CHAPTER 1

Cercospora zeina, a foliar pathogen of maize in South Africa

1. INTRODUCTION

Maize (*Zea mays*) is globally considered as an important cereal crop, and a major staple food in developing countries such as Africa (WARD *et al.* 1999). In South Africa, maize is considered the most important grain crop as it is the main feed grain used for animals and a staple food for the population (FAO 2012). Maize can also be used for the production of maize-based ethanol, which can be used as a bio-fuel. In the USA, approximately 40% (11 million tonnes) of maize produced in 2012 was used for the production of bio-fuel (FAO 2012). Maize production in Africa was estimated to be less than two and on average 1.4 tons per hectare and remains below world average (FAO 2012). It was expected that South African crop production would decrease by approximately six percent during the 2012/2013 growing season as droughts during February and March 2013 in the North West and Free State provinces led to below-average maize yields in these production areas (FAO 2012; USDA 2013). Over the last few years maize production output has not been increasing together with the increasing population growth rate and thus puts pressure on commercial farmers to produce more maize for food security purposes and economical growth. The FAO states that agricultural production still needs to increase by up to 60% (80% in developing countries) within the next four years to be able to cope with an estimated global population growth of 39% by 2050.

One of the major factors negatively influencing maize production is destructive fungal diseases, such as Grey Leaf Spot (GLS), that only contribute to the increasing food scarcity in African nations. GLS disease is considered as one of the main factors negatively influencing maize production in southern Africa (DERERA *et al.* 2008). GLS can be found in many countries where maize is cultivated. However, GLS disease in Africa has been shown to be caused predominantly by *Cercospora zeina* whereas GLS disease in the US is predominantly caused by *Cercospora zeae-maydis* (CROUS *et al.* 2006; DUNKLE and LEVY 2000; MEISEL *et al.* 2009; OKORI *et al.* 2003). In South Africa, yield losses of up to 65% have been reported as a result of GLS infection of maize (WARD and NOWELL 1998). The KwaZulu-Natal Department of Agriculture estimates that annual losses in the province amount to more than R500 million (DERERA *et al.* 2008). Current efforts to combat GLS disease include resistant hybrids, crop rotations, tillage practices and fungicides (LIPPS 1989; STROMBERG 1986; WARD *et al.* 1997a; WARD *et al.* 1997b; WARD *et al.* 1997c; WARD *et al.* 1997d). None of these efforts have effectively resolved the problem (WARD *et al.* 1999), highlighting the importance of research in this field.

Whereas limited information is available regarding the genetic and biochemical basis of pathogenesis in *C. zeae-maydis*, virtually nothing is known about the morphological or molecular processes underlying infection by *C. zeina*. In order to effectively combat the economically important disease, fundamental insight into how these two distinct species of pathogens cause disease, and how maize responds to their attack, is needed. The ultimate goal of this research would be to establish long-term, stable disease resistance to GLS of maize in order to maintain nutritious, high-yielding maize to sustain this valuable food resource. This review will summarize literature on GLS disease and the causal agents, as well as plant pathosystems and the strategies fungal pathogens use to evade detection by their plant hosts. I will also discuss effector genes identified in different fungal pathogens as well as the role they play in pathogen virulence. At the end of the chapter, I will introduce my research project and the proposed aims I will be addressing in the following research chapters.

2. GREY LEAF SPOT (GLS) DISEASE OF MAIZE

2.1 Overview

The foliar disease Grey Leaf Spot (GLS) got its name from the characteristic grey lesions it causes after infection. These grey lesions found on maize leaves reduce the photosynthetic ability of the host, which results in decreased grain fill and severe yield losses. Today, GLS can be found in many maize growing regions worldwide (CLEMENTS *et al.* 2000; LATTERELL and ROSSI 1983; WANG *et al.* 1998; WARD *et al.* 1999) and GLS is regarded as a very serious yield-limiting disease of maize (LIPPS 1998; NUTTER and JENCO 1992; WARD and NOWELL 1998). Yield losses in South Africa as a result of GLS, have been reported to be as high as 50% in hybrids with moderate resistance and 65% in susceptible maize hybrids (WARD and NOWELL 1998).

The casual agents of GLS of maize have been identified as *C. zeae-maydis* (originally named C*. zeae-maydis* Group I) and *C. zeina* (originally named *C. zeae-maydis* Group II). The only known host of these genetically distinct sibling species is maize and a better understanding is needed into how these pathogens cause disease and how maize responds to their attack. Although insight into the molecular infection strategy of the pathogen causing GLS will possibly lead to the development of effective management strategies, current efforts should be made to minimize the initial inoculum present in maize fields in order to reduce the disease severity (WARD *et al.* 1999).

2.2 History and impact of GLS disease

After GLS disease was first reported in Illinois in 1925, it was determined by Charles Chupp that the causative agent was *C. zeae-maydis* (CHUPP 1953; TEHON and DANIELS 1925). GLS spread through maize growing regions in the United States, but only became epidemic in the 1970's (LATTERELL and ROSSI 1983). GLS was detected much later in South Africa in 1988 (WARD *et al.* 1997d; WARD *et al.* 1999), where after it was also detected in northern maize growing regions of the rest of the African continent. It was later determined that two genetically distinct sibling species are associated with GLS in the U.S., namely *C. zeae-maydis* Group I and Group II (DUNKLE and LEVY 2000; GOODWIN *et al.* 2001; WANG *et al.* 1998). Isolates from Group I were found to be distributed throughout maize growing regions in the U.S., whereas isolates of Group II were confined to the eastern third of the country (WANG *et al.* 1998). In some locations in the eastern third of the U.S., isolates from both groups could be found in the same fields (WANG *et al.* 1998). These two groups of isolates were shown to be taxonomically identical, but genetically distinct (WANG *et al.* 1998). Isolates causing GLS in Africa showed identical disease symptoms as seen in isolates of

both groups in the U.S., but the African isolates showed similar culture characteristics as seen with isolates from Group II (DUNKLE and LEVY 2000). It was later determined, through taxonomic measures, that Group I is *C. zeae-maydis* that causes GLS mainly in the U.S. and Group II is *C. zeina* which causes GLS mainly in Africa, but is also found in the U.S. to a lesser extent (CROUS *et al.* 2006).

Even though these two species cannot be distinguished by the disease symptoms they cause (Figure 1A), they are genetically distinct and can be distinguished on a molecular level (Figure 1B). *C. zeina*, and an unidentified *Cercospora* sp. can also be distinguished from *C. zeae-maydis* through a PCR-based test incorporating species-specific primers as seen in Figure 1B (CROUS *et al.* 2006). Additionally, *C. zeae-maydis* is not found in Africa, whereas *C. zeina* can be found mainly in Africa.

