts pyruvate to glucose or carbohydrates through, glucogenesis. Research by Wang et al. (2003) proved that the *ICL* enzyme is a requirement for the virulence of rice blast causing fungi, *Magnaporthe grisea* (Rauyaree et al., 2001). The *ICL1* gene expression was higher in blast infected rice (Howard and Valent, 1996; Talbot and Foster, 2001). This confirmed *ICL1* having a pathogenicity activity for fungal development (Ward et al., 2003).

Additional studies indicated the significance of the glyoxylate cycle in the virulence of the *Candida albicans*, a human pathogenic fungus (Cheah et al., 2014). Results indicated the significance of the glyoxylate cycle in most human pathogenic fungus. Targeted mutation of *ICL1* (without the *ICL* gene) led to the expression of symptoms of the disease and spore germination being significantly delayed (Cheah et al., 2014). The other malate synthase enzyme, which is the second key enzyme in glyoxylate pathway showed to have a defence

mechanism against fungal pathogens but in other studies, the *MS* fungal gene was responsible for fungal pathogenicity. However, the role of isocitrate and malate synthase maize genes is not fully known and the expression in infected maize genotypes still needs to be understood.

Aims and objectives of the study

The aim of the study was to investigate the expression levels of maize primary glyoxylate metabolism genes malate synthase and isocitrate lyase to understand their role in fungal pathogenicity or maize defence against *C. zeina*.

Hypothesis

- The glyoxylate pathway genes found in different GLS disease progression stage B73 maize genotype would be differentially expressed at different disease progression stages

Null hypothesis

- The expression of the glyoxylate pathway genes would remain the same between the leave samples collected at different disease progression stages compared to the B73 maize genotypes that were un-inoculated.

3.3. Materials and methods

The susceptible (B73) and resistant (B73 QTL) maize genotypes that were inoculated with *C. zeina* developed GLS in both the 2016 and 2017 glasshouse trials. The resistant maize genotype did not show a huge decrease in the development of the disease compared to the susceptible maize line (the development of the disease in both glasshouse trials did not have a significant difference). Therefore, the B73 genotype was used in this chapter to observe the expression of the selected maize primary metabolism genes in response to *C. zeina* inoculation in the glasshouse trials (should increase to facilitate infection).

3.3.1. Inoculation and harvesting of susceptible maize genotype at two-time points (first glasshouse trial)

Sample description

The leaf samples (leaf B and leaf C) of B73 collected at 0 and 32 days' post inoculation (0 dpi and 32 dpi) in the first glasshouse trial was used for extracting total RNA.

3.3.1.1. RNA extraction and purity assessment

This RNA was tested for purity and integrity. The quantity and quality were measured using the Thermo Scientific ™ 2000 spectrophotometer (ThermoFisher Scientific) looking at the concentration and absorbance ratio at 206/280. The integrity or quality was further analysed on a 1.2% agarose electrophoresis gel marked with ethidium bromide (EtBr) (0.5 µg/ml) and run at 100 V and 400 mA for 45 minutes. The agarose gel was visualised under ultra-violet light using the Molecular Imager ® Gel Doc XR System that is equipped with the Image Lab ™ software. This RNA was used for synthesizing cDNA.

3.3.1.2. First strand copy DNA synthesis

The cDNA was synthesized using ThermoFisher Scientific, Maxima H Minus First Strand CDNA synthesis kit (K1651) following the instructions of the manufacturer, to confirm that the designed primers amplified the selected maize primary metabolism genes specifically.

The RNA samples and reagents were thawed on ice. Total RNA (0.27 µg) was added into a PCR tube with 1 µl oligo $(dT)_{18}$ primers (100 pmol) which are good for initiating cDNA synthesised from total RNA populations and poly-A tail rich RNA. To the mixture, 1 µl of deoxyribonucleotide triphosphates (dNTPs) mix (10 mM) was added and nuclease water adding up to 15 µl of the mixture (15-x µl). A volume of 4 µl 5X reverse transcriptase buffer was added to the 15 µl mixture. The Maximum H Minus Enzyme Mix (1 µl) was also added. The solution was mixed and centrifuged briefly for 20 seconds at 10 000 rpm. This was incubated for 30 minutes at 50°C. The reaction was terminated using heating (5 minutes at 85°C), and then chilled on ice. The first strand cDNA was used further in reverse-transcriptase PCR reactions.

3.3.1.3. Reverse transcriptase Polymerase Chain Reaction

The first stand cDNA was used in reverse-transcriptase PCR (RT-PCR) reactions as the template for amplifying the banana actin (*BA*) gene as a control (these primers flank the intron region therefore if there is contamination with gDNA will be detected), the isocitrate lyase (*ICL*) gene and malate synthase (*MS*) genes as the selected metabolism genes that respond to *C. zeina*. The primers used for these genes are shown in Table 3.1. The synthesized cDNA for the maize genotype B73_7 which had the best quality DNA, was used to amplify the *ICL* (flanking intron 2) and *MS* (flanking intron 2) genes.

Sample preparation for reverse-transcriptase PCR (*RT-PCR*)

A volume of 2 µl (less than 10% of the total PCR reaction) of the cDNA synthesized was added to the master mix (8 µl double distilled water, 12.5 µl of 1x KAPA2G Robust Hot Start Ready-mix (KapaBiosystems, Wilmington, Massachusetts) and 1.25 µl (0.5 µM) reverse/forward primers.

RT- PCR step reaction

The samples were placed in a PCR machine (Applied Bio Systems 2720 Thermal cycler) for 2 minutes at 94˚C and 30 cycles for (15 seconds at 94˚C, 15 seconds at 58.3˚C, 20 seconds at 72˚C) and 20 seconds at 72˚C for *BA*. The samples were run for 2 minutes at 95˚C for 2 minutes and 35 cycles for [15 seconds at 95˚C for 15 seconds, 30 seconds at 55˚C for 30 seconds, 20 seconds at 72˚C for 20 seconds] and 1 minute for at 72˚C for 1 minute for *MS*. The samples were run for 2 minutes at 95˚C for 2 minutes and 35 cycles for [15 seconds at 95˚C for 15 seconds, 30 seconds at 55˚C for 30 seconds, 20 seconds at 72˚C for 20 seconds] and 72˚C for 1 minute for *ICL*.

Visualization of the amplified banana actin, malate synthase and isocitrate lyase genes: agarose gel electrophoresis

A 1.2% agarose gel (100 ml 1x TAE buffer and 1 µl ethidium bromide) was prepared. An amount of 3 µl of the PCR product or 3 µl of CML444 or B73_10 maize genotype genomic

DNA (as the positive control with banana actin primers) was added to 1 µl of 1X loading dye. These were run for 45 minutes at 120 V. The agarose gel was visualised under ultra-violet light using the Molecular Imager ® Gel Doc XR System that is equipped with the Image Lab ™ software. The amplified reverse transcriptase PCR products were sequenced to confirm that the amplified products were *ICL* and *MS* genes.

Table 3.1: The sequences of PCR primers used for this study

Name of primer	Sequence (5' -3')	Primer length (nt)	Gene target	Amplicon size (base pairs-bp)	Programme used to design the primer
B Actin F	ACCGAAGCCCCTCTTAACCC	20	CML444 or B73 gDNA	260 (gDNA) 170 (cDNA)	Van den Berg et al., 2004
B Actin R	GTATGGCTGACACCATCACC	20			
ZmICL F	GAAGGGTTCTACCGCTTCAG	20	B73 cDNA	0 (gDNA) 418 (cDNA)	Perl Primer design version 1.1.21
ZmICL R	CTCTTCAGTAACTCCTTTGCC	21			
ZmMST03 F	ACGCGTACAACCTCATCGTG	20	B73 gDNA B73 cDNA	275 (gDNA) 186 (cDNA)	Primer Designer version 4
ZmMST03 R	CCCGAGAGTTCAACAGAAGC	20			

3.3.1.4. Sanger sequencing of the isocitrate lyase and malate synthase cDNA products

The amplified reverse transcriptase PCR products were sequenced at the University of Pretoria's Sequencing Facility. The reagents used in the PCR steps are shown in Table 3.2 for each primer.

Table 3.2: Cycles for isocitrate lyase and malate synthase gene amplification

Step	Temperature	Time	Cycles
Initial denaturation	95	2 minutes	1
Denaturation	95	15 seconds	35
Annealing	55	30 seconds	
Extension	72	20 seconds	
Final extension	72	1 minute	1

After RT-PCR, the products were used for sequencing where 1.5 µl exonuclease I, alkaline phosphatase, shrimp alkaline phosphatase (ThermoFisher Scientific) were added. Digestion was performed where the product was incubated for 15 minutes at 37˚C and for another 15 minutes at 85˚C, this was done to remove the primers and only have the DNA of interest. The BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, CA, USA) was used for the Sanger sequencing where 10 µl of the *ICL* or *MS* PCR products, separately, were added to 1 µl of each primer (reverse or forward primer), 2 µl of the sequencing buffer and 1 µl of the BigDye (Rosenblum et al., 1997). The sequencing reaction was run (for 10 seconds at 96˚C, for 5 seconds at 55˚C and for 4 minutes at 60˚C) for 35 cycles.

The products were cleaned using the Sephadex G-50 clean up column system (Sigma-Aldrich, St. Louis, USA). The column was prepared by adding 750 µl of Sephadex into the columns and centrifuging them for 3 minutes at 1200 rpm. The PCR product (14µl) was loaded into the column and centrifuged for 3 minutes at 1200 rpm.

Sanger sequencing analysis of the isocitrate lyase and malate synthase

The sequences that were obtained from the sequencing facility shown in the supplementary information were assembled and analysed using the Sequencher v.4.5 sequencing tool (Sequencher, USA). All the sequences (forward and reverse sequences for each primer) were selected and assembled automatically. Contigs were assembled. The file was closed and renamed with the specific reverse and forward primer for the gene. The contig sequences were opened (Staden, 1979), bases were selected, and the chromatograms option was selected, and this showed the picks with the bases. The bases were edited per the codes for sequences shown below where, T: red, G: black, C: blue and A: green shown Table 3.3. The edited trace files shown in Appendix B (in FASTA text formats) were analysed.

Table 3.3: Key on editing sequences on sequencing tools from the sequencing facility (http://bioinformatics.uthsc.edu/classes/module9/sequencher2.html)

Base letter	Description	Converted to this base
Y	pYrimidine	C/T
R	puRine	A/G
W	Weak	A/T
S	Strong	C/G
K	Keto	T/G
M	aMino	C/A
B	Not A	G/T/C
D	Not C	A/G/T
H	Not G	A/C/T
V	Not T	A/G/C
X or N or?	Unknown	A/G/G/T
O or: or -	Deletion	Nothing

The FASTA (txt) sequences for the contigs were aligned in BLASTn under NCBI (Altschul et al., 1990), where *Zea mays* was chosen as the organism shown in Appendix B, Table 3.4. BLAST search helps to compare a query sequence (consensus for isocitrate lyase and malate synthase) with a library or database of sequences and identify library sequences that resemble the query sequence above a certain parameter. The BLAST analysis gives the score value, query cover, E-value and percentage identity. The score is a numerical value that defines the total quality of an alignment. High numbers correspond to higher percentage similarities.

While the E-value, which is associated to a score (S); is the number of distinct alignments, with a score equivalent to or better than S, that are expected to occur in a database search by chance. The lower the E-value, the more significant the score is. The confirmation of the sequences was further confirmed by using multiple sequence alignment where the genomic DNA, cDNA was aligned to the consensus sequences (ZmICL:ICL_Zimbili cDNA and ZmMST03: MS_Zimbili cDNA) using Clustal Omega Multiple Sequence Alignment tool (Sievers et al., 2011). The MS: malate synthase Zimbili is the owner of the sequence and Zm: *Zea mays* and cDNA: the sequence results.

3.3.2. Inoculation and harvesting of susceptible maize genotype at different disease progression stages (second glasshouse trial)

Sample description

The leaf samples (leaf B and leaf C) B73 (LGT 60393) that were harvested at 0 dpi and 31 dpi (SU: un-inoculated, S1: no lesions, S2: chlorotic spots, S4: several lesions and S5: coalesced lesions) from the 2017 glasshouse trial were used for RNA extraction.

3.3.2.1. RNA extraction and cDNA synthesis of the susceptible maize genotype at different disease progression stages

The Qiagen, RNeasy® Plant Mini Kit (cat. nos. 74903 and 74904) was used to extract total RNA of maize leaf samples collected at different disease progression stages. The cDNA synthesized with ThermoFisher Scientific, Maxima H Minus First Strand CDNA synthesis kit (K1651) following the instructions of the manufacturer was used for RT-PCR. The template for amplifying the maize actin gene as the control (these primers flank the intron region therefore if there is contamination with gDNA will be detected) and malate as the selected metabolism gene that respond to *C. zeina*. The *BA* gene was amplified using flank the intron region therefore if there is contamination with gDNA will be detected (forward primer 5’ ACCGAAGCCCCTCTTAACCC 3’ and reverse primer 5’ GTATGGCTGACACCATCACC 3’) (Van den Berg et al., 2004) with cycling conditions: 2 min at 94˚C followed by 30 cycles (15 secs at 94˚C, 15 secs at 58.3˚C, 20 secs at 72˚C) and 20 secs at 72˚C. The malate synthase gene was amplified using the designed primers (forward primer 5’ ACGCGTACAACCTCATCGTG 3’and reverse primer 5’ CCCGAGAGTTCAACAGAAGC 3’ with cycling conditions: 2 min at 95˚C followed by 35 cycles for (15 secs at 94˚C, 15 secs

at 58.3˚C, and final extension for 20 secs at 72˚C) and final extension for 1 min at 72˚C). The products were subsequently electrophoresed in 1.2% (w/v) agarose gels.

3.3.2.2. Semi-quantitative expression analysis of the malate synthase gene at different disease progression stages (2017 glasshouse trial)

The RT-PCR can identify the absence or presence of messenger RNA (mRNA) in a specific species however; the gene expression quantitative measurement cannot be provided because of the plateau effect. Quantitative methods such as the semi-quantitative RT-PCR (semi-qRTPCR) modifies the normal RT-PCR technique and helps to easily measure mRNA the levels in a sample or give an indication of the comparative expression levels in response to external stimuli at different amplification cycles (Yoshimura et al., 1998). Therefore, this technique was used to quantify the expression levels of malate synthase gene in response to *C. zeina* infection at different disease progression stages. The *ICL* was discontinued as the band intensity was not strong enough to measure quantify the ethidium bromide (EtBr) thus; further optimization of the gene needs to be done in future studies.

Cycle selection for banana actin and malate synthase gene (Fine-tuning)

The nuclease free diluted cDNA (1:10) of B73 (LGT 60393) (SU, S1, S2, S4 and S5) was used to amplify the malate synthase gene performed at different cycles using the malate synthase primers to selectively amplify genes at an optimal semi-qRTPCR cycle. The banana actin gene was amplified where the cycling conditions were as follows: 2 min at 94˚C followed by 15, 20, 25, 30, or 35 cycles (15 secs at 94˚C, 15 secs at 58.3˚C, and 20 secs at 72˚C) and final extension for 20 secs at 72˚C. The malate synthase gene was amplified for 2 min at 95˚C followed by 35 cycles for (15 secs at 94˚C, 15 secs at 58.3˚C, and final extension for 20 secs at 72˚C) and final extension for 1 min at 72˚C).

Banana actin and malate synthase semi-quantitative RT-PCR (Kinetic analysis)

The semi-qRTPCR for *BA* and *MS* was performed by amplifying the respective genes at the optimal cycle conditions using the nuclease free and DEPC diluted cDNA (1:10) for maize leaf at different disease progression stages (SU, S1, S2, S4 and S5). The samples were placed in a PCR machine (Applied Bio Systems 2720 Thermal cycler) where the selected cycles for banana actin was 30 cycles. And, the samples (diluted in nuclease free water) were placed in a PCR machine (Applied Bio Systems 2720 Thermal cycler) where the selected optimal cycle

was 34 and 35 cycles for malate synthase. The products were subsequently electrophoresed in 1.2% (w/v) agarose gels.

Semi-quantitative RTPCR analysis

The band intensity of different replicates at different disease progression stages for banana actin and malate synthase were used to quantify the expression using ImageJ public domain, Java-based image processing programme version 2 (Rasband, 1997-2016). The concentration of EtBr (ethidium bromide) was measured in each band for the replicate at different disease progression stages respectively. The band intensities were represented as peaks which the area and percentage area was measured from. The percentage area for *MS* (SU, S1, S2, S4 and S5) was normalised against the percentage area of banana actin (SU, S1, S2, S4 and S5) respectively. The malate synthase (SU, S1, S2, S4 and S5) normalized mean values were used to draw the bar graphs and the standard deviation was used to draw the error bars on each column.

Results

3.4.1. Inoculation and harvesting of susceptible maize genotype at two-time points (2016 glasshouse trial)

RNA extraction and purity assessment

RNA harvested from B73 was successfully extracted for 0 dpi and 32 dpi shown by the Nano drop concentration and absorption ratio at 280/260 in which is above 1.9 and distinct bands for the 28S and 18S ribosomal RNA (rRNA) were visible from the denaturing by electrophoresis image in Figure 3.1. The purified total RNA was used for cDNA synthesis, RT-PCR, sequencing and alignment analysis of the glyoxylate pathway genes.

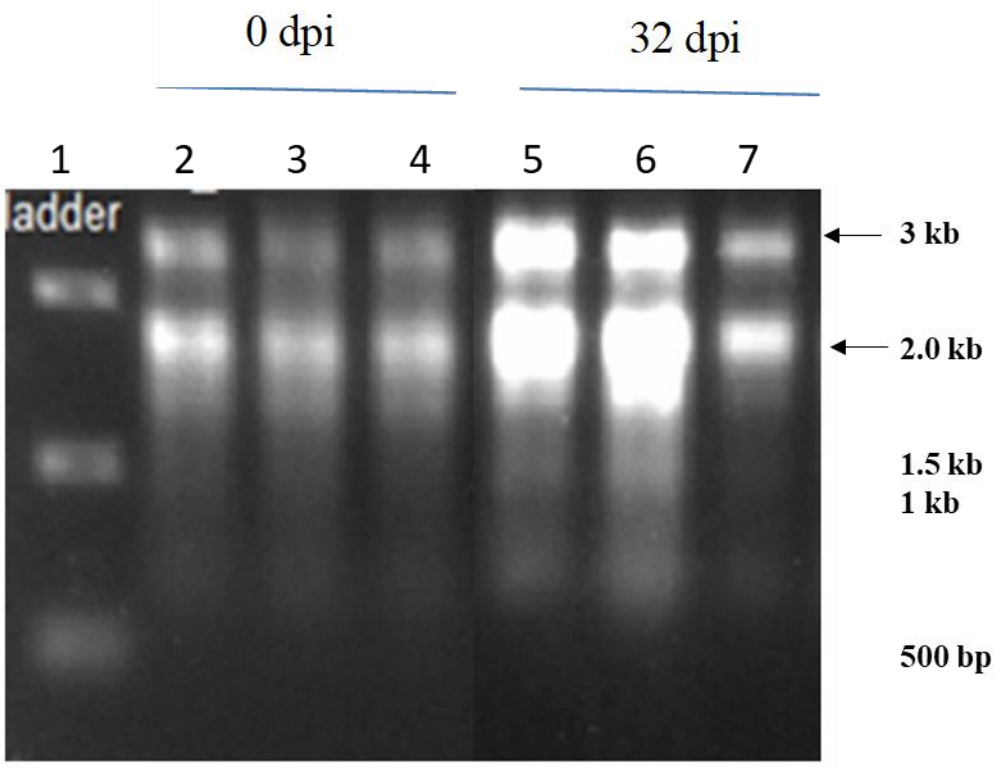

Figure 3.1: RNA extraction on un-inoculated and *C. zeina* inoculated (32 days post inoculation) B73 maize genotype. Lane 1**:** 1 kb Quick Load DNA ladder, lane 2: B73_9, lane 3: B73_10, lane 4: B73_11, lane 5: B73_1, lane 6: B73_7 and lane 9: B73_8 where the 28s RNA is approximately 3 kilobases (kb) in size and the 18s RNA is approximately 2 kb. The RNA was extracted successfully for maize genotypes harvested at for 0 dpi (lane 2-4) and 32 dpi (lane 4-7).