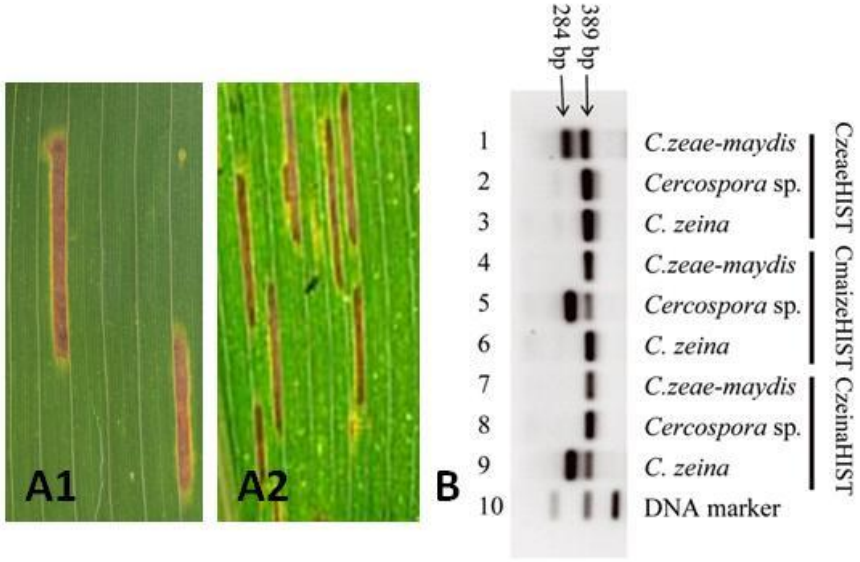

Figure 1. Distinguishing disease symptoms caused by *C. zeina* and *C. zeae-maydis* (Figure adapted from Crous *et al.* 2006 (B). Photo credit: B. Meisel (A1) and www.plantpath.cornell.edu (A2)). Grey Leaf spot disease symptoms caused by *C. zeina* (A1) and *C. zeae-maydis* (A2) are identical and therefore indistinguishable on phenotypic level. The two pathogens can be distinguished on a molecular level by means of a PCR-based test that was developed with species specific primers (B). Two sets of species specific primers can distinguish between *C. zeae-maydis* and *C. zeina*. Species specificity depending on the primer set used is indicated by the 284 bp band and the 398 bp band acts as a positive control and is present in all reactions.

By examining and comparing the cultural characteristics of *C. zeae-maydis* and *C. zeina*, it was determined that the two sibling species can also be distinguished on this level. *C. zeae-maydis* has a faster growth rate, produces cercosporin (red pigment) *in vitro* (See Figure 2B), and has longer conidiophores with more broadly fusiform conidia when compared to *C.*

zeina (CROUS *et al.* 2006; DUNKLE and LEVY 2000; WANG *et al.* 1998). *C. zeina* was not shown to produce the cercosporin toxin *in vitro* as seen in Figure 2A (CROUS et al. 2006; DUNKLE and LEVY 2000).

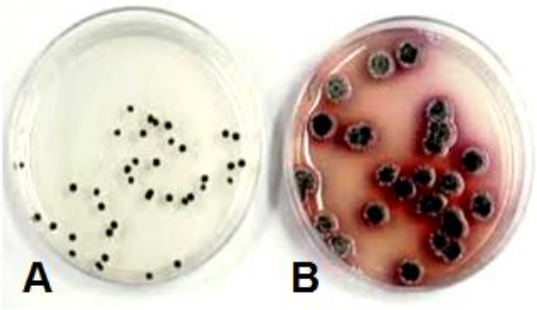

Figure 2. *In vitro* cercosporin production by *C. zeae-maydis* (USDA 2009). Sporulating cultures of *C. zeina* (A) and *C. zeae-maydis* (B) on 0.2 X PDA (Potato Dextrose Agar) media grown in the presence of light. The red pigment, as seen in (B), is characteristic of the toxin cercosporin produced by *C. zeae-maydis*, but not by *C. zeina*, as seen in (A). *Photo credit: USDA 2009.*

C. zeae-maydis has not been shown to be associated with GLS in Africa, but further sampling from more locations within Africa is needed. Literature also suggested another species complex, the *Cercospora sorghi* complex, that can possibly be associated with GLS as *C. sorghi* has also been isolated from maize (CROUS and BRAUN 2003). No sexual stage or parasexuality has been reported for *C. zeae-maydis* or *C. zeina*. However, it is important to note that a telomorphic stage in *Mycosphaerella* was found associated with GLS lesions in overwintered maize debris (LATTERELL and ROSSI 1983). Also, a possibility for sexual reproduction has been reported for *Cercospora beticola* (BOLTON et al. 2012). A population study, which is currently being undertaken by a researcher in our group, will shed some light on the possibility of a sexual stage in *C. zeina* by looking at genetic variation found within and between populations.

In terms of understanding the spread of GLS and how GLS was first introduced to South Africa, a few postulates have been made. The first being that GLS was introduced to South Africa from infected maize residues that were imported from the U.S. (WARD *et al.* 1999). It was further argued that if the infected maize residues that were imported from the U.S., served as the possible source of inoculum, that these infected residues would most likely have contained *C. zeae-maydis*, as it was shown that *C. zeae-maydis* dominates over *C. zeina* in maize growing regions of the U.S. (DUNKLE and LEVY 2000). The casual agent of GLS in South Africa as well as regions in the rest of the continent is *C. zeina* (CROUS *et al.* 2006; DUNKLE and LEVY 2000; MEISEL *et al.* 2009; WANG *et al.* 1998).

A population study has indicated that *C. zeina* has a higher genetic diversity in Africa when compared to the genetic diversity of *C. zeina* in the U.S. (DUNKLE and LEVY 2000). Also, the genetic distance isolating the two groups (Group I and Group II) found in the U.S., implies that these distinct pathogen groups did not originate at the same location and that neither could have been the immediate ancestor of the other (WANG *et al.* 1998). According to this information, it was postulated that *C. zeina* was introduced to the U.S. (DUNKLE and LEVY 2000).

A third hypothesis proposing the origin of *C. zeina* is that *C. zeina* might rather have been introduced to both countries (Africa and the U.S.) through a different host, as maize is not an indigenous species to Africa (DUNKLE and LEVY 2000). It should also be considered that *C. zeina* might have originated in Africa on another indigenous host, such as sorghum (CHUPP 1953), but that the pathogen jumped from the indigenous host to maize (DUNKLE and LEVY 2000). Although many theories exist, more regions in Africa need to sampled and further population studies should be done in order to determine where *C. zeina* originated from.

2.3 GLS disease cycle and symptoms

2.3.1 Disease symptoms

Interestingly, most *Cercospora* species cause leaf spot of some kind in their host plants. Some of the very closely related species of *C. zeae-maydis* and *C. zeina* are *C. apii*, *C. beticola*, and *C. nicotianae*. *C. apii* causes leaf spot in celery as seen in Figure 3A, *C. beticola* causes leaf spot of sugar beet as seen in Figure 3B, and *C. nicotianae* causes leaf spot in tobacco plants as seen in figure 3C.

Figure 3. Closely related *Cercospora* species causing leaf spot. *C. apii* causes leaf spot in celery plants (Nogueira and Paz-Lima 2010) **(A)**, *C. beticola* causes leaf spot in sugar beet (commons.wikimedia.org) **(B)**, and *C. nicotianae* is known to cause leaf spot in tobacco plants (www.plantwise.org) **(C)**.

C. zeae-maydis and *C. zeina* cause GLS disease in maize and affect the leaves of the infected maize plants. The sibling species *C. zeae-maydis* and *C. zeina* cause identical disease symptoms, but the appearance of *C. zeina* disease symptoms occur later than the times described below for *C. zeae-maydis* infection. The initial observable symptoms of GLS are small, irregular shaped chlorotic spots (as seen in Figure 4A) that first appear on the lower leaves of the maize plants approximately nine days after inoculation (BECKMAN and PAYNE 1982) (see figure 4A). The chlorotic spots can easily be seen when held against the light, but they are easily confused with the initial symptoms of other maize foliar diseases (WARD *et al.* 1999). The chlorotic spots increase in size at 9-12 days after inoculation (BECKMAN and PAYNE 1982). The mature lesions of GLS seen 13-16 days after inoculation are characterized by rectangular shaped, tan lesions that become dark gray lesions as the fungus produces conidiophores and conidia at 16-20 days after inoculation (BECKMAN and PAYNE 1982). Necrosis occurs from the middle of a mature lesion and conidiophores and conidia are only produced in areas of necrotic leaf tissue (BECKMAN and PAYNE 1982). These mature lesions are constricted by the leaf veins as seen in Figure 4B. Depending on the severity of the disease as well as favourable weather conditions, the lesions may coalesce and cause the blighting of entire leaves (see Figure 4C). This may cause the death of a plant before it reaches maturity or may result in serious yield losses. Yield losses may be increased even further as the plants are now pre-disposed to other fungal pathogens and root rot that may result in severe lodging (see Figure 4D).