Reverse transcriptase polymerase chain reaction on synthesized cDNA

The cDNA that was synthesized from the B73 maize genotype harvested at 0 dpi and 32 dpi had no genomic DNA contamination as the PCR product (banana actin) was approximately 169 bp instead of approximately 277 bp as shown in the positive genomic CML444 maize gene shown in Figure 3.2.

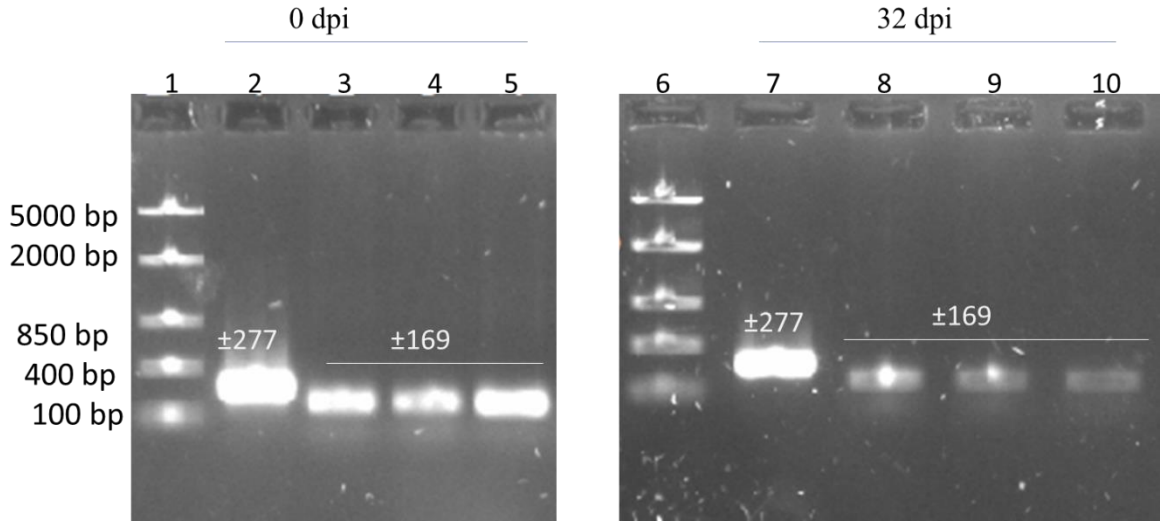

Figure 3.2: RT-PCR for synthesized cDNA. The synthesized cDNA was amplified with *BA* primers which flank an intron in the maize genome to confirm that there was no genomic DNA contamination. Lane 1 and 6: FastRuler medium range ladder, lane 2: CML444 genomic DNA (277 bp amplicon), lane 3: B73_9, lane 4: B73_10, lane 5: B73_11, lane 7: CML444 genomic DNA (277 bp amplicon), lane 8: B73_1, lane 9: B73_7 and lane 10: B73_8.

The synthesized cDNA from B73_7 (32 dpi inoculated) was used to amplify the *ICL* in Figure 3.3 and *MS* in Figure 3.4 genes, using the primers that were designed using the Perl Primer design version 1.1.21 and primer design version 4 tools respectively as shown Appendix B. The B73_10 genomic DNA was used as the positive control to check for genomic DNA contamination. The *ICL* gene was amplified successfully with the expected size (Appendix B) although, the amplification was not specific this could be due to the low annealing temperature. Thus, the annealing temperature was increased in future studies as shown in Figure 3.9.

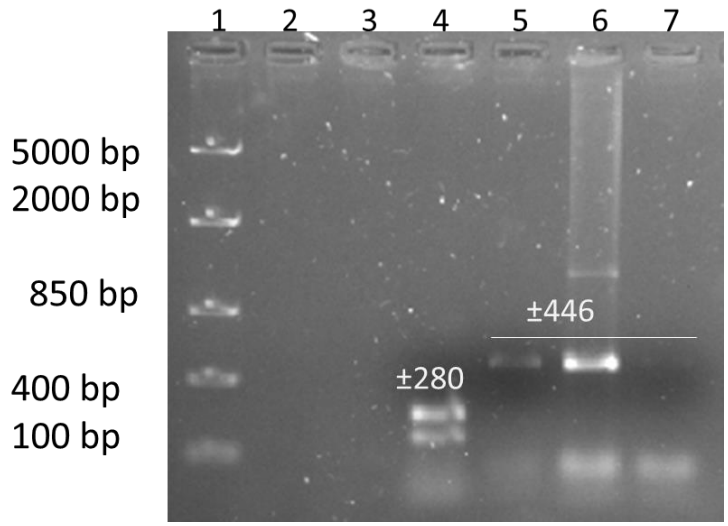

Figure 3.3: RT-PCR of the isocitrate lyase gene. The synthesized cDNA was amplified with ZmICL primers which cut across the second intron in the maize genome genomic DNA contamination is not expected. Lane 1: FastRuler medium range ladder, lane 2: negative template control with just the PCR master mix, lane 3: negative control with water as the template, lane 4: positive control with B73_10 genomic DNA as the template, (280 bp amplicon), lane 5: B73_1, lane 6: B73_7 and lane 7: B73_11 (446 bp amplicon).

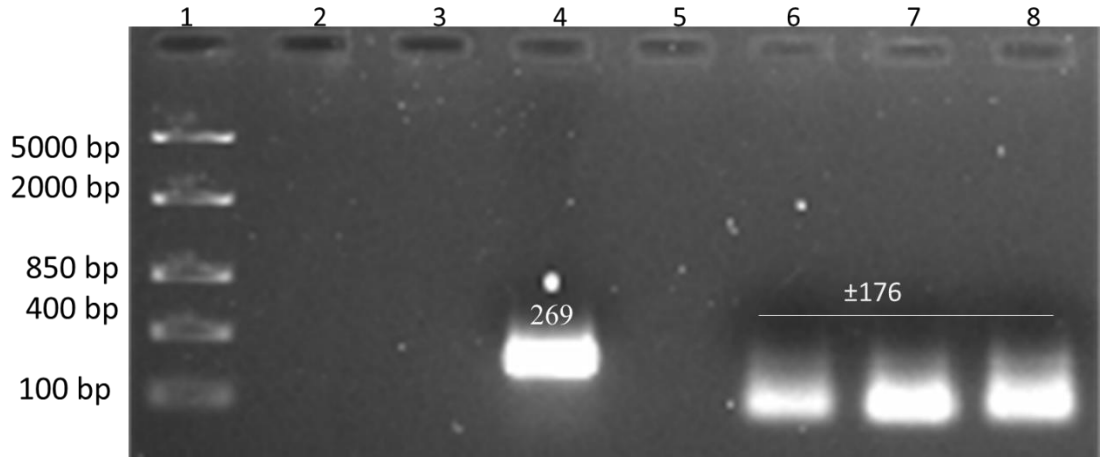

Figure 3.4: RT-PCR of the malate synthase gene. The synthesized cDNA was amplified with ZmT03 primers which flank the second intron in the maize genome where genomic DNA contamination shows an amplicon size of 275 bp which is close to the genomic size of the control banana actin gene. Lane 1: FastRuler medium range ladder, lane 2: negative template control with just the PCR master mix, lane 3: negative control with water as the template, lane 4: positive control with B73_10 genomic DNA as the template, (269 bp amplicon), lane 5: B73_1, 6 B73_7 and lane 7: B73_11 (176 bp amplicon).

Sequencing and alignment analysis

The RT-PCR product of the *ICL* and *MS* gene from the B73_7 cDNA was sequenced and the NCBI alignment data confirmed that the amplified products are indeed the respective genes as shown in Table 3.4. The *ICL* and *MS* consensus sequences (Appendix C) were further aligned using multiple sequence alignment tools to genomic DNA and cDNA sequence. This alignment gave the clear indication of where the respective primers, ZmICL and ZmMST03, bind to each sequence type shown in Figure 3.5 and Figure 3.6. The entire multiple sequence alignment is shown in Appendix D.

Table 3.4: The *Zea mays* isocitrate lyase and malate synthase predicted gene sequences

Gene name	Length (bp)[a]	Intron number	NCBI BLAST n search [b]	Pairwise alignment [c]			
			Best BLASTn match (accession number)	E value	Identity (%)	Similarity (%)	Gap (%)
ICL cDNA	2009	0	*Zea mays* full-length cDNA clone ZM_BFc0096E15 cds (BT040479.1)	0.0	99	99	0.0
ICL cDNA	2075		*Zea mays* isocitrate lyase (LOC103633249) , (NM_001301519.1)	0.0	99	99	0.0
ICL cDNA	2112	0	*Zea mays* clone 1532446 isocitrate lyase (EU955445.1)	0.0	99	99	0.0
ICL cDNA	2294	0	*Zea mays* full-length cDNA clone ZM_BFc0069F13 cds (BT085826.1)		99	97	0.0
MS cDNA	1098	0	*Zea mays* full-length cDNA clone ZM_BFc0057A12 mRNA, complete cds (BT066174.1)	3e-134	99	98	0.0
MS cDNA	2247	0	*Zea mays* PCO110469 mRNA sequence (AY108138.2)	3e-134	99	98	0.0
MS cDNA	1981	0	*Zea mays* malate synthase 1 (mas1) , mRNA (NM_001111845.1)		94	94	3
MS cDNA	2084	0	*Zea may* malate synthase 1 (mas 1), transcript variant X1, mRNA (XM_008670416.2)	4e-68	97	68	0.0

[a] The predicted coding DNA sequence. [b] Predicted *Zea mays ICL* or *MS* gene sequences used as the query sequences in the NCBI BLASTn search within the non-redundant nucleotide sequence (nr) database (E value). The best hit of the *Zea mays ICL* and *MS* genes subjected to a pairwise sequence alignment with identity, gaps and similarities percentages indicated.

The sequences were further analysed by alignment, where the genomic DNA (Reference), cDNA was aligned to the consensus sequence. Where the dash (-) is where the sequences do not align as they are not similar. The region where the sequences have similar is have a * which shows that there is a match at that position.

Figure 3.5: The isocitrate lyase multiple sequence alignment showing the position (in red) of the forward and reverse primers ICL (cutting across intron 2 shaded in grey) in the genomic, cDNA and consensus genome.

Figure 3.6: The malate multiple sequence alignment showing the position (in red) of the forward and reverse primers *MS* (flanking the intron 2 shaded in grey) in the genomic, cDNA and consensus genome.

3.4.2. Inoculation and harvesting of susceptible maize genotype at different disease progression stages (2017 glasshouse trial)

Studies showed a fold increase in the expression of the two glyoxylate pathway genes, isocitrate lyase and malate synthase, between maize genotypes that were susceptible and resistant to GLS at two-time points. Therefore, the study was done to determine how the expression of the glyoxylate pathway genes changed during GLS development and whether these genes could be detected. Leaf material that was inoculated with *C. zeina* was harvested and total RNA was extracted from it. Good quality and pure RNA was obtained for all the samples concentration and absorption ratio at 280/260 shown in Table 3.6 which are above 1.9 and clear bands for the 28S and 18S rRNA were visible from the denaturing by the gel electrophoresis image in Figure 3.7. The purified total RNA was used for cDNA synthesis and semi-qRTPCR analysis.

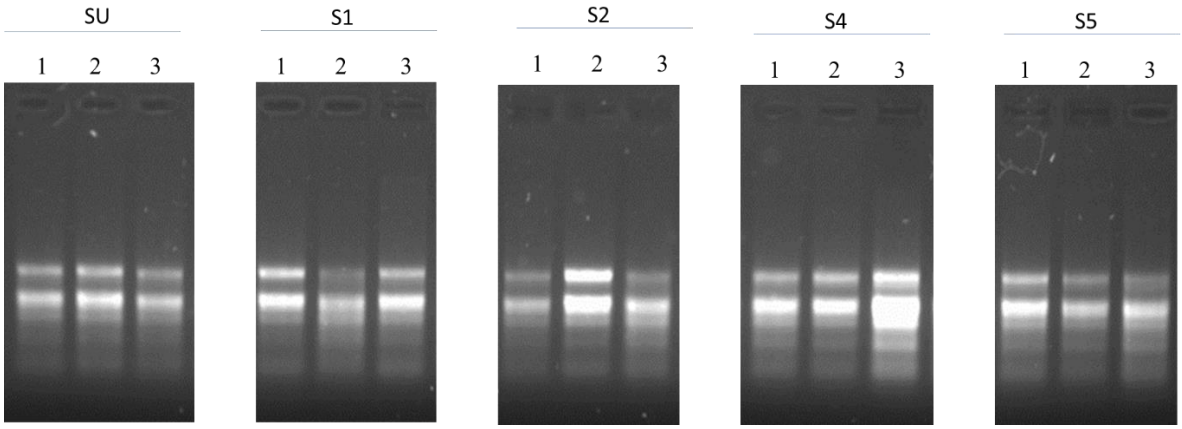

Figure 3.7: The integrity of RNA was evaluated by using denatured gel electrophoresis. Three biological replicates for the B73 maize genotype leaf samples in lanes 1-3 were included for each of the disease progression stages (SU, S1, S2, S4 and S5). The RNA was of good quality as it was intact for all the samples showing the 28S and 18S plant rRNA bands distinctly.

Reverse transcriptase Polymerase Chain Reaction on synthesized cDNA

The cDNA that was synthesized from the B73 maize genotype harvested at the different disease progression stages did not have gDNA contamination as, the cDNA sizes of the PCR products (SU, S1, S2, S4 and S5) and cDNA (B73_9, 10 and _11) amplified with banana actin shown in Figure 3.8 were less than 275 bp (gDNA PCR product). The pure DNA was further used for RT-PCR for *ICL* (Figure 3.9) and *MS* (Figure 3.10). The *ICL* RT-PCR had

strong primer dimers and the bands were too faint to quantify properly. There could be a possibility that there was not enough amount of the sample for the reaction thus, the quantification of the gene at different disease progression stages was measured on the *MS* gene only. The *ICL* gene needs re-optimisation for future studies (could not be done for this study due to time constraints) or other sensitive experiments such as quantitative PCR using SYBR green.

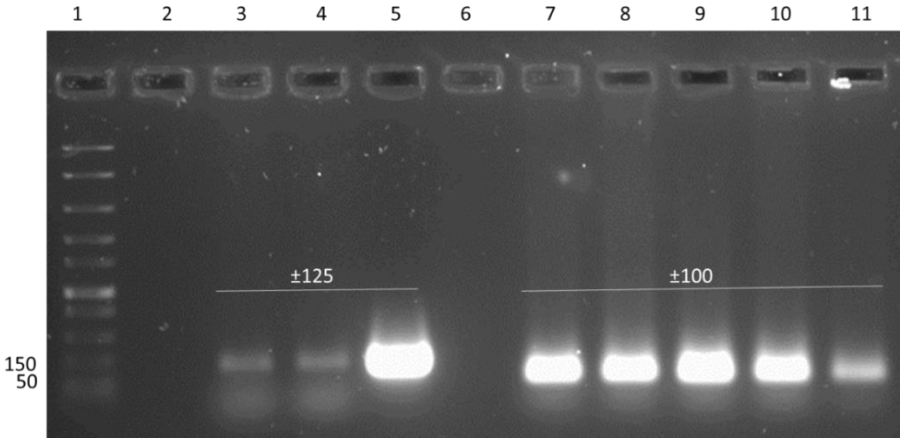

Figure 3.8: RT-PCR synthesized cDNA. The synthesized cDNA was amplified with *BA* primers which flank an intron in the maize genome to prove that there was no genomic DNA. Lane 1: NEB Fast DNA ladder, lane 2: negative control with water as the template, lane 3: B73_9 cDNA, lane 4: B73_10 cDNA, lane 5: B73_11 cDNA (125 bp), lane 7: SU_ cDNA, lane 8: S1_cDNA, 9 S2_cDNA, lane 10: S4_ cDNA and lane 11: S5_ cDNA (100 bp).

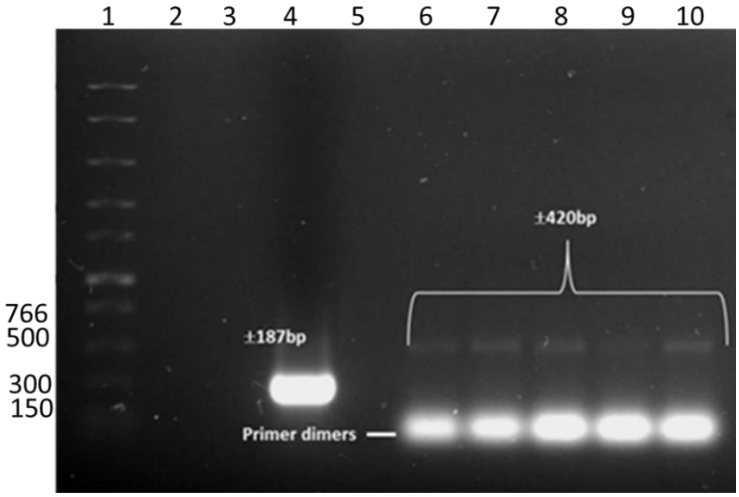

Figure 3.9: RT-PCR of the isocitrate lyase gene. The B73 maize genotype synthesized cDNA was amplified with ZmICL primers which cut across the second intron in the maize genome and genomic DNA contamination was not expected. Lane 1: NEB Fast DNA ladder, lane: 2 negative template control with just the PCR master mix, lane: 3 negative control with water as the template, lane: 4 positive control with B73_10 cDNA as the template amplified with banana actin, (187 bp amplicon), lane 6: SU_cDNA, lane 7: S1_cDNA, lane 8: S2 cDNA, lane 9: S4_cDNA and lane 10: S5_cDNA (420 bp amplicon).