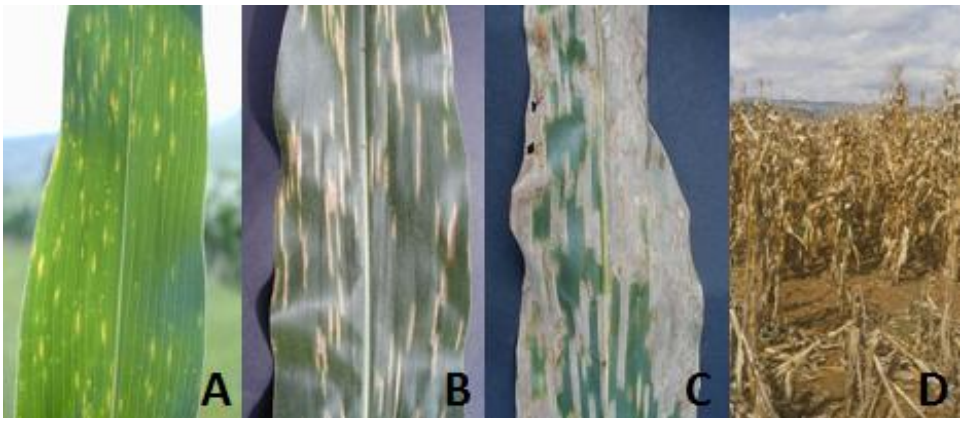

Figure 4. Progressive disease symptoms of GLS disease (Figure adapted from Ward *et al.* (1999). The initial symptoms of GLS are chlorotic spots (A) which can easily be seen when the affected leaf is exposed to sunlight. Chlorotic spots can easily be confused with the initial symptoms of other foliar pathogens. The mature GLS lesions (B) are the characteristic symptoms indicative of GLS. Under favourable conditions such as high humidity and high temperatures, the mature GLS lesions can coalesce causing the blighting of entire leaves (C). In severe conditions, GLS may predispose maize to root rot causing lodging of maize plants and major yield losses (D).

2.3.2 Disease cycle

Once a maize plant has been infected with *C. zeae-maydis* or *C. zeina*, GLS disease is able to spread and infect more maize plants. *C. zeae-maydis* and *C. zeina* are not seed borne (McGee 1988; Richardson 1990), but are able to overwinter in infested residues of maize crops from the previous season. The GLS disease cycle in maize is outlined in figure 5. The spores (conidia) produced in the crop debris are wind-blown onto the lower leaves of the newly planted maize and cause primary infection. The conidia can be dispersed by either wind or rain splash from the lower leaves to the rest of the plant causing a secondary infection (Ward *et al.* 1999). The most serious infections occur in cases where primary infections are seen on the middle to upper canopy leaves as a result of wind-blown conidia from neighbouring plants. This type of infection occurs a bit later in the planting season and causes devastating yield losses as the upper canopy contributes the most to the photosynthate needed for grain fill (Allison 1966). Figure 5 shows a detailed overview of the disease cycle of GLS.

One of the most important factors playing a vital role in the development of GLS disease is favourable environmental conditions. For a successful infection, prolonged and frequent periods of high humidity and warm temperatures are needed (Ward *et al.* 1999). When the weather conditions are unfavourable, conidia are able to stay dormant until the weather conditions become favourable again (Latterell and Rossi 1983).

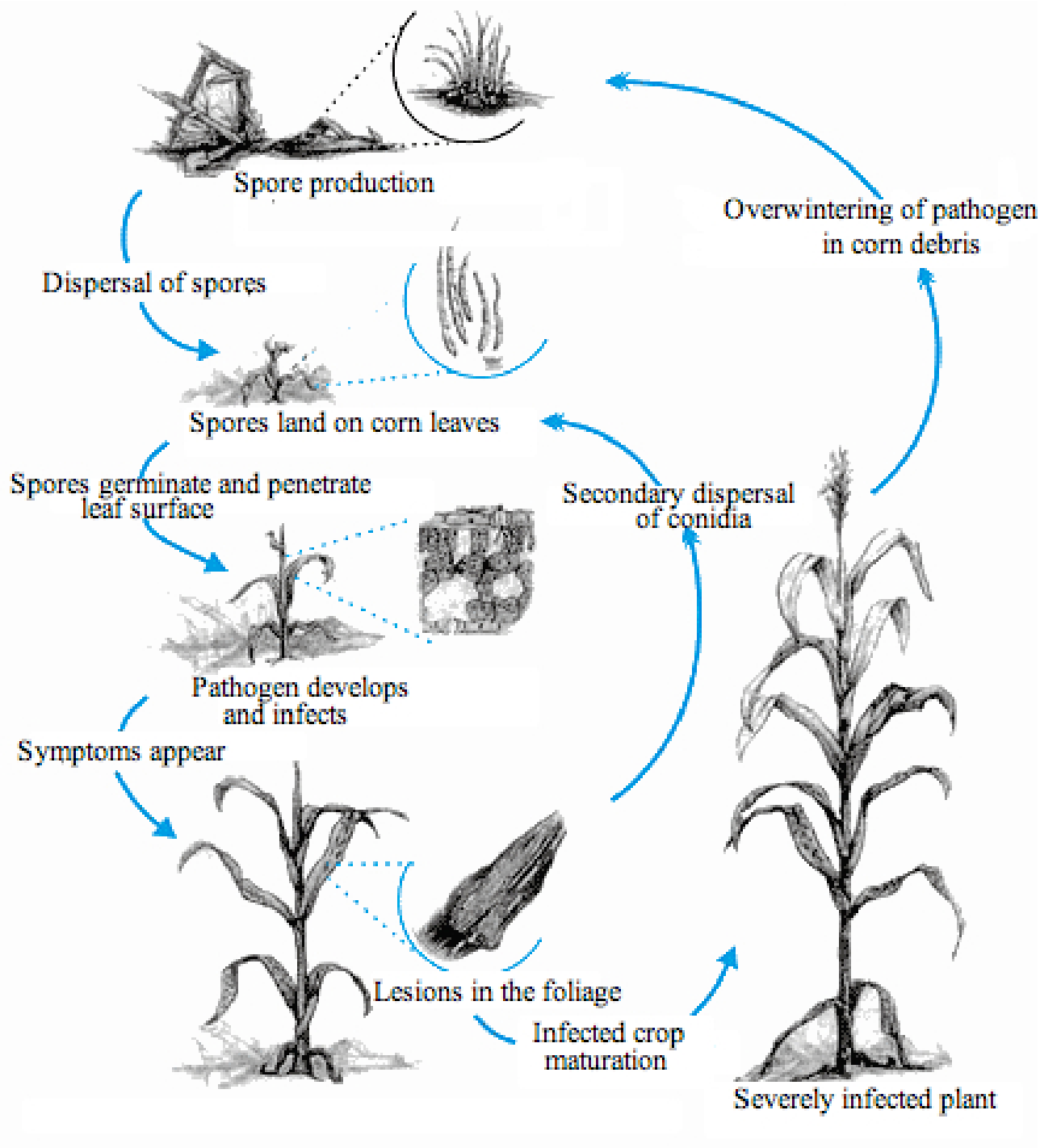

Figure 5. The disease cycle of Grey Leaf Spot (GLS) in maize. The figure shows the disease cycle of GLS in maize as well as the possibility of secondary infection which causes severe yield loss. Most importantly, the pathogen has the ability to overwinter in maize debris and cause infection during the next season (WARD *et al.* 1999).

2.3.3 Infection strategy of *Cercospora zeae-maydis* and *Cercospora zeina*

C. zeae-maydis and *C. zeina* are the casual agents of GLS disease. These two foliar pathogens are from the Dothideomycete class of ascomycete fungi which includes many plant pathogens, and both *C. zeae-maydis* and *C. zeina* are haploid and share hemibiotrophic lifestyles. The infection strategy of *C. zeae-maydis* was described by Beckman and Payne in 1982. Beckman and Payne showed that *C. zeae-maydis* infects maize leaves through a process of external growth on the maize leaf surface followed by penetration of the stomata, internal colonization that is confined to the intercellular spaces of the mesophyll cell layer, and finally sporulation (BECKMAN and PAYNE 1982). This process of infection is similar to the infection strategy used by many *Cercospora* species infecting other plant species. Some examples include *C. beticola* infecting sugar beet (RATHAIAH 1977) and *Mycosphaerella fijiensis* causing Black Sigatoka disease of banana (MEREDITH 1970).

The *C. zeae-maydis* infection from the point of inoculation to mature, sporulating lesions takes about three weeks. Under environmental conditions of high relative humidity and warm temperatures between 22-30ºC, fungal spores (conidia) are able to germinate on the maize leaf surface and form germ tubes within 24 hours after inoculation. The germ tubes show positive stomatal tropism (Figure 6) as they grow towards the stomata in the absence of free water on the leaf surface. Abundant appressorial formation over stomatal openings can be seen after 4-5 days and is followed by penetration of the stomata through penetration pegs at 6-7 days. A single conidium can form up to eight appressoria over different stomatal openings and more than one appressorium can be formed over a single stomatal opening (BECKMAN and PAYNE 1982). A single penetration peg can be formed by each appressorium and penetration only occurs through stomatal openings (BECKMAN and PAYNE 1982). From experience obtained in our research group, *C. zeina* infection shows a similar rate of infection as reported for *C. zeae-maydis*, with infection from the point of inoculation to mature, sporulating lesions also taking about three weeks. These disease stages for *C. zeina* were noted from microscopy observations of lesions obtained in the glasshouse. However electron and confocal light microscopy is needed to confirm specific disease stages for *C. zeina*.

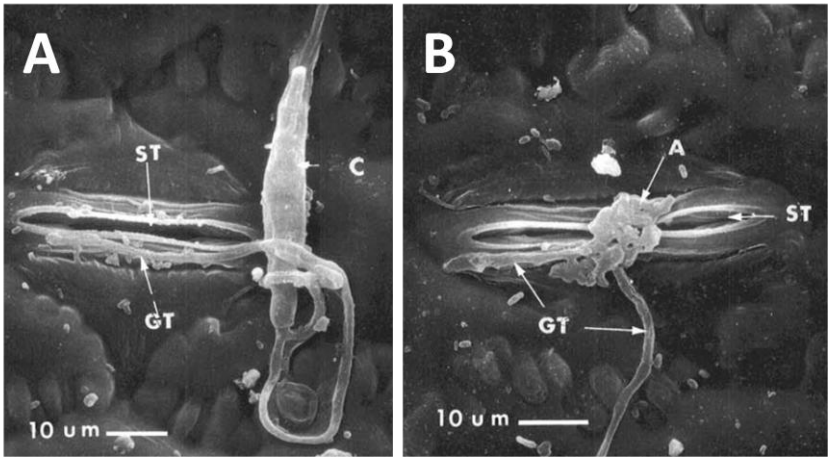

Figure 6. Stomatal tropism and appressorial formation (Figure adapted from Beckman and Payne 1982). After conidia lands on the maize leaf surface, the germ tubes that form show positive stomatal tropism by growing towards the stomata (A) and forming appressoria (B) over the stomatal openings. The figures are scanning electron micrographs. A= appressorium, C= conidium, GT= germ tube, and ST= stomata.

The internal colonization of *C. zeae-maydis* after penetration is characterized by intercellular growth of fungal hyphae that is restricted to the air spaces and intercellular spaces of the mesophyll cell layer (BECKMAN and PAYNE 1982). This form of growth gives rise to the characteristic GLS disease lesions that are confined by and parallel to the leaf veins (BECKMAN and PAYNE 1982). Fungal conidiophores grow through the stomatal openings to form conidia. The mature lesions become grey in colour as the fungus sporulates and produces conidia for secondary infections of neighbouring maize plants (BECKMAN and PAYNE 1982). The fungal mass is able to remain alive in the necrotic, dying leaf material and can overwinter in the leaf debris to produce the primary inoculum in the next growing season (BECKMAN and PAYNE 1982). *C. zeae-maydis* has been shown to be able to grow on the maize leaf surface for at least seven days before infection occurred. This latent period is due to the pathogen's hemibiotrophic lifestyle. Other *Cercospora* species also have latent periods as reported for *C. musae* which has a latent period of up to 120 days (MEREDITH 1970).

I would propose a similar infection mechanism for *C. zeina*, as *C. zeae-maydis* as *C. zeina* causes indistinguishable disease symptoms and is very closely related. Preliminary evidence from our research laboratory with a GFP-labelled *C. zeina* strain suggests that this is the case (A. Visser, personal communication). The proposed infection strategy for *C. zeina* is outlined in Figure 7, where the biotrophic as well as the necrotic growth stages of this hemibiotroph are shown.

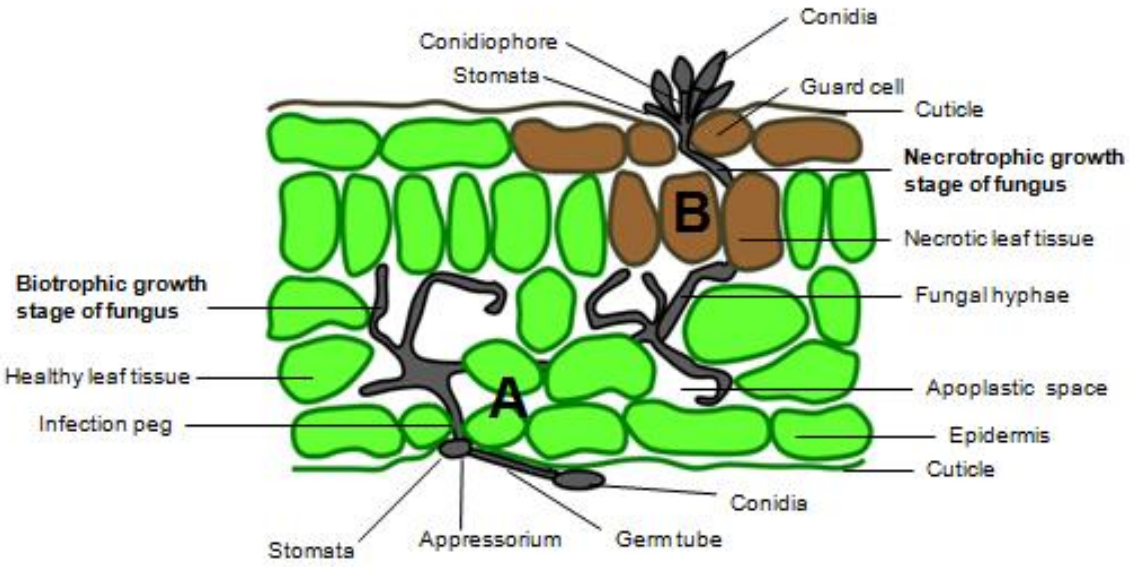

Figure 7. Proposed mechanism of infection for *C. zeina* adapted from the infection strategy described by Beckman and Payne (1982). It is proposed that *C. zeina* infects maize leaves in a similar way as described for *C. zeae-maydis*. After conidial germination on the maize leaf surface, a germ tube forms that will grow towards and over the stomata where it will form an appressorium. An infection peg will grow out of the appressorium and through the stomatal opening into the apoplastic space. The fungal hyphae will grow intercellularly without causing damage to the maize leaf during the initial biotrophic growth stage (A) of the fungus. The necrotrophic growth stage (B) is characterized by dying leaf material. The fungus is able to remain alive during this second growth stage and conidiophores will grow out of the stomata and produce conidia for secondary infections of neighbouring maize plants. Figure drawn by B. Lombard.