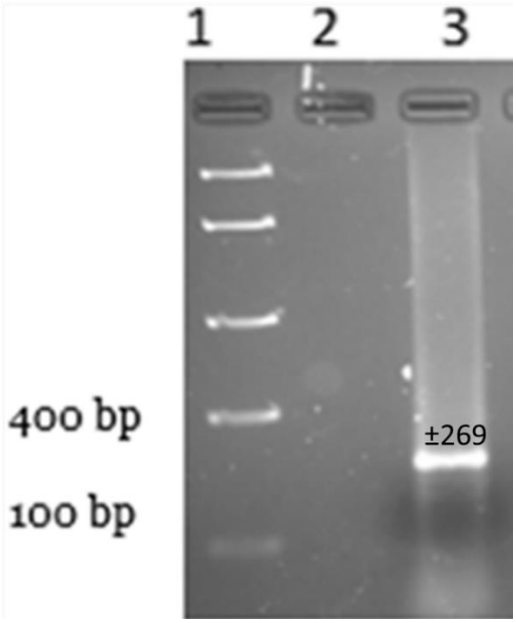

Figure 3.10: RT-PCR of malate synthase. The B73 maize genotype synthesized cDNA was amplified with the ZmMST03 primers which flank the second intron in the maize genome where genomic DNA contamination shows an amplicon size of 275 bp. Lane 1: FastRuler medium range ladder, lane 2: negative template control with just the PCR master mix and lane 3: cDNA amplified with ZmMST03. The *MS* was for the T02 transcript (269 bp) instead of the T03 transcript (169 bp).

3.4.3. Section 3: Semi-quantitative expression analysis of the malate synthase gene at the different disease progression stages (2017 glasshouse trial)

Banana actin and malate synthase fine-tuning

The optimal cycle for semi-qRTPCR had to be chosen from a range of cycles (15, 20, 25, 30 and 35) for the *BA* internal control, and *MS* (30, 31, 32, 33, 34 and 35) gene after amplifying them from the B73_S5 cDNA replicate 1 (diluted 1:10) samples. The optimal cycle for *BA* (Figure 3.11) was 30 and 34 or/ 35 *MS* (Figure 3.12).

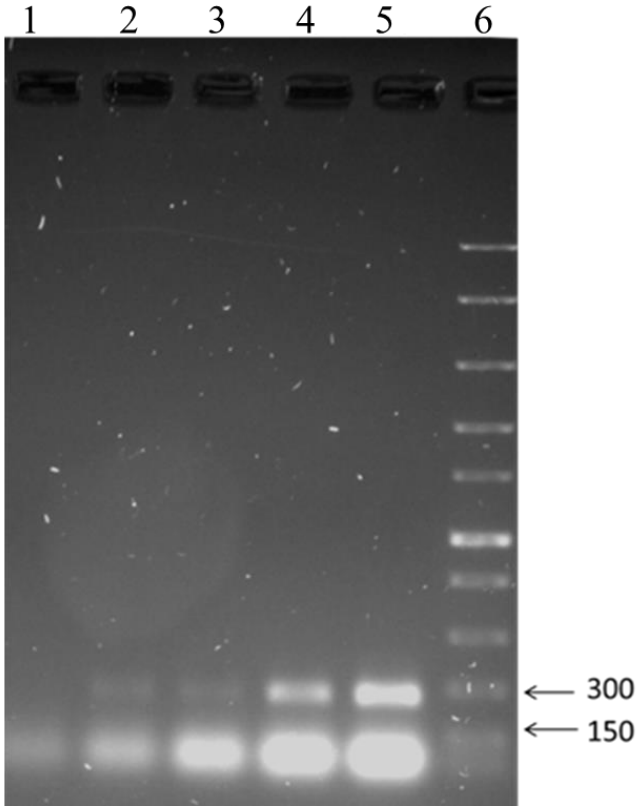

Figure 3.11: RT-PCR cycle optimisation of banana actin. The B73 maize genotype coalesced lesion sample leaf replicate cDNA was used to select the optimal cycle from a range of cycles. Lane 1: B73_S5 cDNA at cycle 15, lane 2: B73_S5 cDNA at cycle 20, lane 3: B73_S5 cDNA at cycle 25, lane 4: B73_S5 cDNA at cycle 30, lane 5: B73_S5 cDNA at cycle 35 and lane 6: NEB Fast DNA ladder. The optimal cycle was 30 for the *BA* gene.

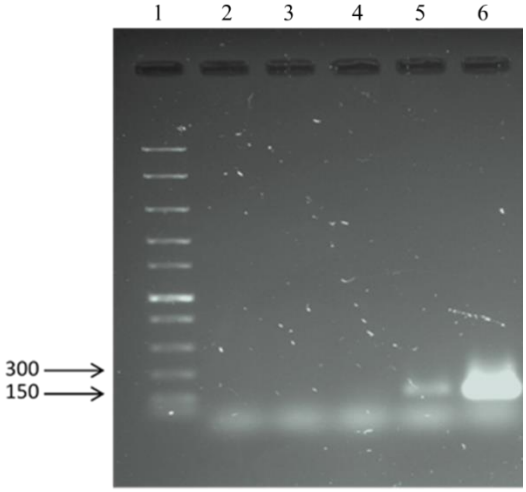

Figure 3.12: RT-PCR cycle optimisation of malate synthase. The B73 maize genotype coalesced lesion sample leaf replicate cDNA was used to select the optimal cycle from a range of cycles. lane 1: NEB Fast DNA ladder, lane 2: B73_S5 cDNA at cycle 31, lane 3: B73_S5 cDNA at cycle 32, lane 4: B73_S5 cDNA at cycle 33, lane 5: B73_S5 cDNA at cycle 34, lane 5: B73_S5 cDNA at cycle 35. The optimal cycle was 34 or/ 35 for the *MS* gene.

Banana actin and malate synthase RT-PCR for the different replicates at different disease progression stages

The optimal cycle for the semi-qRTPCR was chosen for the *BA* (Figure 3.13) internal control at 30 cycles. And, *MS* gene at 34 or/ 35 cycles for DEPC (Figure 3.14 and Figure 3.15) and nuclease free water (Figure 3.16 and Figure 3.17) diluted (1:10) samples. All *MS* diluted in the DEPC and nuclease water ran at 34 or 35 cycles had two transcripts of *MS* T02 (269 bp) and T03 (169 bp) except, *MS* diluted in DEPC and ran at 35 cycles. The concentration of EtBr was measured in each band for the replicate at different disease progression stages respectively with ImageJ tool and the expression of the *MS* gene was analysed.

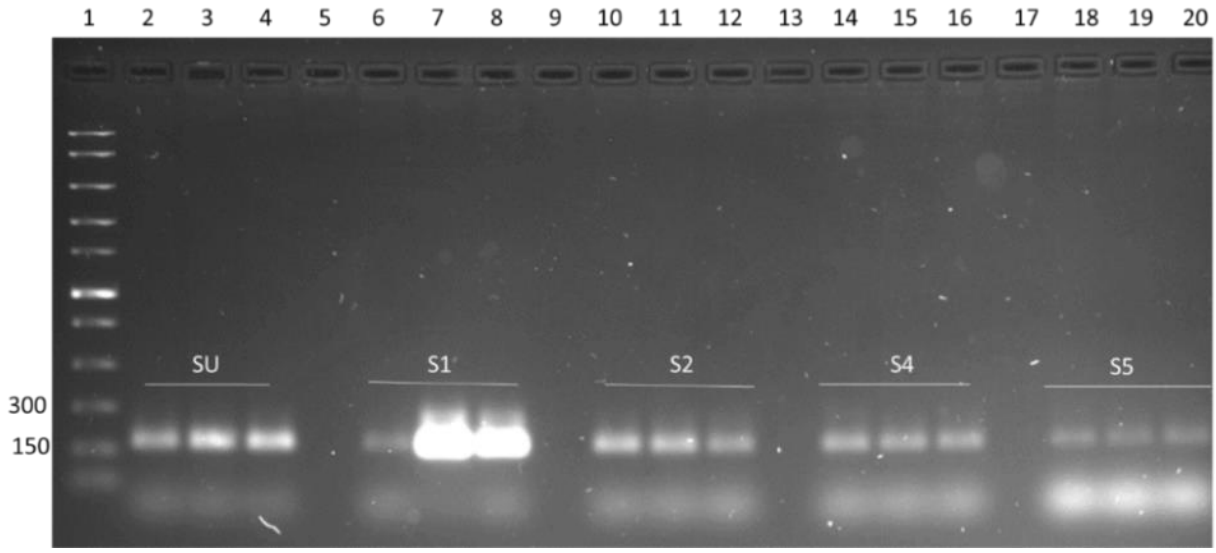

Figure 3.13: RT-PCR of banana actin at optimised cycle for semi-qRTPCR. The replicates of B73 maize genotype were amplified with the *BA* primers under respective optimal PCR previously described in 76.

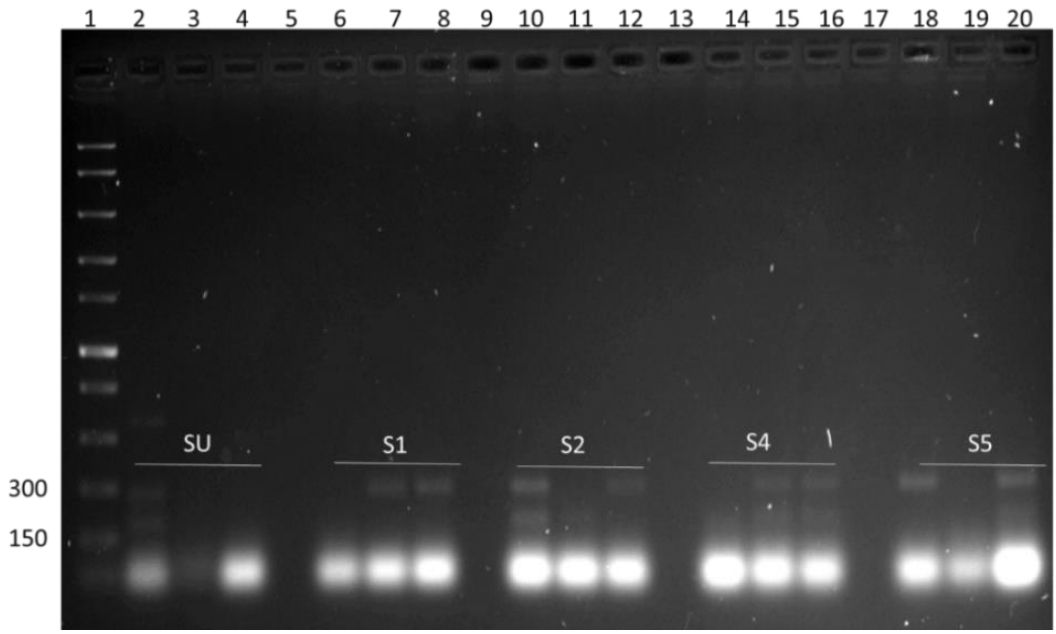

Figure 3.14: Reverse transcriptase polymerase chain reaction of the malate synthase diluted in DEPC (1:10) at optimised cycle for semi-qRTPCR. The replicates (1-3) were included for each of the disease progression stages (SU, S1, S2, S4 and S5) of the B73 maize genotype were amplified with the ZmMST03 primers for 34 cycles under respective optimal PCR conditions described previously described in 76. The *MS* had two transcripts T02 (269 bp) and T03 (169 bp).

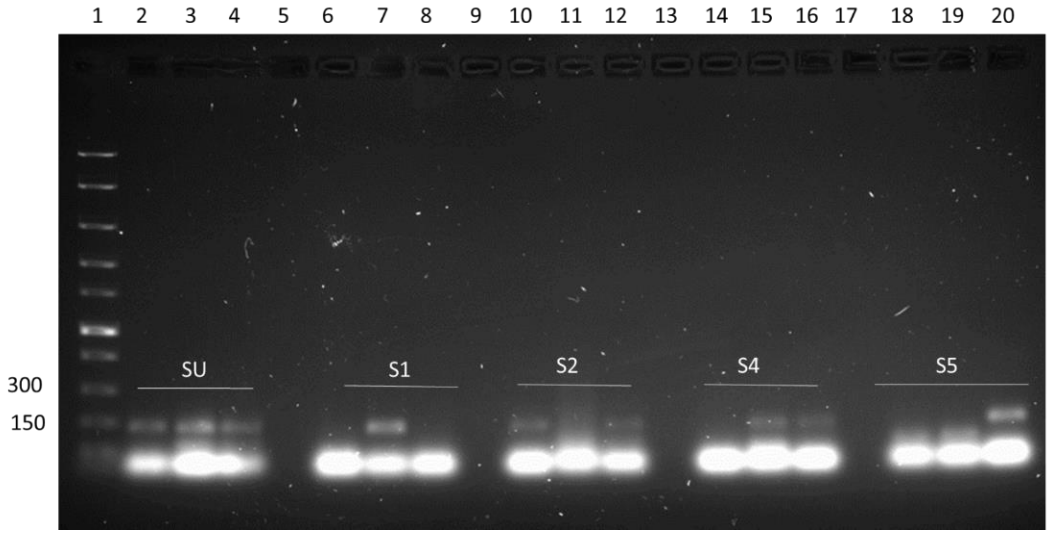

Figure 3.15: Reverse transcriptase polymerase chain reaction of the malate synthase diluted in DEPC (1:10) at optimised cycle for semi-qRTPCR. The replicates (1-3) were included for each of the disease progression stages (SU, S1, S2, S4 and S5) of the B73 maize genotype were amplified with the ZmMST03 for 35 cycles under respective optimal PCR conditions previously described in 76. The *MS* had only one transcript T03 (169 bp).

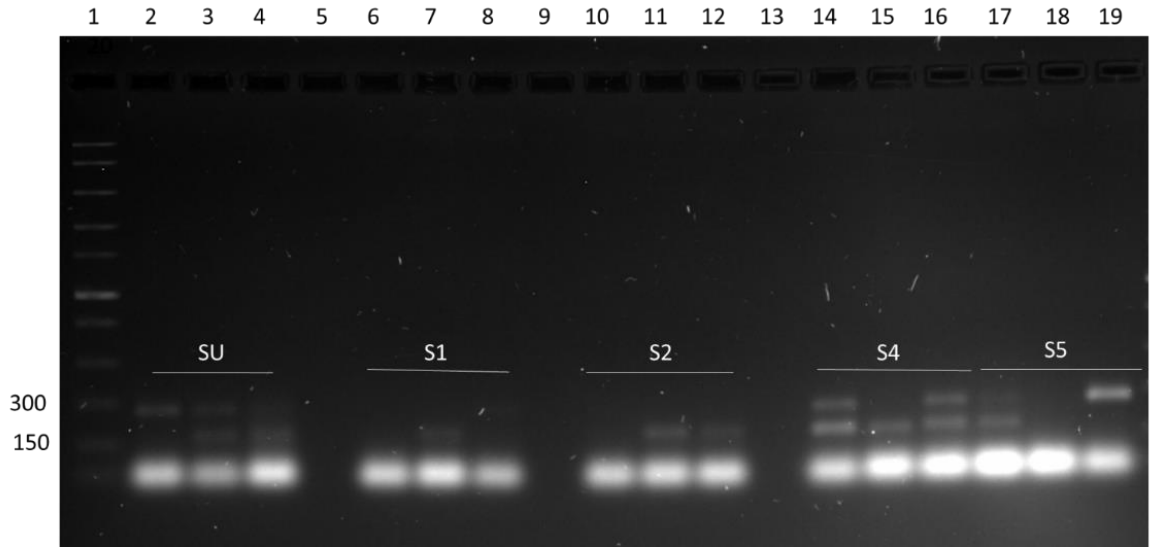

Figure 3.16: Reverse transcriptase polymerase chain reaction of the malate synthase diluted in nuclease free water (1:10) at optimised cycle for semi-qRTPCR. The replicates (1-3) were included for each of the disease progression stages (SU, S1, S2, S4 and S5) of the B73 maize genotype were amplified with the ZmMST03 for 34 cycles under respective optimal PCR conditions described on page 76. The *MS* had two transcripts T02 (269 bp) and T03 (169 bp).

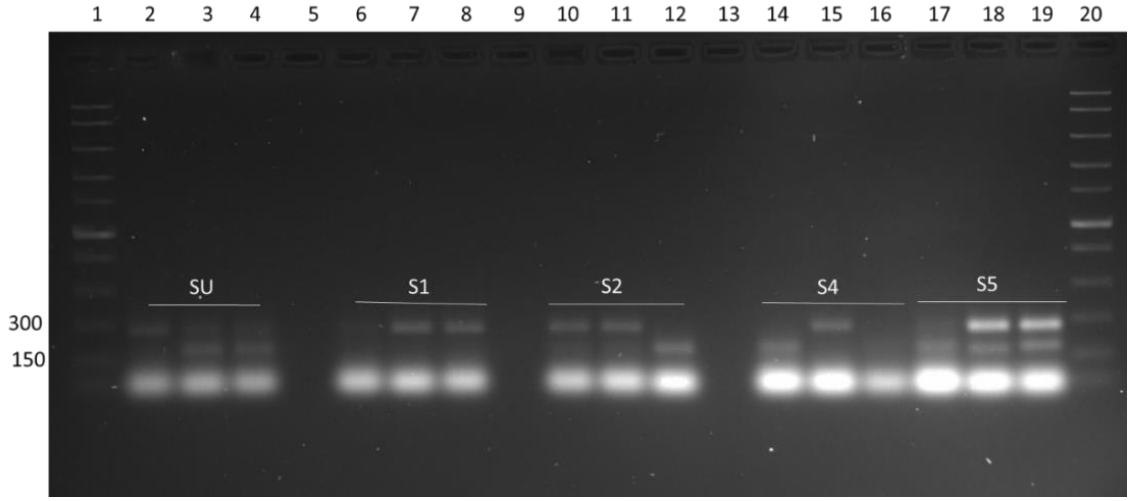

Figure 3.17: Reverse transcriptase polymerase chain reaction of malate synthase diluted in nuclease free water (1:10) at optimised cycle for semi-qRTPCR. The replicates (1-3) were included for each of the disease progression stages (SU, S1, S2, S4 and S5) of B73 maize genotype, and were amplified with ZmMST03 primers for 35 cycles under respective optimal PCR conditions described on page 76. The *MS* had two transcripts T02 (269 bp) and T03 (169 bp).

Semi-quantitative Reverse Transcriptase Polymerase chain reaction analysis for the malate synthase at different disease progression stages diluted in different solutions

The *MS* at the different disease progression stages (SU, S1, S2, S4 and S5) for the B73 genotype cDNA samples diluted in DEPC (Figure 3.18) and nuclease free water (Figure 3.19) were normalised against the internal gene, *BA*. The normalised expression values of the *MS* were then used to draw the bar graphs and the standard deviation was used to draw the error bars on each column. One-way ANOVA was used for statistical analysis with a Tukey's Multiple Comparison test on the diluted samples at the different amplification cycles. There was no statistical significance difference (α:0.05) in the expression of *MS* at the different GLS disease progression stages in all the transcripts at the different DEPC and nuclease free water at different cycle conditions (34 and 35). The reason for no significance could be due to the small sample size that was used in the study (only 3 biological replicates for each disease progression stage), as is shown in Appendix E, which shows all the samples with the semi-qRTPCR data.