2.4 GLS management strategies

Although some *Cercospora* species have been found to infect a broad host range, *Cercospora* species are generally limited to a specific host genus (GROENEWALD *et al.* 2013). *C. zeae-maydis* and *C. zeina* have only one known host namely maize (STROMBERG and CARTER 1991). The only source of inoculum is the spores that can stay dormant in maize debris during unfavourable weather conditions. Although a lot of disease management practices exist, none of these methods have effectively solved the disease problem (WARD *et al.* 1999). Existing management strategies can be divided into three main categories namely: agronomic practices, fungicides and genetic resistance.

2.4.1 Agronomic practices

Some of the agronomic practices used to manage GLS include tillage practices and crop rotation. Tillage practices reduce the amount of initial inoculum that is able to overwinter in maize debris by burying the previously infected maize material. It has also been shown to be effective in regions with lower levels of inoculum and where GLS has not been established

(LATTERELL and ROSSI 1983; PERKINS *et al.* 1995). In areas where GLS is established, primary inoculum may be dispersed from one field to a neighbouring field by means of the wind. One of the disadvantages of tillage practices is that the moisture content of the soil is lowered. Lower moisture levels create favourable conditions for spores to germinate and cause infection. Therefore, tillage practices may have no or little effect in managing GLS. Another problem found in South Africa where primary inoculum can be increased, is during the production practice where farmers let maize dry down before harvesting. This allows infected leaf tissue to be dispersed by winds to neighbouring fields, which may act as a source of inoculum during the next planting season (WARD *et al.* 1996).

Crop rotation is when a season of planting maize for example, is followed by a season of planting alternate crops. This provides a lot of benefits such as the improvement of soil quality, soil moisture stability and the reduction of maize soil pathogens (WARD *et al.* 1999). Other agronomic factors that might also decrease GLS severity are the time of planting, plant density and time of irrigation (WARD *et al.* 1999).

2.4.2 Genetic resistance

Resistant hybrids provide a cost-effective management strategy. Resistant germplasm can be developed through the incorporation of resistance factors from a donor into the selected maize germplasm (GORDON *et al.* 2004). However, the higher the level of resistance required, the more time consuming this practice becomes and these hybrids have been shown to have lower yield potential (WARD *et al.* 1999).

Disease resistance to GLS in maize tends to be quantitative rather than qualitative (CLEMENTS *et al.* 2000; GEVERS *et al.* 1994; LEHMENSIEK *et al.* 2001; WISSER *et al.* 2006). The qualitative response mediated by resistance (*R*) genes is associated with highly effective, complete resistance; however a resistance response is only temporary and race-specific (CHUNG *et al.* 2010). *R*-gene resistance is characterized by a gene-for-gene reaction where the host *R*-gene recognizes the pathogen effector through a direct or indirect interaction causing a host response (JONES and DANGL 2006). No gene-for-gene resistance has been shown for GLS. The report of a major gene for GLS resistance (GEVERS *et al.* 1994) was actually two quantitative trait loci (QTLs) that conferred the resistance (GORDON *et al.* 2004).

A lot of research has gone into how resistance to GLS is controlled. Previous studies have reported that GLS resistance is inherited quantitatively (GEVERS *et al.* 1994; HUFF *et al.*

1988; MANH 1977; THOMPSON *et al.* 1987; ULRICH *et al.* 1990). Quantitative disease resistance (QDR) is controlled by multiple genes with minor effect (ROSS 1986; VAN DER PLANK 1963) and characterized by a more durable, intermediate resistance response (PARLEVLIET 2002; POLAND *et al.* 2011). Many studies have also been undertaken to determine the heritability as well as to analyse QTL of maize resistance to GLS (GORDON *et al.* 2006; ININDA *et al.* 2007; MENKIR and AYODELE 2005). Maize resistance to GLS was shown to be controlled predominantly by additive gene action, with some dominance effects (COATES and WHITE 1998; GEVERS *et al.* 1994) and showed moderate to high inheritance patterns (CLEMENTS *et al.* 2000; GORDON *et al.* 2006; HUFF *et al.* 1988; THOMPSON *et al.* 1987). Since the first publication of a QTL associated with GLS (BUBECK *et al.* 1993), more QTLs for GLS resistance have been identified and mapped in many studies of different populations under diverse environmental conditions. Recently identified QTLs for GLS resistance are summarised in Table 1. Even though many QTLs for GLS resistance have been identified, there is still a lack of information on the underlying genetic basis and mechanisms of defence involved (GEIGER and HEUN 1989; KELLY and VALLEJO 2006; YOUNG 1996). A review on recent advances in research on quantitative disease resistance (QDR) has compiled a few plausible hypotheses with evidence on the mechanisms underlying QDR (POLAND *et al.* 2009). Mechanisms proposed in this review included QDR regulation through genes affecting phenotypic characteristics, and QTLs being involved in different aspects such as defence signal transduction, being weak forms of *R*-genes, forming part of previously unidentified genes, being components of chemical warfare, and representing mutations of receptors involved in basal defence.

Table 1. A summary of the major QTLs identified and validated for GLS resistance that have been mapped to all ten maize chromosomes. The table gives information on the chromosome locations, the effect the individual QTLs have on the phenotypic variance associated with GLS severity, as well as the authors that described the QTLs.

QTLs	Chromosome (Bin location if available)	Phenotypic variance component[a]	Authors
3	2.04, 4.07, 4.08	4-26%	(BUBECK *et al.* 1993)
3	1, 4, & 8	56, 14 & 11%	(MAROOF *et al.* 1996)
5	2.05, 4.03	51-58.7%	(CLEMENTS *et al.* 2000)
4	1.05/1.06, 3.04, 5.03/5.04 & 5.05/5.06	37, 8-10, 11, & 11%	(LEHMENSIEK *et al.* 2001)
2	2.09 & 4.08	40-47%	(GORDON *et al.* 2004)
5	1.05, 2.04, 4.05, 9,03, & 9.05	<12%	(BALINT-KURTI *et al.* 2008)
2	2.06 & 2.08	Not available	(DANSON *et al.* 2008)
4	1,2,5 & 8	2.53-23.90%	(JULIATTI *et al.* 2009)
3	1.05, 1.07, & 3.07	<10%	(POZAR *et al.* 2009)
3	2.02-2.03, 7.02 & 10.05	11.2, 9.9 & 16%	(ZWONITZER *et al.* 2009)
2	1.04 & 8.05	Not available	(CHUNG *et al.* 2011)
4	1, 2, 5, & 8	2.53-23.90%	(ZHANG *et al.* 2012)
2	4.08 & 2.09	Not available	(ASEA *et al.* 2012)
7	1.10, 4.08, 9.04/9.05, 10.06/10.07, 6.06/6.07, 7.02/7.03, 9.06	>11%	(BERGER *et al.* 2014)

[a] Phenotypic variance as a result of genetic factors and environmental factors

2.4.3 Fungicides

Fungicides and resistant hybrids go hand in hand as a management strategy of GLS. Effective fungicide control can improve maize yield potential significantly. In order to effectively control GLS, fungicides consisting of a combination of different chemical groups are used. Some of the registered chemical groups used are triazoles and benzimidazoles (WARD *et al.* 1999). The reason for using combinations of fungicide chemical groups to manage GLS is to prevent or delay the occurrence of pathogen resistance to a specific chemical.

The most effective fungicides applied presently, include these listed below.