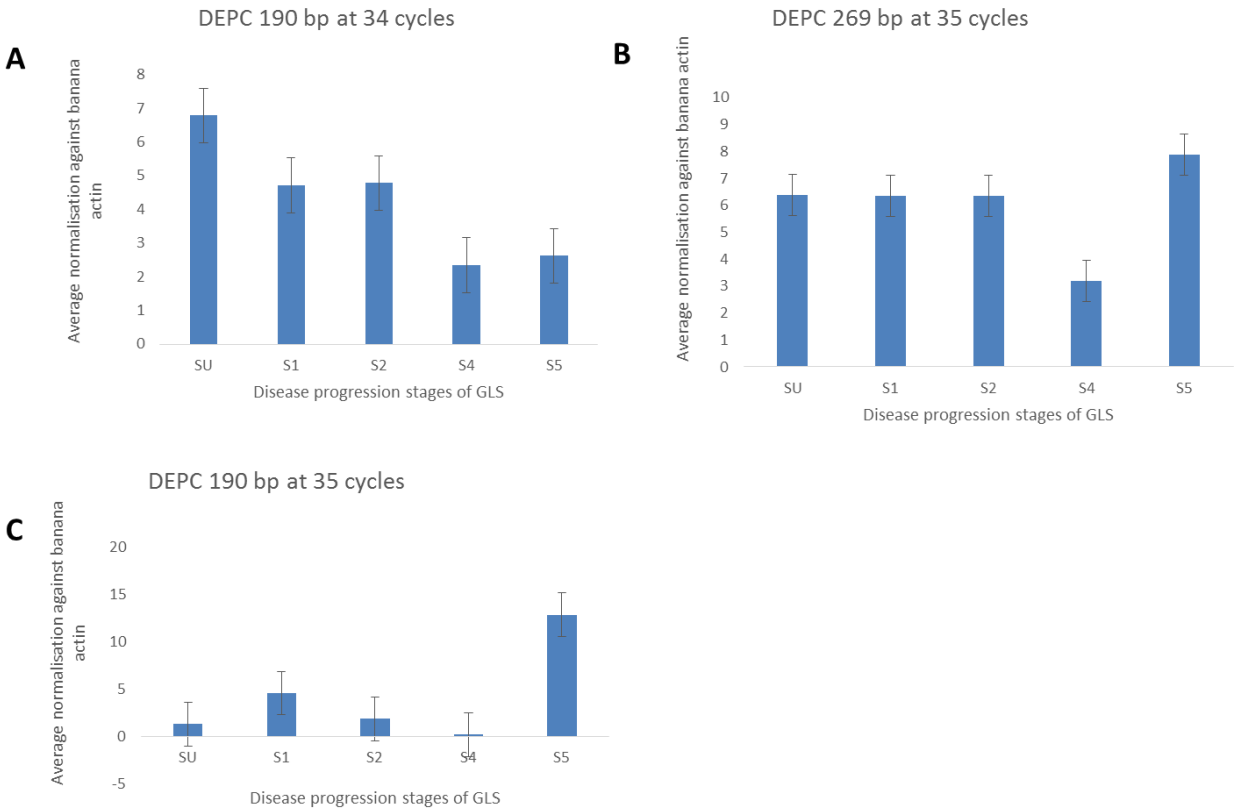

Figure 3.18: Malate synthase gene expressed in the B73 maize at different disease stages after glasshouse inoculation with *C. zeina* CMW 25467. Semi-qRTPCR was carried out on cDNA diluted in DEPC from maize collected at the different disease progression stages (SU, S1, S2, S4 and S5. The obtained data was then used to calculate the average normalised mean of the expression percentages of the *MS* against the stably expressed reference gene banana actin for (A) DEPC 269 bp *MS* ran at 34 cycles, (B) DEPC 269 bp *MS* ran at 35 cycles and (C) DEPC 190 bp *MS* ran at 35 cycles. The standard error bars are included in the graphs and the statistical analysis was done using one-way ANOVA analysis with a Tukey's and Dunn's Multiple Comparison test. There was no statistical significance difference (α:0.05) in the expression of *MS* at the different GLS disease progression stages.

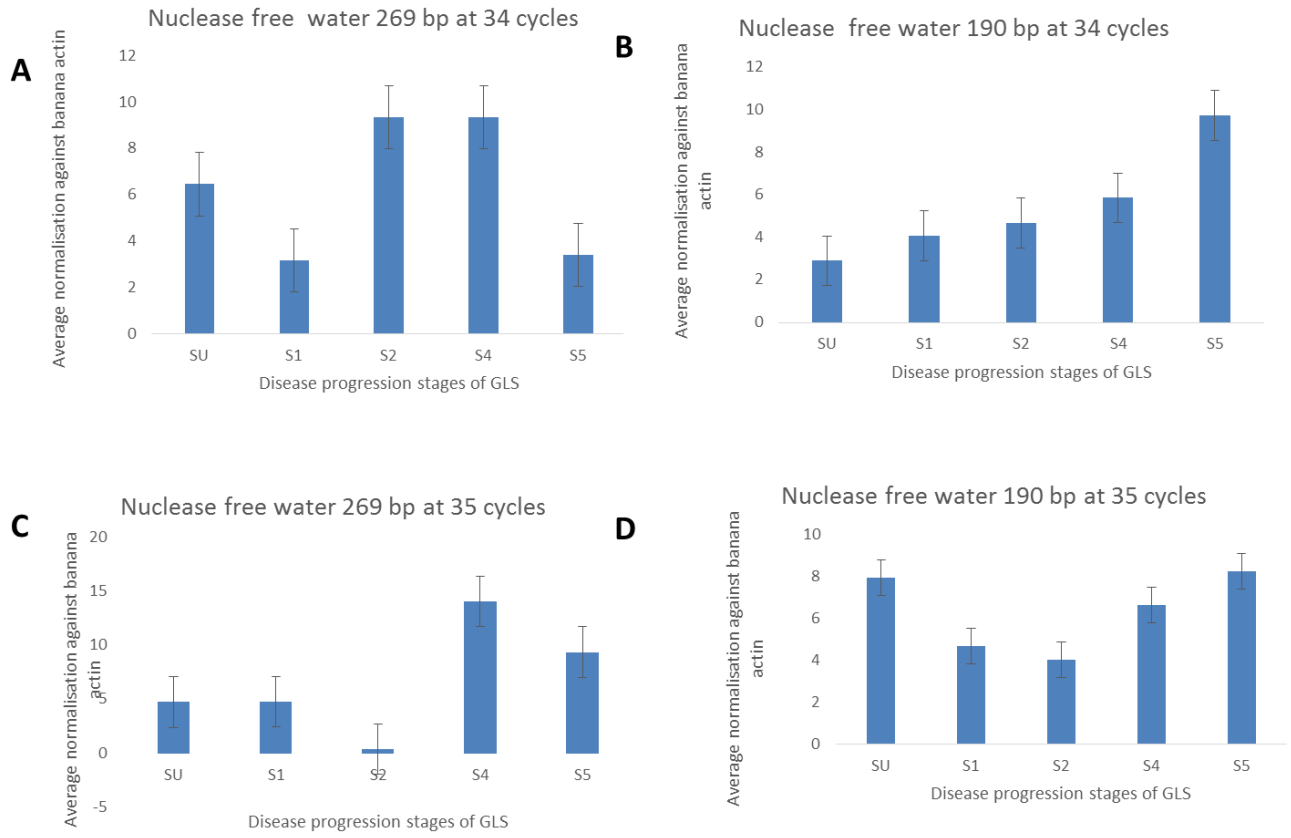

Figure 3.19: Malate synthase gene expressed in B73 maize at different disease stages after glasshouse inoculation with *C. zeina* CMW 25467 strain. Semi-qRTPCR was carried out on cDNA diluted in nuclease free water from maize collected at different disease progression stages (SU, S1, S2, S4 and S5. The obtained data was then used to calculate the average normalised mean of the expression percentages of the *MS* against the stably expressed reference gene banana actin for (A) nuclease free water 269 bp *MS* ran at 34 cycles, (B) nuclease free water 190 bp *MS* ran at 34 cycles, (C) nuclease free water 269 bp *MS* ran at 35 cycles and (D) nuclease free water 190 bp *MS* ran at 35 cycles. The standard error bars are included in the graphs and the statistical analysis was done using one-way ANOVA analysis with a Tukey's Multiple Comparison test. There was no statistical significance difference (α:0.05) in the expression of *MS* at the different GLS disease progression stages.

3.5. Discussion

Plant diseases caused by foliar fungal pathogens such as the grey leaf spot disease causing fungi, *C. zeina* species begin when conidial spores attach to host surfaces and germinate. This results in the formation of germ tubes which attach and penetrate the leaf surface through the stomata (Mendgen and Deising, 1993; Mendgen et al., 1996). A subdivision of fungal pathogens evolved specialized infection structures, appressorium, that can directly penetrate the plant cuticle (Dean, 1997). A study done by Wang et al. (2003) stated that the conidial germination and appressorium formation occur in a nutrient-free environment using lipid metabolism (Thines et al., 2000). Lipid metabolism may contribute to the generation of ATP and produces secondary metabolic pathways, which are an induction of the glyoxylate cycle to provide a mechanism of generating glucose (Idnurm and Howlett, 2002). The glyoxylate pathway genes are temporally expressed during infection of the host plant for germination, penetration and hyphae development (Wang et al., 2003). Thus, a differential expression of the genes, *ICL* and *MS*, was expected at different disease progression stages.

The expression levels of *ICL* could not be measured as the band intensities were too low as shown in Figure 3.9, since the expression was measured using the semi-qRTPCR technique which measures the amount of ethidium in each visible intense band. The expression of isocitrate lyase in other studies was found during conidial germination and appressorium formation, which indicated that the glyoxylate pathway was stimulated at that time (Solomon et al., 2003; Wang et al., 2003). Additionally, the isocitrate lyase gene was found to be highly expressed during the invasion of the hyphae (Bowyer et al., 2000). Inhibition studies of isocitrate lyase showed a decrease in fungal invasion and hyphae development (Idnurm and Howlett, 2002; Cheah et al., 2014). Overall, this means that the *ICL* is crucial for fungal pathogenicity.

Further studies of targeting the *ICL* in *C. zeina* or the maize genotype can be done and tested on the field. Additionally, a RNA sequencing study done by one of the Molecular and Plant Pathology Interaction Group, University of Pretoria (Unpublished) showed that the *MS* gene had a high fold increase together with the isocitrate lyase gene during which the inoculated and un-inoculated B73 maize genotypes were compared.

The expression levels of malate synthase increased at the different GLS disease progression stages compared to the un-inoculated B73 maize genotype as is shown in Figure 3.18 and Figure 3.19. This meant that as *C. zeina* was infecting, penetrating and invading the glyoxylate pathway way temporally activated. This can also be seen in studies of the *ICL*, also a glyoxylate pathway gene like the *MS*, is increased in expression, which could mean that the malate synthase is differentially expressed at different development stages of GLS shown in Figure 3.18 and Figure 3.19, however the expression is not statistically significant ($\alpha \geq 0.05$ instead of $\alpha \leq 0.05$) as proven by the ANOVA multiple comparison tests shown in Tables in Appendix E. However, there was a study showing that the malate gene can be triggered not only by the malate synthase enzyme but also by the NADP-ME which is crucial for the C_4 cereal crop defence against fungi, which is in contradiction to the possibility that *MS* is responsible for fungal pathogenicity (Lorenz and Fink, 2001).

The NADP-ME activity was examined in both healthy tissues taken from outside the lesions and from within the lesions themselves. The activity of the NADP-ME in lesions outside the tissues was 7.4 µmol m^{-2} s^{-1}, and the activity inside the lesion increased up to 12.6 µmol m^{-2} s^{-1}, approximately double the activity compared to the healthy tissues. Therefore, they associated this result with a need for the infected tissue to increase biosynthetic capacity to protect itself against the pathogen. The NADP-ME may further contribute to generate reductive power from stored (Pryke and Rees 1977). The study by Casati et al. (1999) concluded that the NADP-ME functions in mechanisms that produce NADPH. This will result in the formation of stimulated oxygen species which are responsible for killing or damaging pathogens and an increase in the levels of NADP-ME builds up energy for the biosynthesis of defence compounds, proposing that this *MS* metabolite functions in plant defence. A study can be further done to confirm the specific role of malate synthase and the relation it has to the NADP-ME enzyme and whether it will have a defence mechanism or induce *C. zeina* pathogenicity.

3.6. Conclusion

The total RNA from both glasshouse trials was successfully extracted from the B7 maize genotype. The cDNA that was synthesized from the B73 maize genotype had no genomic DNA contamination for both experimental trials. The *MS* and *ICL* genes were amplified and sequenced successfully with the expected size although the amplification of *ICL* was not intense enough, this needs to be improved in future studies where different sets of primers can be designed or dilutions of the primers with annealing conditions used. Therefore, the expression analysis was measured on the *MS* gene that was ran at different cycles and diluted in different solutions.

The hypothesis stated that the expression levels of *MS* at different disease progression stages would be differentially different compared to the untreated or un-inoculated B73 maize genotypes. On average, there was a difference in the expression of the gene was observed at the different stages (SU, S1, S2, S4 and S5) however, the expression was not significantly different. The p value was greater than 0.05, therefore we rejected to fail the null hypothesis, meaning that the expression of the glyoxylate pathway genes will remain the same between the leaf samples collected at the different disease progression stages compared to the B73 maize genotypes that were un-inoculated.

This result is due to the small sample size, since three biological replicates were used for each time point. A larger sample size with more biological replicates should be used in future studies. In the future, further research can be done to quantify the expression of the *MS* gene by using quantitative-RTPCR which is more stringent and uses SYBR green as the marker to detect expression of the gene. Southern Blots can also be used to determine the expression of the genes at different GLS disease progression stages. A study on the inhibition of the *ICL* or *MS* genes can also be done in the future to test for the antifungal effects it will have on B73 maize genotype. This study can be done on the model plant, *Nicotiana benthamiana*, which has been used in many studies for the plant-pathogen interactions. This will help understand the role of these metabolism genes in the pathogenicity of *C. zeina* and in case of *MS*, the role in maize defence.

3.7. References

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Appendix A

Table 3.5: RNA extraction concentration and purity check using Nanodrop spectrophotometry for leaf samples collected at two-time points namely 0 dpi (B73_9, 10 and 11) and 32 dpi (B73_1, 7 and 10).

#	Sample ID	Nucleic acid (ng/µl)	A260	A280	260/280	A260/230
1	B73_9	87,5	2,19	1,03	2,13	0,33
2	B73_10	55,4	1,39	0,66	2,1	0,24
3	B73_11	55,9	1,4	0,66	2,11	0,52
4	B73_1	115,2	2,88	1,39	2,07	1,83
5	B73_7	147,8	3,69	1,76	2,1	1,04
6	B73_8	38,3	0,96	0,46	2,08	0,91

A260: absorbance at 260 nm, A280: absorbance at 280 nm, 260/280: ratio of absorbance between 260 nm and 280 nm, 260/230: ration of absorbance at 260 nm and 230 nm.

Table 3.6: RNA extraction concentration and purity check using Nanodrop spectrophotometry for leaf samples collected at different disease progression stages namely 0 dpi (Uninoc) and 31 dpi (S1:no lesions, S2: chlorotic spots, S4: several lesions and S5: coalesced lesions).

#	Sample ID	Nucleic acid (ng/µl)	A260	A280	260/280	A260/230
1	B73-Uninoc-1	388,3	9,71	4,67	2,08	2,01
2	B73-Uninoc-2	389,8	9,74	4,67	2,09	2,02
3	B73-Uninoc-3	153,8	3,85	1,89	2,03	2,1
4	B73-S1-1	556,9	13,92	6,5	2,14	2,19
5	B73-S1-2	383,5	9,59	4,63	2,07	2,05
6	B73-S1-3	509,2	12,73	5,99	2,12	1,48
7	B73-S2-1	309,1	7,73	3,7	2,09	1,81
8	B73-S2-2	767,2	19,18	8,84	2,17	2,28
9	B73-S2-3	652,2	16,31	7,7	2,12	1,97
10	B73-S4-1	1070,9	26,77	12,49	2,14	2,27
11	B73-S4-2	700,4	17,51	8,14	2,15	1,84
12	B73-S4-3	538,7	13,47	6,32	2,13	2,08
13	B73-S5-1	739,4	18,49	8,54	2,17	2,09
14	B73-S5-2	716,2	17,9	8,35	2,14	2,28
15	B73-S5-3	379,4	9,48	4,49	2,11	2,03

A260: absorbance at 260 nm, A280: absorbance at 280 nm, 260/280: ratio of absorbance between 260 nm and 280 nm, 260/230: ration of absorbance at 260 nm and 230 nm.

Appendix B

Primer design and position for isocitrate lyase and malate synthase

Isocitrate lyase

The genomic DNA sequence (Maize Genome Database GRMZM2G056369 shown in Figure 3.20 was taken from NCBI GenBank (LOC103633249). The genomic DNA was aligned to the cDNA from the GenBank amino acid sequence (LOC103633249) shown in the supplementary information to design primers for isocitrate lyase. PerlPrimer version 1.1.21 design tool was used to design the primers for isocitrate lyase shown in Figure 3.20.

MaizeGDB code: GRMZM2G056369

NCBI gene code: LOC103633249

GRMZM2G056369 is between **1, 54226775** and **1,54231208** on Chromosome **7**

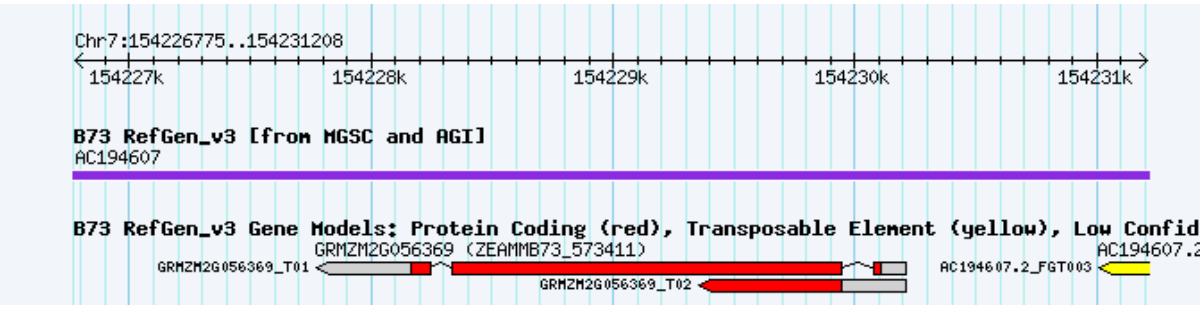


Figure 3.20: The illustration of isocitrate lyase gene from Maize Genome Database (GRMZMG056369) and the National Centre of Biotechnology Institute GenBank gene code (LOC103633249). The isocitrate lyase is found in chromosome 7 of the maize genome between 1, 54226775 and 1, 54231208 bases. The gene direction is from the flat side (5') to the sharp ended side (3'). The grey part indicated the untranslated regions of the gene, the red indicates the exon part of the gene (coding regions) and the lines between the exons indicate the introns. There are two transcripts of the isocitrate lyase gene namely, transcript 1 (T01) and transcript 2 (T02). T01 has 3 exons indicated in red, T02 has 1 exon. The T01 transcript was used to design primers.

The isocitrate lyase primer pair designed with PerlPrimer is 509 bp long including the intron length shown in However, the primers were designed on the cDNA which does not have an intron thus, the expected genomic DNA size is 0 and the cDNA size is 418 bp (509- 89 intron: 420 bp) shown in Table 3.7 and in the schematic diagram showing the introns in Figure 3.21.The position of the primers in relation to the genomic DNA and cDNA are shown in Figure 3.22.

Primer set 2 (product size=509)

Forward primer: 5'GAAGGGTTCTACCGCTTCAG 3'		Reverse primer: 5'TCTTCAGTAACTCCTTTGCCCA	
Tm: 60.59°C	Length: 20 bases	Tm: 61.82°C	Length: 22 bases
dS°: -434.63 eu	dH°: -156.20 kcal/mol	dS°: -462.46 eu	dH°: -166.10 kcal/mol
dG°37: -21.40 kcal/mol		dG°37: -22.67 kcal/mol	
GC% = 55		GC% = 45	

- dG= represents the quantity of energy needed to fully break a secondary DNA structure. The lower dG values (more negative values), the higher the quantity of energy needed to "separate" the DNA strands if a secondary structure
- dH and dS = It is for helix formation (more negative values the higher the quality of the primers should be higher than -5 kcal/mol)

Figure 3.21: Isocitrate lyase primer pair designed using PerlPrimer version 2.1.21 design tool. The primer was designed using the genomic DNA and cDNA obtained from NCBI (OC103633249. The designed primer was 509 base pairs long in length.

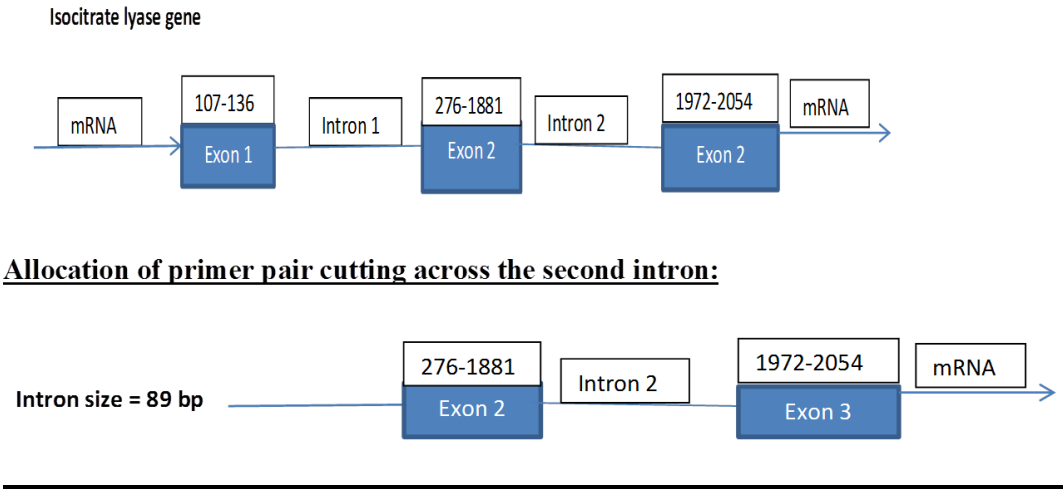


Figure 3.22: The schematic diagram showing the isocitrate lyase gene and the position of the exons and introns. The primer pair was designed to cut across the second intron which is 89 base pairs in length.

Table 3.7: Expected genomic DNA and cDNA product size using isocitrate lyase primer pair cutting across the second intron

Intron size	Genomic DNA size	Isocitrate lyase cDNA
91 base pairs	0 base pairs	418 base pairs

```
   1 ACGCAACCAAGGCACGCTTCGTACTTGCCATAGTTCGTCATCTCCAAGTGCGTGCCCTGG

  61 CCGCCCCCTATCCCGCCGGCCCTGTGTGTGTTCCCCGCTACCAGCCATGGCGTCGCCGTT
                                                  1
 121 CTCCGTGCCTTCGCTGGTCGGTTTCGATTTACTAGTTCTATAGACAGTTGCTAATGCTAG
                    30
 181 GCGCCTAGGCGTACGTGTGCATGATGATCTCGTACGTACGTAACTGATAATAACTCACTT

 241 ATTCGTCCCGTGTGGACGTGTGTGCATGCATGCAGATCATGGAGGAGGAAGGGCGGTTCG

 301 AGGCGGAGGTGGCGGAGGTGGAGTCGTGGTGGGGCACGGAGCGGTTCCGCCTCACCAAGC
     56
 361 GCCCCTACACCGCCCGCGACGTGGTCCTCCTCCGGGGCACGCTCCGGCAGAGCTACGCGT
     116
 421 CGGGGGAGATGGCCAAGAAGCTGTGGCGCACGCTCAAGGCGCACCAGGCCGGCGGCACCG
     176
 481 CGTCCCGCACCTTCGGCGCGCTCGACCCCGTCCAGGTGACCATGATGGCCAAGCACCTGG
     236
 541 ACACCATCTACGTCTCCGGGTGGCAGTGCTCGTCCACGCACACCTCCACCAACGAGCCCG
     296
 601 GCCCGGACCTCGCCGACTACCCCTACGACACCGTGCCAAACAAGGTGGAGCACCTCTTCT
     356
 661 TCGCGCAGCTCTACCACGACCGCAAGCAGCGGGAGGCGCGCATGTCGCTGCCCCGCGCCG
     416
 721 AGCGCGCGCGGGCGCCGTACGTCGACTTCCTCAAGCCCATCATCGCCGACGGCGACACGG
     476
 781 GCTTCGGCGGCGCCACGGCCACCGTCAAGCTCTGCAAGCTCTTCGTGGAGCGCGGGGCGG
     536
 841 CCGGGGTCCACCTCGAGGACCAGTCCTCCGTCACCAAGAAGTGCGGCCACATGGCGGGCA
     596
 901 AGGTGCTCGTCGCCGTCTCCGAGCACGTGAACCGCCTCGTCGCCGCGCGGCTCCAGTTCG
     656
 961 ACGTCATGGGCGTCGAGACCGTCCTCGTCGCGCGCACCGACGCCGTCGCCGCCACGCTCA
     716
1021 TCCAGACCAACGTCGACGCGCGCGACCACCAGTTCATAGTCGGCGCCACCAACCCGGGCC
     776
```

```
1081 TCAGGGGCCAGAGCCTCGCGGCCGTGCTCTCGGCCGGCATGTCGGCGGGCAAGAGCGGCA
     836
1141 GGGAGCTCCAGGCCATCGAGGACGAGTGGCTGGCCGCGGCGCAGCTCAAGACCTTCTCCG
     896
1201 AGTGCGTCAGGGACGCCATCGCGGGCCTCGGCGTCGCGGCCAAGGAGAAGCAGCGCAGGC
     956
1261 TCCAGGAGTGGGACCGCGCCACCGGCGGCTACGACAGGTGCGTGTCCAACGACCAGGCGC
     1016
1321 GCGACATCGCCGCCAGCCTCGGCGTCACGTCCGTGTTCTGGGACTGGGACCTGCCGCGGA
 1076 5'>>gaagggttctaccgcttcag>>3'FP2   within Exon 2
1381 CCAGGGAAGGGTTCTACCGCTTCAGGGGGTCCGTGGCGGCGGCCGTGGTCCGGGGGCGCG
    1136
1441 CCTTCGCGCCGCACGCCGACGTGCTGTGGATGGAGACGTCCAGCCCCAACGTGGCCGAGT
     1196
1501 GCACCGCCTTCTCCGAGGGCGTCAAGGCGGCGTGCCCCGAGGCGATGCTCGCCTACAACC
     1256
1561 TCTCGCCGTCCTTCAACTGGGACGCGTCCGGGATGACGGACGCCGAGATGGCCGCCTTCA
     1316
1621 TCCCGAGCGTCGCGCGCCTCGGCTACGTCTGGCAGTTCATCACGCTCGCCGGGTTCCACG
     1376
1681 CCGACGCGCTCGTCACCGACACGTTCGCGCGGGACTTCGCACGCCGCGGCATGCTGGCCT
     1436
1741 ACGTGGAGAGGATCCAGAGGGAGGAGAGGATCAACGGCGTGGAGACGCTGGAGCATCAGA
     1496
1801 AGTGGTCGGGCGCCAATTTCTACGACAGGGTGCTCAAGGCTGTGCAGGGTGGCATCTCCT
     1556

              Exon 2                Intron 2
1861 CGACTGCAGCCATGGGCAAAGGCAAAGTTCCTCATTTCCCAGCGTTTTTTTTTGTTTGG
     1616      3'<<ccgtttc      1636
               3' part of ICL R primer
5' CTCTTCAGTAACTC-CTTTGCC 3'

          Intron 2                                              Exon 3
1921 AAAAAAATAAACCATCGTTCGTACACAGTTTTGATGTTGTTTTGTTTACAGGAGTTACTG
```

Figure 3.23: The location of the isocitrate lyase forward and reverse primers in the genomic DNA and cDNA (coding DNA sequence) sequences. The introns and exons are shown where the yellow represents the exons and grey represents the introns. The CDS which is the DNA region coding for proteins is highlighted in green.

Malate Synthase

The genomic DNA sequence (Maize Genome Database GRMZM2G094328 shown in Figure 3.24 was taken from NCBI GenBank (LOC542252). The genomic DNA was aligned to the cDNA from the GenBank amino acid sequence (LOC542252) shown in the supplementary information to design primers for malate synthase. Primer Designer 4 tool was used to design the primers for malate synthase shown in Figure 3.25.

MaizeGDB code: GRMZM2G102183
NCBI gene code: LOC542252
GRMZM2G094328 is between **9,547,650** and **9,549,271** on Chromosome **2**

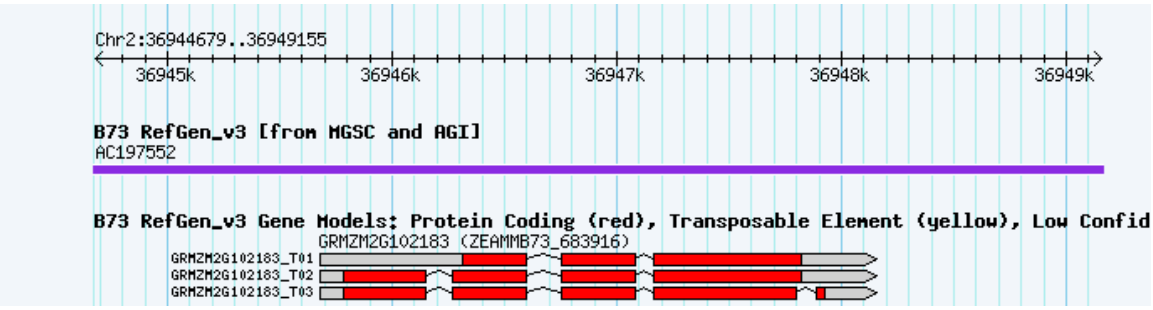
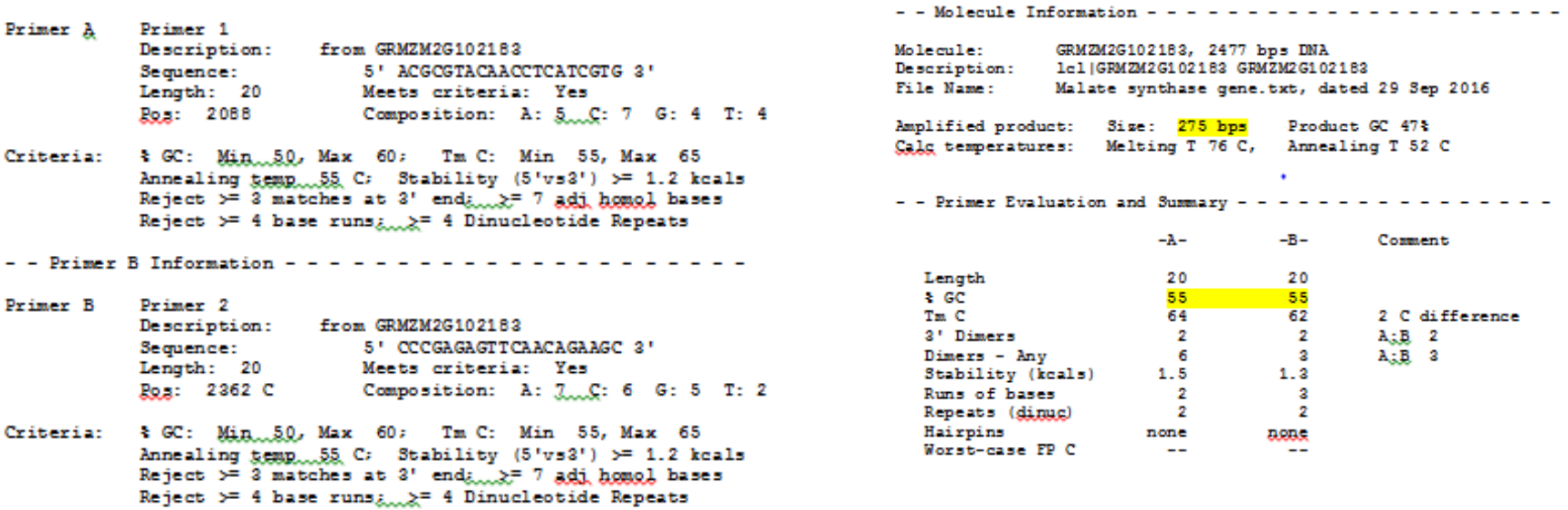


Figure 3.24: The illustration of malate synthase gene from Maize Genome Database (GRMZM2G094328) and the National Centre of Biotechnology Institute GenBank gene code (LOC542252). The malate synthase is found in chromosome 2 of the maize genome between 9,547,650 and 9,549,271 bases. The gene direction is from the flat side (5') to the sharp ended side (3'). The grey part indicated the untranslated regions of the gene, the red indicates the exon part of the gene (coding regions) and the lines between the exons indicate the introns. There are three transcripts of the malate synthase gene namely, transcript 1 (T01), transcript 2 (T02) and transcript 3 (T03). T01 has 3 exons indicated in red, T02 has 4 exons and T0 has 5 exons. The T03 transcript was used to design primers

The malate synthase primer pair designed with Primer Designer 4 is 275 base pairs (bp) long including the intron length shown in Figure 3.25. The expected genomic DNA size is 275 base pairs and the cDNA size is 186 bp (275-89 intron: 186 bp) shown in Table 3.8 and in the schematic diagram showing the introns in Figure 3.26. The position of the primers in relation to the genomic DNA and cDNA are shown in Figure 3.27.



```
Primer A    Primer 1
            Description:    from GRMZM2G102183
            Sequence:           5' ACGCGTACAACCTCATCGTG 3'
            Length:  20         Meets criteria:  Yes
            Pos:  2088          Composition:  A: 5  C: 7  G: 4  T: 4

Criteria:   % GC:  Min  50, Max  60;   Tm C:  Min  55, Max  65
            Annealing temp  55 C;  Stability (5'vs3') >= 1.2 kcals
            Reject >= 3 matches at 3' end;  >= 7 adj homol bases
            Reject >= 4 base runs;  >= 4 Dinucleotide Repeats

- - Primer B Information - - - - - - - - - - - - - - - - - - - - -

Primer B    Primer 2
            Description:    from GRMZM2G102183
            Sequence:           5' CCCGAGAGTTCAACAGAAGC 3'
            Length:  20         Meets criteria:  Yes
            Pos:  2362 C        Composition:  A: 7  C: 6  G: 5  T: 2

Criteria:   % GC:  Min  50, Max  60;   Tm C:  Min  55, Max  65
            Annealing temp  55 C;  Stability (5'vs3') >= 1.2 kcals
            Reject >= 3 matches at 3' end;  >= 7 adj homol bases
            Reject >= 4 base runs;  >= 4 Dinucleotide Repeats
```

```
- - Molecule Information - - - - - - - - - - - - - - - - - - - - -

Molecule:       GRMZM2G102183, 2477 bps DNA
Description:    lcl|GRMZM2G102183 GRMZM2G102183
File Name:      Malate synthase gene.txt, dated 29 Sep 2016

Amplified product:   Size:  275 bps    Product GC 47%
Calc temperatures:   Melting T 76 C,   Annealing T 52 C

- - Primer Evaluation and Summary - - - - - - - - - - - - - - - -

                          -A-          -B-       Comment

  Length                   20           20
  % GC                     55           55
  Tm C                     64           62       2 C difference
  3' Dimers                 2            2       A:B  2
  Dimers - Any              6            3       A:B  3
  Stability (kcals)       1.5          1.3
  Runs of bases             2            3
  Repeats (dinuc)           2            2
  Hairpins               none         none
  Worst-case FP C          --           --
```

Figure 3.25: Malate synthase primer pair designed using Primer Designer 4 tool. The primer was designed using the genomic DNA and cDNA obtained from NCBI (LOC542252). The designed primer was 275 base pairs long in length with a melting temperature of 64°C for the forward and 62°C for the reverse primer.