- AMISTAR® is the world's current top fungicide and is produced by Syngenta. Amistar® has Azoxystrobin as its active ingredient and has fungicidal activity. This broad-spectrum fungicide inhibits fungal mitochondrial respiration and results in consistent plant yield and quality benefits.

- ABACUS® produced by BASF is a broad-spectrum fungicide that has pyraclostrobin and epoxiconazole as its active ingredients and also inhibits fungal respiration, but in a site-specific manner.
- CELEST® XL also produced by Syngenta, which can be applied to maize seeds as a broad-spectrum fungicide with the active ingredient fludioxonil. The fungicide interferes at various points in the fungal life cycle, inhibiting conidial germination, germ tube formation, mycelia growth etc.

Even though fungicides can be an effective method of control, they are expensive and pathogens become resistant over a certain period of time (WARD *et al.* 1999). They are also not readily available to subsistence farmers in Africa.

The present conventional management practices to control GLS have been inefficient and unsustainable (BIGIRWA *et al.* 2001; DANSON *et al.* 2008). In order to effectively control GLS epidemics, conservation tillage would need to be reduced globally (LIPPS *et al.* 1996). Fungicides have been effective in a certain way, but are costly and not a durable control method as the fungus can build resistance towards these chemical compounds (WARD *et al.* 1997c). As maize is mostly cultivated by small-scale farmers (FAO 2012), there is a need for resistant hybrids. Resistant hybrids are an economical management strategy to control GLS, but hybrids do not possess high enough resistance to survive GLS epidemics (LIPPS *et al.* 1996). The main limitation of breeding for GLS resistance is the frequent interactions seen between the genotype and the environment (DANSON *et al.* 2008). Moreover, hybrids still have poor agronomic characteristics which include low yield potential, susceptibility to other diseases, and slower development, which complicates the breeding process (BUBECK *et al.* 1993; COATES and WHITE 1994; ELWINGER 1990; GEVERS *et al.* 1994; GRAHAM *et al.* 1993; HILTY *et al.* 1979; THOMPSON *et al.* 1987; ULRICH *et al.* 1990). As no effective form of control for GLS is available, it is important to gain insight into the molecular mechanisms of fungal infection in order to establish a more specific and effective method to control GLS and to be able to uphold high yields of maize.

3. PLANT-FUNGAL INTERACTIONS

3.1 Plant-fungal interactions and resistance

During a plant-pathogen interaction, the plant must be able to detect the pathogen and be able to respond to its attack in order to defend itself. The pathogen on the other hand, must be able to manipulate the plant's biology in order to be able to grow and reproduce. This process can be seen as a form of communication between the plant and the pathogen by means of gene products (BOYD *et al.* 2013). The plant is able to defend itself against pathogen attack on two levels (JONES and DANGL 2006). The first level of defence is PAMP-triggered immunity (PTI) which involves the recognition of pathogen associated molecular patterns (PAMPs or MAMPs) which, upon recognition by plant receptors, are able to trigger a plant defence response. The pathogen has evolved to overcome this first level of defence by means of effectors that are able to suppress an immune response. The second level of plant defence is thus referred to as effector-triggered immunity (ETI) and involves the recognition of effector proteins (avirulence (Avr) proteins) by specific host resistance genes (*R* genes) and the subsequent stronger immune response that is associated with ETI. This constant arms race between the fungus and the plant was proposed by Jones and Dangl in the form of a zig-zag model (See Figure 8) (JONES and DANGL 2006). The Avr/R-gene specific recognition interaction was described by means of the gene-for-gene model for resistance proposed by Flor (FLOR 1942).

Pathogen associated molecular patterns (PAMPs) are pathogen molecules that are critical for pathogen survival, unable to withstand modification, and are conserved across a broad range of isolates (BOYD *et al.* 2013). Examples of PAMPs include oomycete heptoglucans (MONAHAN and ZIPFEL 2012), fungal xylanase, and most importantly chitin which is one of the major constituents of fungal cell walls (see Figure 9) and one of the best studied fungal PAMPs (FELIX 1993). PAMPs are recognized by means of plant pattern recognition receptors (PRR proteins) (CHISHOLM *et al.* 2006) and this recognition interaction is characterized by PTI (BENT and MACKEY 2007; BOLLER and HE 2009; MONAHAN and ZIPFEL 2012). For example, PTI is initiated when chitin is recognized by rice LysM domain-containing receptor-like protein (RLP) CEBiP (KAKU *et al.* 2006). An interesting finding was that PRRs have been identified that are able to detect released peptides or cell wall fragments as a result of wounding or infection. These peptides and cell wall fragments are referred to as damage-associated molecular patterns (DAMPs) (DE LORENZO *et al*. 2011)

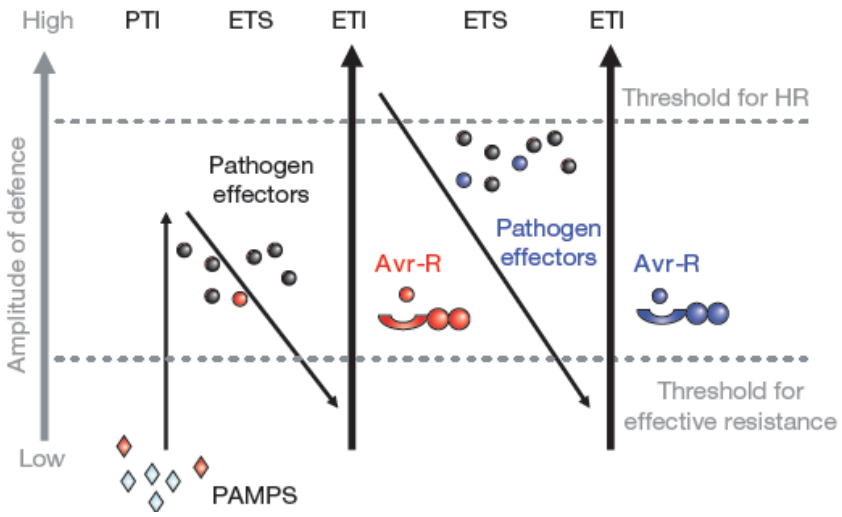

Figure 8. The proposed zig-zag model of plant-pathogen interactions (Jones and Dangl 2006). Plants are able to detect pathogen associated molecular patterns (PAMPs), indicated as red diamonds, by means of PAMP recognition receptors (PRRs) and are able to trigger PAMP-triggered immunity (PTI) in the host. Pathogens are able to evolve in order to escape recognition by means of effector proteins (indicated in red), causing effector-triggered susceptibility (ETS) in the host. The plant, in turn, is able to evolve by means of developing resistance (*R*)-genes that are able to detect pathogen effectors and cause an effector-triggered immune response (ETI) in the host. ETI is often a stronger immune response than PTI, and passes the threshold of induction of a hypersensitive response (HR) in the host. Pathogens evolve to produce new effectors (indicated in blue) that are able to suppress ETI. Plants can keep on evolving to produce new *R*-genes that are able to detect new effectors and cause ETI again.

In response to PRRs, pathogens have developed effectors which target plant defence pathways in order to achieve successful infection. Effectors provide a way by which the pathogen can manipulate the plant's cellular environment in order to suppress a defence response and to create a favourable environment to enable infection and the future existence of its kind (KOECK *et al.* 2011). An example is the rice blast fungus *Magnaporthe oryzae* that secretes the Slp1 effector protein that is able to sequester chitin oligosaccharides, in a similar fashion as seen with *Cladosporium fulvum* Ecp6, to prevent its detection and the subsequent host immune response (MENTLAK *et al.* 2012).