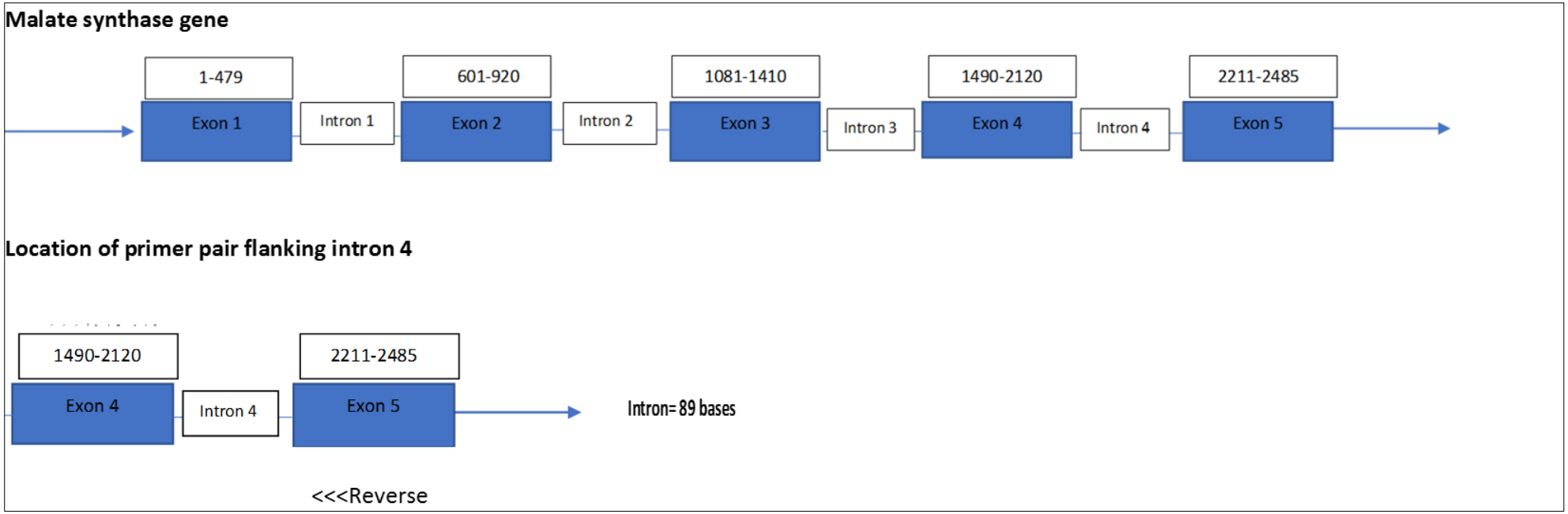


Figure 3.26: The schematic diagram showing the malate synthase gene and the position of the exons and introns. The primer pair was designed to cut across the second intron which is 89 base pairs in length.

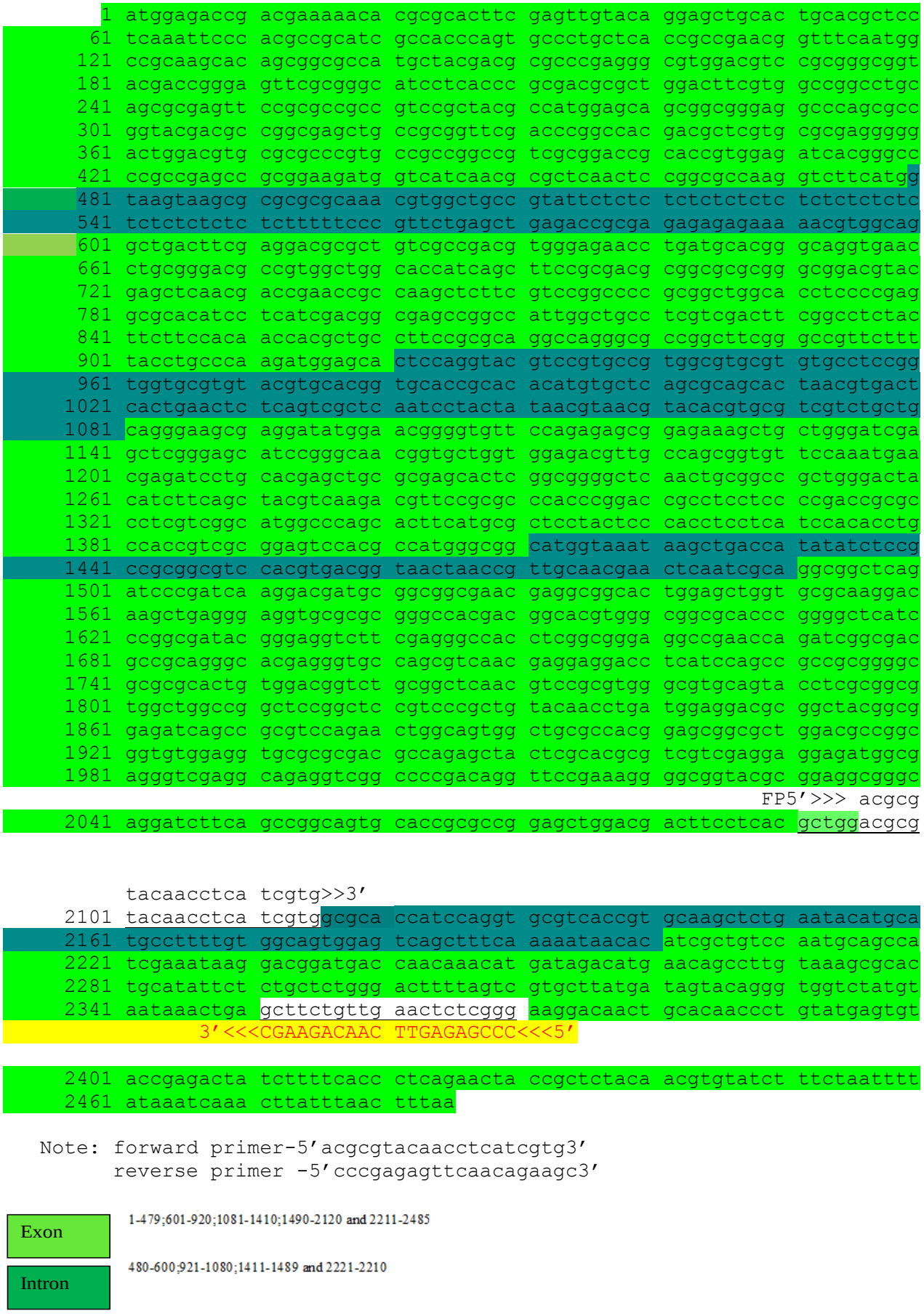


Figure 3.27: The location of the malate synthase forward and reverse primers in the genomic DNA and cDNA sequences. The introns and exons are shown, where the dark green represents the exons and light green represents the introns. The primers designed will attach to the underlined region of the sequence.

Table 3.8: Expected genomic DNA and cDNA product size using malate primer pair flanking the forth intron.

Intron size	Genomic DNA size	Malate synthase cDNA
89 base pairs	275 base pairs	186 base pairs

Appendix C

>ICL_FP_&_RP
AAGGGTTCTACCGCTTCAGGGGGTCCGTGGCGGCGGCCGTGGTCCGGGGG
CGCGCCTTCGCGCCGCACGCCGACGTGCTGTGGATGGAGACGTCCAGCCC
CAACGTGGCCGAGTGCACCGCCTTCTCCGAGGGCGTCAAGGCGGCGTGCC
CCGAGGCGATGCTCGCCTACAACCTCTCGCCGTCCTTCAACTGGGACGCG
TCCGGGATGACGGACGCCGAGATGGCCGCCTTCATCCCGAGCGTCGCGCG
CCTCGGCTACGTCTGGCAGTTCATCACGCTCGCCGGGTTCCACGCCGACG
CGCTCGTCACCGACACGTTCGCGCGGGACTTCGCACGCCGCGGCATGCTG
GCCTACGTGGAGAGGATCCAGAGGGAGGAGAGGATCAACGGCGTGGAGAC
GCTGGAGCATCAGAAGTGGTCGGGCGCCAATTTCTACGACAGGGTGCTCA
AGGCTGTGCAGGGTGGCATCTCCTCGACTGCAGCCATGGGCAAAGGAGTT
ACTGAAGAGA

>MS_FP_&_RP
AGTTTACGCGTACAACCTCATCGTGGCGCACCATCCAGGTGCGTCACCGT
GCAAGCTCTGAATACATGCATGCCTTTTGTGGCAGTGGAGTCAGCTTTCA
AAAATAACACATCGCTGTCCAATGCAGCCATCGAAATAAGGACGGATGAC
CAACAAACATGATAGACATGAACAGCCTTGTAAAGCGCACTGCAAATTCT
CGGGTTTGGGACTTTTAGTCGTGCTTATGATAGTACAGGGTGGTCTATGT
AATAAACTGAGCTTCTGTTGAACTCTCGGGA

Appendix D

Multiple sequence alignment of isocitrate lyase and malate synthase using Clustal Omega Multiple Sequence alignment tool:

1. Isocitrate lyase alignment

```
CLUSTAL O(1.2.4) multiple sequence alignment Isocitrate lyase

ICL_REF            ACGCAACCAAGGCACGCTTCGTACTTGCCATAGTTCGTCATCTCCAAGTGCGTGCCCTGG     60
ICL cDNA           ------------------------------------------------------------     0
ICL_ZIMBILI CDNA   ------------------------------------------------------------     0
                                                 Start codon of ICL     Exon 1
ICL_REF            CCGCCCCCTATCCCGCCGGCCCTGTGTGTGTTCCCCGCTACCAGCCATGGCGTCGCCGTT     120
ICL_CDNA           ------------------------------------------------------------     0
ICL_Zimbili cDNA   ------------------------------------------------------------     0

ICL_REF            CTCCGTGCCTTCGCTGGTCGGTTTCGATTTACTAGTTCTATAGACAGTTGCTAATGCTAG     180
ICL_CDNA           ------------------------------------------------------------     0
ICL_Zimbili cDNA   ------------------------------------------------------------     0

ICL_REF            GCGCCTAGGCGTACGTGTGCATGATGATCTCGTACGTACGTAACTGATAATAACTCACTT     240
ICL_CDNA           ------------------------------------------------------------C    1
ICL_ZIMBILI CDNA   ------------------------------------------------------------     0

ICL_REF             ATTCGTCCCGTGTGGACGTGTGTGCATGCATGCAGATCATGGAGGAGGAAGGGCGGTTCG     300
ICL_CDNA            CAGCCATGGCGTCGCCGTTCTCCGTGCCTTCGCTGATCATGGAGGAGGAAGGGCGGTTCG     61
ICL_Zimbili cDNA    ------------------------------------------------------------     0
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ICL_REF           AGGCGGAGGTGGCGGAGGTGGAGTCGTGGTGGGGCACGGAGCGGTTCCGCCTCACCAAGC     360
ICL_CDNA          AGGCGGAGGTGGCGGAGGTGGAGTCGTGGTGGGGCACGGAGCGGTTCCGCCTCACCAAGC     121
ICL_Zimbili cDNA  ------------------------------------------------------------     0

ICL_REF           GCCCCTACACCGCCCGCGACGTGGTCCTCCTCCGGGGCACGCTCCGGCAGAGCTACGCGT     420
ICL_CDNA          GCCCCTACACCGCCCGCGACGTGGTCCTCCTCCGGGGCACGCTCCGGCAGAGCTACGCGT     181
ICL_Zimbili cDNA  ------------------------------------------------------------     0

ICL_REF           CGGGGGAGATGGCCAAGAAGCTGTGGCGCACGCTCAAGGCGCACCAGGCCGGCGGCACCG     480
ICL_CDNA          CGGGGGAGATGGCCAAGAAGCTGTGGCGCACGCTCAAGGCGCACCAGGCCGGCGGCACCG     241
ICL_Zimbili cDNA  ------------------------------------------------------------     0

ICL_REF           CGTCCCGCACCTTCGGCGCGCTCGACCCCGTCCAGGTGACCATGATGGCCAAGCACCTGG     540
ICL_CDNA          CGTCCCGCACCTTCGGCGCGCTCGACCCCGTCCAGGTGACCATGATGGCCAAGCACCTGG     301
ICL_Zimbili cDNA  ------------------------------------------------------------     0

ICL_REF           ACACCATCTACGTCTCCGGGTGGCAGTGCTCGTCCACGCACACCTCCACCAACGAGCCCG     600
ICL_CDNA          ACACCATCTACGTCTCCGGGTGGCAGTGCTCGTCCACGCACACCTCCACCAACGAGCCCG     361
ICL_ZIMBILI CDNA  ------------------------------------------------------------     0

ICL_REF           GCCCGGACCTCGCCGACTACCCCTACGACACCGTGCCAAACAAGGTGGAGCACCTCTTCT     660
ICL_CDNA          GCCCGGACCTCGCCGACTACCCCTACGACACCGTGCCAAACAAGGTGGAGCACCTCTTC-     420
ICL_ZIMBILI CDNA  ------------------------------------------------------------     0

ICL_REF           TCGCGCAGCTCTACCACGACCGCAAGCAGCGGGAGGCGCGCATGTCGCTGCCCCGCGCCG     720
ICL_CDNA          TCGCGCAGCTCTACCACGACCGCAAGCAGCGGGAGGCGCGCATGTCGCTGCCCCGCGCCG     480
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ICL_ZIMBILI CDNA    ------------------------------------------------------------     0

ICL_REF             AGCGCGCGCGGGCGCCGTACGTCGACTTCCTCAAGCCCATCATCGCCGACGGCGACACGG     780
ICL_CDNA            AGCGCGCGCGGGCGCCGTACGTCGACTTCCTCAAGCCCATCATCGCCGACGGCGACACGG     540
ICL_ZIMBILI CDNA    ------------------------------------------------------------     0

ICL_REF             GCTTCGGCGGCGCCACGGCCACCGTCAAGCTCTGCAAGCTCTTCGTGGAGCGCGGGGCGG     840
ICL_CDNA            GCTTCGGCGGCGCCACGGCCACCGTCAAGCTCTGCAAGCTCTTCGTGGAGCGCGGGGCGG     600
ICL_ZIMBILI CDNA    ------------------------------------------------------------     0

ICL_REF             CCGGGGTCCACCTCGAGGACCAGTCCTCCGTCACCAAGAAGTGCGGCCACATGGCGGGCA     900
ICL_CDNA            CCGGGGTCCACCTCGAGGACCAGTCCTCCGTCACCAAGAAGTGCGGCCACATGGCGGGCA     660
ICL_ZIMBILI CDNA    ------------------------------------------------------------     0

ICL_REF             AGGTGCTCGTCGCCGTCTCCGAGCACGTGAACCGCCTCGTCGCCGCGCGGCTCCAGTTCG     960
ICL_CDNA            AGGTGCTCGTCGCCGTCTCCGAGCACGTGAACCGCCTCGTCGCCGCGCGGCTCCAGTTCG     720
ICL_ZIMBILI CDNA    ------------------------------------------------------------     0

ICL_REF             ACGTCATGGGCGTCGAGACCGTCCTCGTCGCGCGCACCGACGCCGTCGCCGCCACGCTCA    1020
ICL_CDNA            ACGTCATGGGCGTCGAGACCGTCCTCGTCGCGCGCACCGACGCCGTCGCCGCCACGCTCA     780
ICL_ZIMBILI CDNA    ------------------------------------------------------------     0

ICL_REF             TCCAGACCAACGTCGACGCGCGCGACCACCAGTTCATAGTCGGCGCCACCAACCCGGGCC    1080
ICL_CDNA            TCCAGACCAACGTCGACGCGCGCGACCACCAGTTCATAGTCGGCGCCACCAACCCGGGCC     840
ICL_ZIMBILI CDNA    ------------------------------------------------------------     0
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ICL_REF           TCAGGGGCCAGAGCCTCGCGGCCGTGCTCTCGGCCGGCATGTCGGCGGGCAAGAGCGGCA     1140
ICL_CDNA          TCAGGGGCCAGAGCCTCGCGGCCGTGCTCTCGGCCGGCATGTCGGCGGGCAAGAGCGGCA     900
ICL_ZIMBILI CDNA  ------------------------------------------------------------     0

ICL_REF           GGGAGCTCCAGGCCATCGAGGACGAGTGGCTGGCCGCGGCGCAGCTCAAGACCTTCTCCG     1200
ICL_CDNA          GGGAGCTCCAGGCCATCGAGGACGAGTGGCTGGCCGCGGCGCAGCTCAAGACCTTCTCCG     960
ICL_ZIMBILI CDNA  ------------------------------------------------------------     0

ICL_REF           AGTGCGTCAGGGACGCCATCGCGGGCCTCGGCGTCGCGGCCAAGGAGAAGCAGCGCAGGC     1260
ICL_CDNA          AGTGCGTCAGGGACGCCATCGCGGGCCTCGGCGTCGCGGCCAAGGAGAAGCAGCGCAGGC     1020
ICL_ZIMBILI CDNA  ------------------------------------------------------------     0

ICL_REF           TCCAGGAGTGGGACCGCGCCACCGGCGGCTACGACAGGTGCGTGTCCAACGACCAGGCGC     1320
ICL_CDNA          TCCAGGAGTGGGACCGCGCCACCGGCGGCTACGACAGGTGCGTGTCCAACGACCAGGCGC     1080
ICL_ZIMBILI CDNA  ------------------------------------------------------------     0

ICL_REF           GCGACATCGCCGCCAGCCTCGGCGTCACGTCCGTGTTCTGGGACTGGGACCTGCCGCGGA     1380
ICL_CDNA          GCGACATCGCCGCCAGCCTCGGCGTCACGTCCGTGTTCTGGGACTGGGACCTGCCGCGGA     1140
ICL_ZIMBILI CDNA  ------------------------------------------------------------     0
            ICL2 FP 5' GAAGGGTTCTACCGCTT 3' within Exon 2
ICL_REF           CCAGGGAAGGGTTCTACCGCTTCAGGGGGTCCGTGGCGGCGGCCGTGGTCCGGGGGCGCG     1440
ICL_CDNA          CCAGGGAAGGGTTCTACCGCTTCAGGGGGTCCGTGGCGGCGGCCGTGGTCCGGGGGCGCG     1200
ICL_ZIMBILI CDNA  -----AAGGGTTTCTACCGCTTCAGGGGGTCCGTGGCGGCGGCCGTGGTCCGGGGGCGCG     55
                        * ** **************************************************
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ICL_REF            CCTTCGCGCCGCACGCCGACGTGCTGTGGATGGAGACGTCCAGCCCCAACGTGGCCGAGT     1500
ICL_CDNA           CCTTCGCGCCGCACGCCGACGTGCTGTGGATGGAGACGTCCAGCCCCAACGTGGCCGAGT     1260
ICL_ZIMBILI CDNA   CCTTCGCGCCGCACGCCGACGTGCTGTGGATGGAGACGTCCAGCCCCAACGTGGCCGAGT     115
                   ************************************************************

ICL_REF            GCACCGCCTTCTCCGAGGGCGTCAAGGCGGCGTGCCCCGAGGCGATGCTCGCCTACAACC     1560
ICL_CDNA           GCACCGCCTTCTCCGAGGGCGTCAAGGCGGCGTGCCCCGAGGCGATGCTCGCCTACAACC     1320
ICL_ZIMBILI CDNA   GCACCGCCTTCTCCGAGGGCGTCAAGGCGGCGTGCCCCGAGGCGATGCTCGCCTACAACC     175
                   ************************************************************

ICL_REF            TCTCGCCGTCCTTCAACTGGGACGCGTCCGGGATGACGGACGCCGAGATGGCCGCCTTCA     1620
ICL_CDNA           TCTCGCCGTCCTTCAACTGGGACGCGTCCGGGATGACGGACGCCGAGATGGCCGCCTTCA     1380
ICL_ZIMBILI CDNA   TCTCGCCGTCCTTCAACTGGGACGCGTCCGGGATGACGGACGCCGAGATGGCCGCCTTCA     235
                   ************************************************************

ICL_REF            TCCCGAGCGTCGCGCGCCTCGGCTACGTCTGGCAGTTCATCACGCTCGCCGGGTTCCACG     1680
ICL_CDNA           TCCCGAGCGTCGCGCGCCTCGGCTACGTCTGGCAGTTCATCACGCTCGCCGGGTTCCACG     1440
ICL_ZIMBILI CDNA   TCCCGAGCGTCGCGCGCCTCGGCTACGTCTGGCAGTTCATCACGCTCGCCGGGTTCCACG     295
                   ************************************************************

ICL_REF            CCGACGCGCTCGTCACCGACACGTTCGCGCGGGACTTCGCACGCCGCGGCATGCTGGCCT     1740
ICL_CDNA           CCGACGCGCTCGTCACCGACACGTTCGCGCGGGACTTCGCACGCCGCGGCATGCTGGCCT     1500
ICL_ZIMBILI CDNA   CCGACGCGCTCGTCACCGACACGTTCGCGCGGGACTTCGCACGCCGCGGCATGCTGGCCT     355
                   ************************************************************

ICL_REF            ACGTGGAGAGGATCCAGAGGGAGGAGAGGATCAACGGCGTGGAGACGCTGGAGCATCAGA     1800
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ICL_CDNA            ACGTGGAGAGGATCCAGAGGGAGGAGAGGATCAACGGCGTGGAGACGCTGGAGCATCAGA     1560
ICL_ZIMBILI_CDNA    ACGTGGAGAGGATCCAGAGGGAGGAGAGGATCAACGGCGTGGAGACGCTGGAGCATCAGA     415
                    ************************************************************

ICL_REF             AGTGGTCGGGCGCCAATTTCTACGACAGGGTGCTCAAGGCTGTGCAGGGTGGCATCTCCT     1860
ICL_CDNA            AGTGGTCGGGCGCCAATTTCTACGACAGGGTGCTCAAGGCTGTGCAGGGTGGCATCTCCT     1620
ICL_ZIMBILI_CDNA    AGTGGTCGGGCGCCAATTTCTACGACAGGGTGCTCAAGGCTGTGCAGGGTGGCATCTCCT     475
                    ************************************************************

                              Exon 2          Intron 2
ICL_REF             CGACTGCAGCCATGGGCAAAGCAAAGTTCCTCATTTCCCAGCGTTTTTTTTTGTTTGG      1920
ICL_CDNA            CGACTGCAGCCATGGGCAAA----------------------------------------      1650
ICL_ZIMBILI_CDNA    CGACTGCAGCCATGGGCAAA----------------------------------------      512
                ICL2 R primer   3'  CCGTTT-CTCAATGACTTCTC-5'

                    Intron 2                                                Exon 3
ICL_REF             AAAAAAATAAACCATCGTTCGTACACAGTTTTGATGTTGTTTTGTTTACAGGAGTTACTGAAGAG 1985
ICL_CDNA            ----------------------------------------------------GAGTTACTGAAGAG 1655
ICL_ZIMBILI_CDNA    ----------------------------------------------------GAGTTACTGAAGAG 517
                                                ICL2 R primer   3'   CCGTTTC-CTCAATGACTTCTC -5'
                                                                                Stop codon of ICL
ICL_REF             CAGTTCAAGGATCCACGGCCCGCAACTGGGAGCAGTGGCCTGCAAGTTATGGCCAAATCCAGGATTTGA  2054
ICL_CDNA            CAGTTCAAGGATCCACGGCCCGCAACTGGGAGCAGTGGCCTGCAAGTTATGGCCA--------------  1650
ICL_ZIMBILI_CDNA    ---------------------------------------------------------------------  512
```

2. Malate synthase alignment

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CLUSTAL O(1.2.4) multiple sequence alignment malate synthase

MS_CDNA           ATGGAGACCGACGAAAAACACGCGCACTTCGAGTTGTACAGGAGCTGCACTGCACGCTCC      60
MS-gDNA           ATGGAGACCGACGAAAAACACGCGCACTTCGAGTTGTACAGGAGCTGCACTGCACGCTCC      60
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           TCAAATTCCCACGCCGCATG-CCACCCAGTGCCCTGCTCACCGCCGAACGGTTTCAATGG      119
MS-gDNA           TCAAATTCCCACGCCGCATCGCCACCCAGTGCCCTGCTCACCGCCGAACGGTTTCAATGG      120
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           CCGCAAGCACAGCGGCGCCATGCTACGACGCGCCCGAGGGCGTGGACGTCCGCGGGCGGT      179
MS-gDNA           CCGCAAGCACAGCGGCGCCATGCTACGACGCGCCCGAGGGCGTGGACGTCCGCGGGCGGT      180
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           ACGACCGGGAGTTCGCGGGCATCCTCACCCGCGACGCGCTGGACTTCGTGGCCGGCCTGC      239
MS-gDNA           ACGACCGGGAGTTCGCGGGCATCCTCACCCGCGACGCGCTGGACTTCGTGGCCGGCCTGC      240
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           AGCGCGAGTTCCGCGCCGCCGTCCGCTACGCCATGGAGCAGG--CGGGAGGCCCAGCGCC      297
MS-gDNA           AGCGCGAGTTCCGCGCCGCCGTCCGCTACGCCATGGAGCAGCGGCGGGAGGCCCAGCGCC      300
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           GGTACGACGCCGGCGAGCTGCCGCGGTTCGACCCGGCCACGACGCTCGTGCGCGAGGGGG      357
MS-gDNA           GGTACGACGCCGGCGAGCTGCCGCGGTTCGACCCGGCCACGACGCTCGTGCGCGAGGGGG      360
MS_ZIMBILI CDNA   ------------------------------------------------------------      0
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MS_CDNA           ACTGGACGTGCGCGCCCGTGCCGCCGGCCGTCGCGGACCGCACCGTGGAGATCACGGGCC      417
MS-gDNA           ACTGGACGTGCGCGCCCGTGCCGCCGGCCGTCGCGGACCGCACCGTGGAGATCACGGGCC      420
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           CCGCCGAGCCGCGGAAGATGGTCATCAACGCGCTCAACTCCGGCGCCAAGGTCTTCATG-      476
MS-gDNA           CCGCCGAGCCGCGGAAGATGGTCATCAACGCGCTCAACTCCGGCGCCAAGGTCTTCATGG      480
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           ------------------------------------------------------------      476
MS-gDNA           TAAGTAAGCGCGCGCGCAAACGTGGCTGCCGTATTCTCTCTCTCTCTCTCTCTCTCTC      540
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           ------------------------------------------------------------      476
MS-gDNA           TCTCTCTCTCTCTTTTTCCCGTTCTGAGCTGAGACCGCGAGAGAGAGAAAAACGTGGCAG      600
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           GCTGACTTCGAGGACGCGCTGTCGCCGACGTGGGAGAACCTGATGCACGGGCAGGTGAAC      536
MS-gDNA           GCTGACTTCGAGGACGCGCTGTCGCCGACGTGGGAGAACCTGATGCACGGGCAGGTGAAC      660
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           CTGCGGGACGCCGTGGCTGGCACCATCAGCTTCCGCGACGCGGCGCGCGGGCGGACGTAC      596
MS-gDNA           CTGCGGGACGCCGTGGCTGGCACCATCAGCTTCCGCGACGCGGCGCGCGGGCGGACGTAC      720
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           GAGCTCAACGACCGAACCGCCAAGCTCTTCGTCCGGCCCCGCGGCTGGCACCTCCCCG-A      655
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MS-gDNA           GAGCTCAACGACCGAACCGCCAAGCTCTTCGTCCGGCCCCGCGGCTGGCACCTCCCCGAG      780
MS_ZIMBILI CDNA   ------------------------------------------------------------      0


MS_CDNA           GCGCACATCCTCATCGACGGCGAGCCGGCCATTGGCTGCCTCGTCGACTTCGGCCTCTAC      715
MS-gDNA           GCGCACATCCTCATCGACGGCGAGCCGGCCATTGGCTGCCTCGTCGACTTCGGCCTCTAC      840
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           TTCTTCCACAACCACGCTGCCTTCCGCGCAGGCCAGGGCGCCGGCTTCGGGCCGTTCTTT      775
MS-gDNA           TTCTTCCACAACCACGCTGCCTTCCGCGCAGGCCAGGGCGCCGGCTTCGGGCCGTTCTTT      900
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           TACCTGCCCAAGATGGAGCACA-----------------------------------      797
MS-gDNA           TACCTGCCCAAGATGGAGCACTCCAGGTACGTCCGTGCCGTGGCGTGCGTGTGCCTCCGG      960
MS_ZIMBILI CDNA   ----------------------------------------------------      0

MS_CDNA           ------------------------------------------------------------      797
MS-gDNA           TGGTGCGTGTACGTGCACGGTGCACCGCACACATGTGCTCAGCGCAGCACTAACGTGACT      1020
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           ------------------------------------------------------------      797
MS-gDNA           CACTGAACTCTCAGTCGCTCAATCCTACTATAACGTAACGTACACGTGCGTCGTCTGCTG      1080
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           --GGGAAGCGAGGATATGGAACGGGGTGTTCCAGAGAGCGGAGAAAGCTGCTGGGATCGA      855
MS-gDNA           CAGGGAAGCGAGGATATGGAACGGGGTGTTCCAGAGAGCGGAGAAAGCTGCTGGGATCGA      1140
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MS_ZIMBILI CDNA   ----------------------------------------------------------  0
MS_CDNA           GCTCGGGAGCATCCGGGCAACGGTGCTGGTGGAGACGTTGCCAGCGGTGTTCCAAATGAA  915
MS-gDNA           GCTCGGGAGCATCCGGGCAACGGTGCTGGTGGAGACGTTGCCAGCGGTGTTCCAAATGAA  1200
MS_ZIMBILI CDNA   ----------------------------------------------------------  0

MS_CDNA           C----------------------------------------------------------  916
MS-gDNA           CGAGATCCTGCACGAGCTGCGCGAGCACTCGGCGGGGCTCAACTGCGGCCGCTGGGACTA  1260
MS_ZIMBILI CDNA   -----------------------------------------------------------  0

MS_CDNA           -ATCTTCAGCTACGTCAAGACGTTCCGCGCCCACCCGGACCGCCTCCTCCCCGACCGCGC  975
MS-gDNA           CATCTTCAGCTACGTCAAGACGTTCCGCGCCCACCCGGACCGCCTCCTCCCCGACCGCGC  1320
MS_ZIMBILI CDNA   ----------------------------------------------------------  0

MS_CDNA           CCTCGTCGGCATGGCCCAGCACTTCATGCGCTCCTACTCCCACCTCCTCATCCACACCTC  1035
MS-gDNA           CCTCGTCGGCATGGCCCAGCACTTCATGCGCTCCTACTCCCACCTCCTCATCCACACCTG  1380
MS_ZIMBILI CDNA   ----------------------------------------------------------  0

MS_CDNA           CACCGTC-GCGGAGTCCACGCC-------------------------------------  1056
MS-gDNA           CCACCGTCGCGGAGTCCACGCCATGGGCGGCATGGTAAATAAGCTGACCATATATCTCCG  1440
MS_ZIMBILI CDNA   ----------------------0-----------------------------------  0

MS_CDNA           ----------------------------------------ATGGGCGGGGCGGCTCAG  1074
MS-gDNA           CCGCGGCGTCCACGTGACGGTAACTAACCGTTGCAACGAACTCAATCGCAGGCGGCTCAG  1500
MS_ZIMBILI CDNA   ----------------------------------------0-----------------  0

MS_CDNA           ATCCCGATCAAGGACGATGCGGCGGCGAACGAGGCGGCACTGGAGCTGGTGCGCAAGGAC  1134
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MS-gDNA           ATCCCGATCAAGGACGATGCGGCGGCGAACGAGGCGGCACTGGAGCTGGTGCGCAAGGAC      1560
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           AAGCTGAGGGAGGTGCGCGCGGGCCACGACGGCACGTGGGCGGCGCACCCGGGGCTCATC      1194
MS-gDNA           AAGCTGAGGGAGGTGCGCGCGGGCCACGACGGCACGTGGGCGGCGCACCCGGGGCTCATC      1620
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           CCGGCGATACGGGAGGTCTTCGAGGGCCACCTCGGCGGGAGGCCGAACCAGATCGGCGAC      1254
MS-gDNA           CCGGCGATACGGGAGGTCTTCGAGGGCCACCTCGGCGGGAGGCCGAACCAGATCGGCGAC      1680
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           GCCGCAGGGCACGAGGGTGCCAGCGTCAACGAGGAGGACCTCATCCAGCCGCCGCGGGGC      1314
MS-gDNA           GCCGCAGGGCACGAGGGTGCCAGCGTCAACGAGGAGGACCTCATCCAGCCGCCGCGGGGC      1740
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           GCGCGCACTGTGGACGGTCTGCGGCTCAACGTCCGCGTGGGCGTGCAGTACCTCGCGGCG      1374
MS-gDNA           GCGCGCACTGTGGACGGTCTGCGGCTCAACGTCCGCGTGGGCGTGCAGTACCTCGCGGCG      1800
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           TGGCTGGCCGGCTCCGGCTCCGTCCCGCTGTACAACCTGATGGAGGACGCGGCTACGGCG      1434
MS-gDNA           TGGCTGGCCGGCTCCGGCTCCGTCCCGCTGTACAACCTGATGGAGGACGCGGCTACGGCG      1860
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           GAGATCAGCCGCGTCCAGAACTGGCAGTGGCTGCGCCACGGAGCGGCGCTGGACGCCGGC      1494
MS-gDNA           GAGATCAGCCGCGTCCAGAACTGGCAGTGGCTGCGCCACGGAGCGGCGCTGGACGCCGGC      1920
MS_ZIMBILI CDNA   ------------------------------------------------------------      0
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MS_CDNA           GGTGTGGAGGTGCGCGCGACGCCAGAGCTACTCGCACGCGTCGTCGAGGAGGAGATGGCG      1554
MS-gDNA           GGTGTGGAGGTGCGCGCGACGCCAGAGCTACTCGCACGCGTCGTCGAGGAGGAGATGGCG      1980
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           AGGGTCGAGGCAGAGGTCGGCCCCGACAGGTTCCGAAAGGGGCGGTACGCGGAGGCGGGC      1614
MS-gDNA           AGGGTCGAGGCAGAGGTCGGCCCCGACAGGTTCCGAAAGGGGCGGTACGCGGAGGCGGGC      2040
MS_ZIMBILI CDNA   ------------------------------------------------------------      0

MS_CDNA           AGGATCTTCAGCCGGCAGTGCACCGCGCCGGAGCTGGACGACTTCCTCACGCTGGACGCG      1674
MS-gDNA           AGGATCTTCAGCCGGCAGTGCACCGCGCCGGAGCTGGACGACTTCCTCACGCTGGACGCG      2100
                                                              MS FP 5'ACGCG
MS_ZIMBILI CDNA   ------------------------------------------------AGTTTACGCG      10
                                                                *  *****
                  TACAACCTCATCGTG 3'
MS_CDNA           TACAACCTCATCGTGGCGC----------------------------------------      1693
MS-gDNA           TACAACCTCATCGTGGCGCACCATCCAGGTGCGTCACCGTGCAAGCTCTGAATACATGCA      2160
MS_ZIMBILI CDNA   TACAACCTCATCGTGGCGCACCATCCAGGTGCGTCACCGTGCAAGCTCTGAATACATGCA      70
                  ******************
MS_CDNA           ----------------------------------------AATCGCTGTCCAATGCAGCCA      1714
MS-gDNA           TGCCTTTTGTGGCAGTGGAGTCAGCTTTCAAAAATAACACATCGCTGTCCAATGCAGCCA      2220
MS_ZIMBILI CDNA   TGCCTTTTGTGGCAGTGGAGTCAGCTTTCAAAAATAACACATCGCTGTCCAATGCAGCCA      130
                                                          ********************
MS_CDNA           TCGAAATAAGGACGGATGACCAACAAACATGATAGACATGAACAGCCTTGTAAAGCGCAC      1774
MS-gDNA           TCGAAATAAGGACGGATGACCAACAAACATGATAGACATGAACAGCCTTGTAAAGCGCAC      2280
MS_ZIMBILI CDNA   TCGAAATAAGGACGGATGACCAACAAACATGATAGACATGAACAGCCTTGTAAAGCGCAC      190
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                  ************************************************************
MS_CDNA           TGCATATTCTCTGCTCTGGGACTTTTAGTCGTGCTTATGATAGTACAGGGTGGTCTATGT      1834
MS-gDNA           TGCATATTCTCTGCTCTGGGACTTTTAGTCGTGCTTATGATAGTACAGGGTGGTCTATGT      2340
MS_ZIMBILI CDNA   TGCAAATTCTCGGGTTTGGGACTTTTAGTCGTGCTTATGATAGTACAGGGTGGTCTATGT      250
                  **** ****** * * ******************************************