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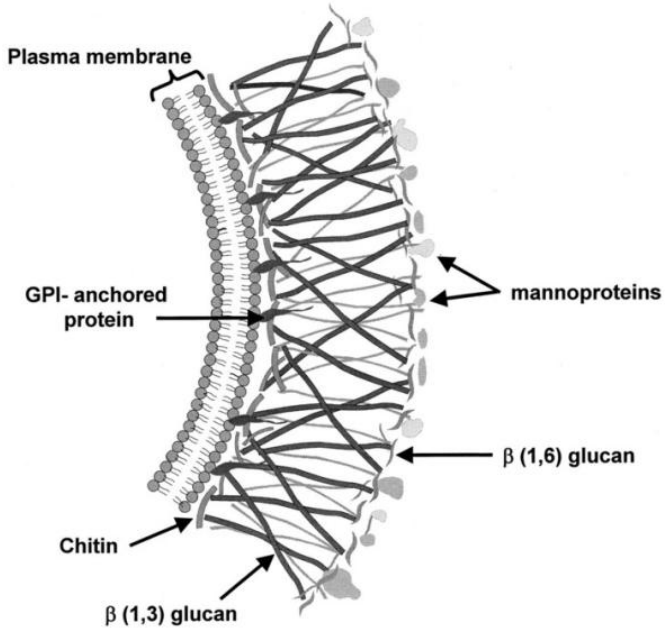

Figure 9. The fungal cell wall (www.glogster.com). The fungal cell wall mainly consists of chitin, glucans and mannoproteins. Chitin is a well-studied fungal pathogen associated molecular pattern (PAMP).

Plants (hosts) in turn, have evolved to develop specific resistance genes (*R* genes) that are able to detect these effector proteins and initiate ETI. To date, there are many *R*-genes that have been cloned from different crop species (GURURANI *et al.* 2012). *R*-genes are able to recognize effector proteins by either directly binding to the effector protein, or by means of an indirect interaction where the *R*-gene interacts with another plant protein, the "guard protein", that is able to detect changes in the virulence target of the effector protein (guard hypothesis) (VAN DER BIEZEN and JONES 1998; VAN DER HOORN and KAMOUN 2008).

3.2 Gene-for-gene model of resistance

The gene-for-gene model of resistance describes an interaction between a host (plant) and a pathogen where the plant's resistance as well as the pathogen's ability to cause disease, is controlled by pairs of matching genes. These genes are plant resistance genes (*R* genes) and pathogen avirulence genes (*Avr* genes). An incompatible interaction is determined by the presence of a complementary *R* gene in the host and can be characterized by a hypersensitive response which serves as a local mechanism to inhibit spread of the pathogen. The host is able to recognize the pathogen *Avr* gene product (race-specific elicitor) through a direct or indirect interaction between the host resistance gene product (receptor) and the complementary pathogen elicitor. On the other hand, a compatible reaction is determined by the presence of a pathogen avirulence gene and the absence of a complementary host resistance gene. The host is therefore susceptible and the pathogen

will be able to cause disease as it is able to remain undetected within its host (SHABAB *et al.* 2008).

3.3 Defence signalling in plants

Plant defence responses can be associated with decreased fitness, as defence strategies require investment and the associated cost from the plant's side. Therefore, inducible plant defence systems are tightly regulated so that they are only induced when necessary. Some of the major plant defence responses include preformed and induced defence responses. Preformed defence responses can be referred to as the basal resistance of the plant and unspecific defences induced as a result of PTI. An example of a basal defence mechanism is chemical barriers (phytoanticipins) that restrict pathogen access (ANDERSON *et al.* 2010; MORRISSEY and OSBOURN 1999). Defences induced as a result of PTI can be in the form of physiological, morphological and molecular changes (ALTENBACH and ROBATZEK 2007). PTI is a broad-spectrum defence mechanism and is effective against most potential pathogens (SHAN 2007). Initial changes include destabilization of ions across the plasma membrane (ionic flux), protein phosphorylation, and an oxidative burst (SCHWESSINGER and ZIPFEL 2008), which leads to changes in transcription. Later physical changes include a barrier to pathogen growth in the form of callose deposition, and the closing of stomata (ZHANG *et al.* 2008), which can be an entry point for many plant pathogens. Defences induced as a result of ETI include a variety of antimicrobial defences that aim to reduce the pathogen's ability to cause disease. Other forms of plant defence are pathogen tissue-degrading enzymes like proteases, chitinases and amylases (CHEN 2008; LAVANIA *et al.* 2006) and reactive oxygen species (ROS) production. Detection of pathogen effectors by host resistance genes trigger a hypersensitive defence response characterized by programmed cell death. Plant hormones also play an important role as plant defence signalling molecules. The defence signalling hormone, salicylic acid (SA), plays an important role during PTI by inducing pathogenesis related gene expression and systemic acquired resistance (SAR) (SATO *et al.* 2007; TSUDA *et al.* 2008). Jasmonic acid (JA) is critical for plant defence against herbivory and has been shown to aid in communication between plants in anticipation of attack by pathogens or other biotic stresses (BALDWIN *et al.* 2006). Ethylene, auxin, cytokinin, absissic acid (ABA), giberillin, and brasinosteroids also play a role in plant defence responses by inhibiting pathogen colonization (BARI and JONES 2009; ROBERT-SEILANIANTZ *et al.* 2007).

3.4 Function and evolution of fungal effectors

This section considers the possible roles of effector proteins during plant-fungal interactions as seen in Figure 10, as well as the diversification processes. Effector proteins produced by

filamentous fungi are synthesized in the endoplasmic reticulum and secreted through vesicles formed by the Golgi apparatus (DE JONGE *et al.* 2011). Effectors can either be secreted directly into the apoplastic space (extracellular space) or they can be delivered by a yet unknown mechanism(s) into the host cell cytoplasm (intracellular space). It is postulated that effector proteins are mainly secreted from fungal hyphal tips in the case of fungi limited to extracellular space, whereas effector proteins from fungi growing intracellularly (such as rusts, powdery mildews etc.) have been shown to be secreted by the haustoria (PANSTRUGA and DODDS 2009). Another interesting mechanism of effector delivery through an interfacial biotrophic complex has been discovered for the intracellular fungus *Magnaporthe oryzae* (KANKANALA *et al.* 2007). Therefore, although all effectors are delivered apoplastically, many are further translocated into intracellular spaces.

After excretion into the apoplastic space (extracellular space), certain effectors may function within this region by suppressing host defence responses, protecting the pathogen from host hydrolytic enzymes that are present in this space, and by scavenging PAMPs in order to prevent a host immune response (Figure 10). These apoplastic effector functions have been characterized into groups. Many apoplastic effectors have been well characterized, for example the *Cochliobolus carbonum SNF1* gene and the NLP virulence factors that function as necrosis and ethylene-inducing effectors (OTTMANN *et al.* 2009; TONUKARI *et al.* 2000). Another group of apoplastic effectors with a cell wall-degrading enzymatic function have also been well characterized and do not show conservation between fungal pathogens (MA *et al.* 2010; SPANU *et al.* 2010). The third group of apoplastic effectors can generally be characterized as secreted proteins that are small in size, cysteine-rich, and play a role in fungal virulence as seen in *Cladosporium fulvum* (Currently known as *Passalora fulva*, but to be consistent with previous literature it will be referred to as *C. fulvum* in this thesis). Cysteine-rich effectors are generally conserved between isolates and even species.

Apoplastic effectors are processed either N- or C-terminally before translocation as seen with the *C. fulvum* Avr and Ecp effectors (STERGIOPOULOS and DE WIT 2009). Other effectors are translocated to the host cytoplasmic space by possibly exploiting host translocation machinery (Figure 10). Cytoplasmic R-proteins that are able to recognize fungal effectors provide evidence for cytoplasmic translocation (ELLIS *et al.* 2007; RAFIQI *et al.* 2010). Insight into a possible method of effector translocation into host cells as reported recently (KALE *et al.* 2010). Several predicted oomycete effectors have been shown to contain an N-terminal RxLR motif (REHMANY *et al.* 2005) that is proposed to be a possible mechanism by which the translocation of oomycete effectors is mediated (DOU *et al.* 2008; WHISSON *et al.* 2007).