MS_CDNA           AATAAACTGAGCTTCTGTTGAACTCTCGGGAAGGACAACTGCACAACCCTGTATGAGTGT      1894
MS-gDNA           AATAAACTGAGCTTCTGTTGAACTCTCGGGAAGGACAACTGCACAACCCTGTATGAGTGT      2400
MS_ZIMBILI CDNA   AATAAACTGAGCTTCTGTTGAACTCTCGGGA----------------------------      281
                  ******************************
                         3'GCTTCTGTTGAACTCTCGGG5' MS RP
MS_CDNA           ACCGAGACTATCTTTTCACCCTCAGAACTACCGCTCTACAACGTGTATCTTTCTAATTTT      1954
MS-gDNA           ACCGAGACTATCTTTTCACCCTCAGAACTACCGCTCTACAACGTGTATCTTTCTAATTTT      2460
MS_ZIMBILI CDNA   ------------------------------------------------------------      281
MS_CDNA           ATAAATCAAACTTATTTAACTTTAA     1979
MS-gDNA           ATAAATCAAACTTATTTAACTTTAA     2485
MS_ZIMBILI CDNA   -------------------------     281
```

Appendix E

Semi-qRTPCR data analysis

ns:non-significant, P value : indicating strong evidence against null hypothesis, F : comparing statistical models that have been fitted to a data set, in order to identify the model that best fits the population from which the data were sampled (F-distribution), R square: measures how close the data is to the fitted regression line, ANOVA: Analysis of variance, Residual: measures the difference between the predicted value and the observed value of the dependent variable , Mean diff: measures statistical dispersion between means, q: is a p-value that has been adjusted for the proportion of false positives you can expect to get from a test , CI of diff: Confidence Interval on the difference between Means, SS= is the variance or the sum of squared differences from the mean, Df= is the degree of freedom which indicates the number of independent values that can differ in an analysis without breaking any restraints and MS=mean square is used to analyse the variance and is calculated as a sum of squares divided by its appropriate degrees of freedom and vs: versus (against).

DEPC 269 bp at 34 cycles

SUMMARY

Table Analysed	Pre-hoc data (normalised data)		
One-way ANOVA			
value of p	0.85		
summary of p value	**ns**		
Is there significance difference between the means? (P < 0.05)	**No**		
Number of groups	5		
F	0.34		
R square	0.12		
ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	51.93	4	12.98
Residual (within columns)	382.9	10	38.29
Total	**434.9**	**14**	

Tukey's test

	Mean Diff.	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
SU vs S1	-1.07	0.30	No	ns	-17.70 to 15.56
SU vs S2	-1.06	0.30	No	ns	-17.68 to 15.57
SU vs S4	-0.13	0.04	No	ns	-16.76 to

					16.49
SU vs S5	-5.08	1.42	No	ns	-21.71 to 11.55
S1 vs S2	0.01	0.00	No	ns	-16.61 to 16.64
S1 vs S4	0.94	0.26	No	ns	-15.69 to 17.56
S1 vs S5	-4.01	1.12	No	ns	-20.64 to 12.62
S2 vs S4	0.92	0.26	No	ns	-15.70 to 17.55
S2 vs S5	-4.02	1.13	No	ns	-20.65 to 12.60
S4 vs S5	-4.95	1.39	No	ns	-21.57 to 11.68

DEPC 269 bp at 35 cycles

SUMMARY			
Table Analysed	Pre-hoc (normalised data)		
One-way ANOVA			
value of p	0.29		
summary of p value	**ns**		
Is there significance difference between the means? (P < 0.05)	**No**		
Number of groups	5		
F	1.45		
R square	0.37		
ANOVA Table	SS	df	MS
Treatment (between columns)	315.6	4	78.9
Residual (within columns)	543.4	10	54.34
Total	859	14	

Tukey's Multiple Comparison Test

	Mean Diff.	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
SU vs S1	-3.28	0.77	No	ns	-23.09 to 16.53
SU vs S2	-0.56	0.13	No	ns	-20.37 to 19.25
SU vs S4	1.12	0.26	No	ns	-18.68 to 20.93
SU vs S5	-11.56	2.72	No	ns	-31.37 to 8.247

S1 vs S2	2.72	0.64	No	ns	-17.09 to 22.53
S1 vs S4	4.40	1.04	No	ns	-15.40 to 24.21
S1 vs S5	-8.28	1.95	No	ns	-28.09 to 11.53
S2 vs S4	1.68	0.40	No	ns	-18.12 to 21.49
S2 vs S5	-11.00	2.59	No	ns	-30.81 to 8.807
S4 vs S5	-12.68	2.98	No	ns	-32.49 to 7.123

DEPC 190 bp at 35 cycles

SUMMARY

Table Analysed	Pre-hoc (normalised data)		
One-way ANOVA			
value of p	0.4868		
summary of p value	**ns**		
Is there significance difference between the means? (P < 0.05)	**No**		
Number of groups	5		
F	0.9254		
R square	0.2702		
ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	70.7	4	17.68
Residual (within columns)	191	10	19.1
Total	261.7	14	

Tukey's Multiple Comparison Test

	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
SU vs S1	3.65	1.45	No	ns	-8.094 to 15.39
SU vs S2	-2.65	1.05	No	ns	-14.39 to 9.094
SU vs S4	0.70	0.28	No	ns	-11.04 to 12.44
SU vs S5	2.50	0.99	No	ns	-9.240 to 14.25
S1 vs S2	-6.30	2.50	No	ns	-18.04 to 5.444
S1 vs S4	-2.95	1.17	No	ns	-14.69 to 8.794
S1 vs S5	-1.15	0.45	No	ns	-12.89 to 10.60

S2 vs S4	3.35	1.33	No	ns	-8.394 to 15.09
S2 vs S5	5.15	2.04	No	ns	-6.590 to 16.90
S4 vs S5	1.80	0.71	No	ns	-9.940 to 13.55

Nuclease free water 269 bp at 34 cycles

SUMMARY

Table Analysed	Pre-hoc data)	(normalised
One-way ANOVA		
value of p	0.1254	
summary of p value	**ns**	
Is there significance difference between the means? (P < 0.05)	**No**	
Number of groups	5	
F	2.34	
R square	0.48	
ANOVA Table	**SS**	**df** **MS**
Treatment (between columns)	327.5	4 81.87
Residual (within columns)	349.5	10 34.95
Total	677	14

Tukey's Multiple Comparison Test

	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
SU vs S1	0	0	No	ns	-15.89 to 15.89
SU vs S2	1.56	0.46	No	ns	-14.32 to 17.45
SU vs S4	-7.12	2.09	No	ns	-23.01 to 8.763
SU vs S5	-10.31	3.02	No	ns	-26.20 to 5.573
S1 vs S2	1.56	0.46	No	ns	-14.32 to 17.45
S1 vs S4	-7.12	2.09	No	ns	0
S1 vs S5	-10.31	3.02	No	ns	-26.20 to 5.573
S2 vs S4	-8.69	2.55	No	ns	-24.57 to 7.199
S2 vs S5	-11.88	3.48	No	ns	-27.76 to 4.009
S4 vs S5	-3.19	0.93	No	ns	-19.08 to 12.70

Nuclease free water 190 bp at 34 cycles

SUMMARY			
Table Analysed	Pre-hoc (normalised data)		
One-way ANOVA			
value of p	0.1254		
summary of p value	**ns**		
Is there significance difference between the means? (P < 0.05)	**No**		
Number of groups	5		
F	2.342		
R square	0.4837		
ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	327.5	4	81.87
Residual (within columns)	349.5	10	34.95
Total	677	14	

Tukey's Multiple Comparison Test

	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
SU vs S1	0	0	No	ns	-15.89 to 15.89
SU vs S2	1.56	0.46	No	ns	-14.32 to 17.45
SU vs S4	-7.12	2.09	No	ns	-23.01 to 8.763
SU vs S5	-10.31	3.02	No	ns	-26.20 to 5.573
S1 vs S2	1.56	0.46	No	ns	-14.32 to 17.45
S1 vs S4	-7.12	2.09	No	ns	-23.01 to 8.763

S1 vs S5	-10.31	3.02	No		ns	-26.20 to 5.573
S2 vs S4	-8.69	2.55	No		ns	-24.57 to 7.199
S2 vs S5	-11.88	3.48	No		ns	-27.76 to 4.009
S4 vs S5	-3.19	0.93	No		ns	-19.08 to 12.70

Nuclease free water 269 bp at 35 cycles

SUMMARY	
Table Analysed	Pre-hoc (normalised data)
One-way ANOVA	
value of p	0.6865
summary of p value	**ns**
Is there significance difference between the means? (P < 0.05)	**No**
Number of groups	5
F	0.58
R square	0.19

ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	177.5	4	44.39
Residual (within columns)	770.4	10	77.04
Total	947.9	14	

Tukey's Multiple Comparison Test

	Mean Diff.	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
SU vs S1	-4.85	0.96	No	ns	-28.44 to 18.73
SU vs S2	-3.07	0.61	No	ns	-26.65 to 20.51
SU vs S4	-1.73	0.34	No	ns	-25.31 to 21.86
SU vs S5	-10.04	1.98	No	ns	-33.62 to 13.55
S1 vs S2	1.78	0.35	No	ns	-21.80 to 25.37
S1 vs S4	3.13	0.62	No	ns	-20.46 to 26.71
S1 vs S5	-5.18	1.02	No	ns	-28.77 to 18.40
S2 vs S4	1.34	0.27	No	ns	-22.24 to 24.93
S2 vs S5	-6.97	1.38	No	ns	-30.55 to 16.62
S4 vs S5	-8.31	1.64	No	ns	-31.89 to 15.27

Nuclease free water 190 bp at 35 cycles

SUMMARY			
Table Analysed	Pre-hoc (normalised data)		
One-way ANOVA			
value of p	0.5561		
summary of p value	**ns**		
Is there significance difference between the means? (P < 0.05)	**No**		
Number of groups	5		
F	0.79		
R square	0.24		
ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	308.9	4	77.23
Residual (within columns)	974.6	10	97.46
Total	1283	14	

Tukey's Multiple Comparison Test

	Mean Diff.	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
SU vs S1	1.74	0.31	No	ns	-24.78 to 28.27
SU vs S2	2.12	0.37	No	ns	-24.41 to 28.64
SU vs S4	-3.82	0.67	No	ns	-30.35 to 22.70
SU vs S5	-10.04	1.76	No	ns	-36.57 to 16.48
S1 vs S2	0.37	0.07	No	ns	-26.15 to 26.90
S1 vs S4	-5.57	0.98	No	ns	-32.09 to 20.96
S1 vs S5	-11.79	2.07	No	ns	-38.31 to 14.74
S2 vs S4	-5.94	1.04	No	ns	-32.47 to 20.59
S2 vs S5	-12.16	2.13	No	ns	-38.69 to 14.37
S4 vs S5	-6.22	1.09	No	ns	-32.75 to 20.31

Chapter 4
Conclusion and prospects

Introduction

Maize is an important staple food in most Southern African countries and serves as fodder for industrialised countries such as the United States of America. The crop is affected by different environmental and living factors. Environmental factors (abiotic) that cause stress to the crop include drought, less availability of nutrients, and living factors (biotic) such as viruses, fungi, cause diseases stress and damage. Diseases such as grey leaf spot (GLS) cause stress and affect the yield, quality and production potential of maize. This disease is because of an infection by the *Cercospora* species. The specific *Cercospora* species causing GLS in Southern Africa, *Cercospora zeina* (*C. zeina*), was reported to result in yield decrease of up to 65% (Ward et al., 1999), posing a problem affecting food security to countries that produce and depend on maize as a staple food. It was estimated that food production needs to increase by 70% by the year 2050 as the global population is increasing. Thus, it is very important to increase the production of maize and prevent maize causing diseases to ensure that food is supplied for the growing population. There are several management strategies of controlling the progression of the disease, however, most are labour intensive, expensive and there is limited knowledge and understanding of the molecular mechanisms that can be used to induce resistance in maize genotypes or hybrids (Levitt, 1980).

A better understanding of the host-pathogen interaction can help prevent the progression of GLS. There has been progress made so far towards understanding the disease epidemiology, biological composition, life cycle and infection strategy of *C. zeina* in maize. Management strategies that aim at inhibiting the occurrence, infection and progression of GLS consist mainly of the application of fungicides that have been shown to have an effect in controlling the progression of GLS when the appropriate procedures had been followed (Ward et al., 1997). However, the constant use of fungicides can lead to the development of fungicide-resistance by the pathogen and additionally, fungicides are very expensive. This financial constraint turns out to be a problem to subsistence farmers who cannot afford to use fungicides to combat GLS on their crops thus; they need seeds that will yield hybrids that are already GLS resistant.

This study was aimed at identifying the role of glyoxylate pathway primary metabolism genes namely, malate synthase (*MS*) and isocitrate lyase (*ICL*), in pathogen-host interactions. The primary metabolism genes have been shown as pathogenicity factors during the pathogen

infection, showing that a fungal ICL mutants failed to induce disease progression in the host. Thus, the expression levels of *ICL* and *MS* play a role in the progression of the pathogen in the host. This helped to better understand their role at the different disease progression stages.

Overview of results

Polymerase chain reaction (PCR) primers were designed from the available *ICL* and *MS* maize sequences that 1) were used for reverse transcriptase quantitative PCR, 2) resulted in products that could be identified by running on an agarose gel, and 3) were specific in amplifying cDNA of the respective metabolism genes. These primers were used on cDNA products for RT-PCR and semi-qRTPCR to analyse the expression of the genes in susceptible maize genotype at two different time points (0 dpi and 32 dpi) and at different disease progression stages (un-inoculated, S1, S2, S4 and S5). The non-significant difference gene expression of *MS* at the different time points indicated that there was no significance gene expression difference for the susceptible maize genotype at the different disease progression stages for this pathway. However, this study showed that two *MS* transcripts (which were not expected) were expressed at the different disease progression stages.

Future studies can look at analysing each transcript by creating primers in the *MS* gene regions illustrated in Figure 4.1. The primers will be designed on transcripts 1 (T01), transcript 2 (T02) and transcript 3 (T03). All the forward primers will be in the exon region while the position of the reverse primers will vary [flank an intron (T01/T02/T03), are in the 3’ UTR (T01/T02), cut across an intron (T01/T02), or one part is in the same exon as the forward and the other portion is in the following exon (T03)] as shown in Figure 4.1 and Table 4.1. The expression levels of *MS* in infected maize will use different methods to measure expression. The primers that have only one type of transcript can be measured using the sensitive SYBR green quantitative PCR method while the primers that can amplify different transcripts can be measured using semi-qRTPCR because the expression will not be easily distinguished using SYBR green. The primers in the 3’ UTR region are not specific for cDNA (used to measure expression) only but can also be used to amplify genomic DNA These are not that ideal for expression analysis however, more specific primers that cut across or flank the intron can be used for expression.

Table 4.1: The predicted primers for malate synthase transcripts namely transcript 1 (T01), transcript 2 (T02) and transcript 3(T03). The types of amplicon that will be generated are shown together with the types of combinations that are possible.

Transcript	Primer name	Position of primer		Expected amplicon	
		Forward	Reverse	Genomic DNA	cDNA
T01	MST01	Exon 3	3' UTR	Yes, same size for cDNA/genomic DNA	
T02	MST01	Exon 4	3' UTR	Yes, same size for cDNA/genomic DNA	
T03	MST01	Exon 4	Split across the intron portion in Exon 5 and portion in Exon 4	None	Yes
T01/T02/T03	MST01/T02/T03	Flanking intron 4 in Exon 4	Exon 5	Yes	Yes
T01/T02	MST01/T02	Exon 3 : T01 and Exon 4 : T02	Cutting across intron 4	None	Yes

Figure 4.1: The proposed primer positions designed for the malate synthase transcripts for gene expression. The illustration of malate synthase gene from Maize Genome Database (GRMZM2G094328) and the National Centre of Biotechnology Institute GenBank gene code (LOC542252). *MS* is found in chromosome 2 of the maize genome between 9,547,650 and 9,549,271 bases. The gene direction is from the flat side (5') to the sharp ended side (3'). The grey part indicated the untranslated regions of the gene (5' UTR and 3' UTR), the red indicates the exon part of the gene (coding regions) and the lines between the exons indicate the introns. There are three transcripts of the *MS* gene namely, transcript 1 (T01), transcript 2 (T02) and transcript 3 (T03). T01 has 3 exons indicated in red, T02 has 4 exons and T03 has 5 exons. The T01, T02 and T03 transcript will be used to design primers in future studies. Primers will be designed (indicated by the arrows) so that the forward primers are in the exon region and the reverse primers either flank an intron (T01/T02/T03), are in the 3' UTR (T01/T02), cut across an intron (T01/T02), or one part is in the same exon as the forward and the other portion is in the following exon (T03).

The expression levels of malate synthase increased at the different GLS disease progression stages compared to the un-inoculated B73 maize genotype however, there was no significant difference in the findings indicating that the glyoxylate pathway genes were not temporally activated as the as *C. zeina* was infecting, penetrating and invading the maize plant. Previously, expression levels of the *MS* were measured for the susceptible maize genotype at different disease progression stages instead of two time points by the Christie et al. (2017). This gives a broad spectrum and helps more to understand how genes are regulated during the development of GLS. In this study, the expression data revealed that there was an additional malate synthase transcript, T02 (unspliced-with intron), being expressed together with T03 (sliced-smaller amplicon) at the different GLS progression stages, which were not seen in the RNASeq data by Christie et al. (2017). The additional T02 transcript was expressed at later stages of GLS progression and the expression levels were lower than those of T03. The T03 expression was higher in leaf samples that had high GLS development (higher in coalesced lesions than in chlorotic spots). Overall, a trend was seen in the expression levels of the malate synthase, the higher the disease progression the higher the malate synthase usage to make glucose for the progression of the fungi.

These results however, are not in agreement with the Casati et al. (1999) and Técsi et al. (1996) studies, which showed that malate synthase helped in plant defence against fungi. Therefore, the hypothesis stating that the expression is different at all the various stages, meaning that the *MS* will have housekeeping and different defence roles, was rejected. These results however, agreed with a previous study by Christie et al. (2017) (which showed an increased expression of *MS* in B73 susceptible maize infected with *C. zeina*) and Solomon et al. (2003) in which *Stagonospora nodorum* is responsible for causing glume blotch and leaf disease had high levels of malate synthase). But, the expression levels were not statistically different at the various disease progression stages, thus future studies need more susceptible maize samples and for more sensitive tests to measure the expression of the transcripts individually to prove that *MS* and *ICL* play a role in fungal pathogenicity.

Further studies, that measure the expression levels of malate synthase and isocitrate lyase in the fungi at the different disease progression stages can be done in future studies to compare the expression levels between the pathogen and host and to further confirm the role of *MS* and *ICL* *in vitro* and *in planta*. Targeting the *ICL* in *C. zeina* or the maize genotype can be done, and tested by planting the maize genotypes in a field trial. Thus, a decrease in expression of the *ICL* in plant may help in reducing the progression of GLS. This can be done by knocking out the *ICL* gene in *C. zeina* or by silencing the gene in maize temporarily since the gene is expressed at different times of disease progression.

Concluding remarks

The glyoxylate gene, malate synthase gene was highly expressed in the infected maize, however, this was not statistically significant because of time constraint (the maize has one growing season thus two growing seasons were already performed for the two years and two glasshouse trials) and the small sample size. In the future, further research can be done to quantify expression of the *MS* gene by using quantitative-RTPCR, which is more stringent and uses SYBR green as the marker to detect the expression of the gene. A study on the inhibition of the *ICL* or *MS* genes can be done in the future to test for the antifungal effects it would have on B73 maize genotype. A study by Cheah et al., 2014 performed an experiment that identified antifungal compounds that targeted *ICL* by using a developed screening approach that reflected the physical environment that the cells of *C. albicans* experience during infection. Cheah et al. (2014) identified three compounds that had antifungal activity against *C. albicans* namely, rosmarinic acid (ROS), apigenin (API) and caffeic acid (CAFF) under glucose-depleted conditions. The study used *ICL* enzyme assays to confirm that these compounds had the ability to inhibit *ICL*. These three compounds rosmarinic acid (ROS), apigenin (API) and caffeic acid (CAFF) can be induced *in planta* to inhibit *ICL* for antifungal effects on *C. zeina* inoculated plants. Thus, this strategy can be further used in maize for defence against *C. zeina* and GLS progression.

A study by Schenk et al. 2003 was aimed at gaining an insight into transcriptional changes that occur in distal tissue during defence responses triggered by an incompatible fungal pathogen.

They used the RT-qPCR technique for a time-course after inoculating with *A. brassicicola* to check for the expression of candidate defence gene transcript PDF1.2 (Penninckx et al., 1996). The results showed an induction in the gene expression of the transcript as early 6 to 12 hours after inoculations. However, accumulation of the PDF1.2 was highest compared to that of the uninoculated control plant. A Northern-blot analysis of the PDF1.2 gene confirmed significant expression in locally inoculated and in distal leaf tissue of two independent inoculation. Therefore, northern blots can be done in future studies to further confirm the expression levels of the transcripts malate synthase transcripts. A study on reducing the expression levels of malate synthase expression in maize can be done (no complete inhibition as malate synthase is required for glucose production and by the plant for normal growth and activity. This study be used as an antifungal effect investigation of B73 maize genotype against *C. zeina*. This study can be done on the model plant, *N. benthamiana*, which has been used in many studies for plant-pathogen interactions.

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