Although more insight is needed into the functions of these translocated effectors (DE JONGE *et al.* 2011), it is believed that these effectors affect cytoplasmic processes involved in host defence response.

A different group of effectors are associated with translocation to the host nucleus and are predicted to interfere with the transcription of target genes (Figure 10). A possible translocation mechanism was shown through a motif that was discovered for crinkler effectors, in oomycetes, which target the host nucleus (SCHORNACK *et al.* 2010). In the biotrophic fungus *M. oryzae*, live-cell imaging was used to show that effectors secreted by invasive hyphae are collected in a specialized structure called the biotrophic interfacial complex (BIC). The BIC localized secreted effectors are then translocated into the host cytoplasm, where they are able to move from cell to cell ahead of the growing hyphae and regulate cellular processes as well as neutralise host defence responses (KHANG *et al.* 2010).

The mechanisms underlying the birth and diversification of Dothideomycete effectors remain unclear. It is expected that mutations are the most prominent source of diversity for an asexually reproducing pathogen such as *C. zeina* (ANDERSON *et al.* 2010). These mutations are in the form of small insertions, deletions, and point mutations which may in turn cause elements in the genome to be transposed to another area of the genome. Effectors might therefore reside in subtelomeric regions of the genome where mutations are more frequent (FARMAN 2007). Another possibility for diversification of effectors is horizontal gene transfer (HGT). During HGT, fungal hyphae undergo anastomosis which provides an opportunity for cytoplasmic or nuclear factors to recombine. These recombination events range from being discrete to whole nuclei that can be exchanged. Unfortunately, little evidence for fungal gene transfer events are available (FRIESEN *et al.* 2006; OLIVER and SOLOMON 2008).

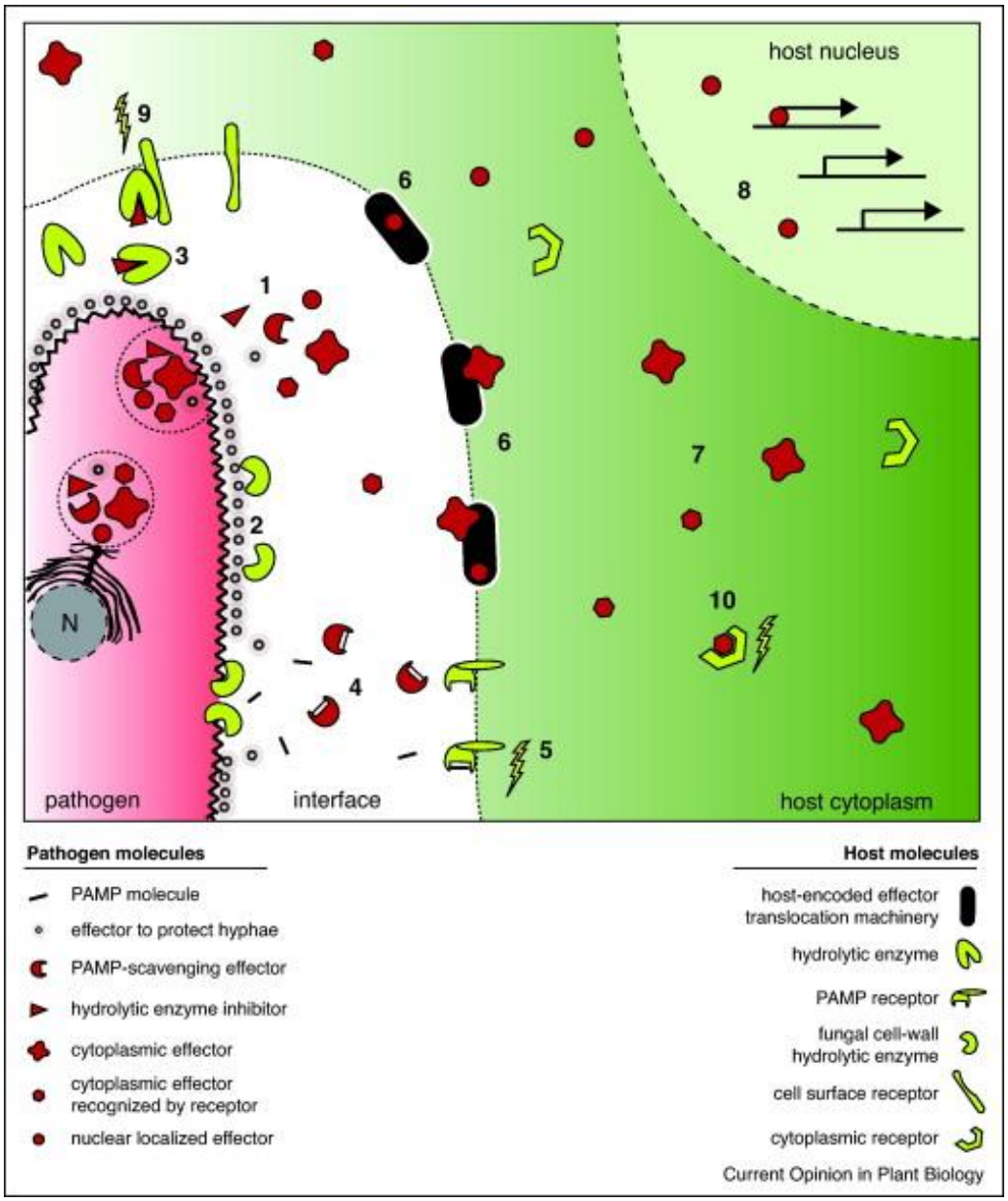

Figure 10. The role of effector proteins during plant-fungal interactions (DE JONGE *et al.* 2011). Fungal effector proteins are secreted into the apoplastic space after the pathogen has penetrated the host (1). Some effectors play a defensive role in fungal virulence by protecting fungi against hydrolytic enzymes present in the apoplastic space (2), by deactivating these plant defensive hydrolytic enzymes (3), or by binding to potential PAMPs that may be released (4) that are able to activate a host defence response (5). Although some effectors remain in the apoplastic space, others are translocated to the host cytoplasm through non-host translocation machinery (6). Although more insight is needed into how the molecular mechanisms of translocation of effector proteins contribute to fungal virulence, some are expected to target cytoplasmic processes that play a role in host defence (7). Recently it was proposed that some fungal effectors are translocated to the host nucleus and are able to regulate targeted gene transcription (8). Pathogen effectors are recognized by host cell surface receptors that are present in the apoplastic space (9), or by NB-LRR-type receptors (10) that are present in the host cytoplasm.

3.4.1 Plant pathogenic Dothideomycete effectors

Plant pathogenic fungi belonging to the Dothideomycete class are generally considered to be extracellular pathogens that are able to infect plants by penetration through stomatal openings and colonization of the apoplastic space. Specialized feeding structures (e.g. haustoria) are not produced; therefore the fungi need to rely on their specific lifestyle (e.g. hemibiotrophic lifestyle) to manipulate the plant extracellularly to be able to retrieve nutrients. In order to manipulate its host, the fungi will produce and secrete effectors to be able to survive. In this section, some well characterized Dothideomycete effectors will be discussed where after the most important ones relative to this study will be discussed in the subsections.

Cladosporium fulvum Avr9 was the first cloned fungal *Avr* gene (VAN KAN *et al.* 1991). *C. fulvum* is the casual agent of leaf mould in tomato. Identification of the *C. fulvum Avr9* effector produced a model species to study plant-pathogen interactions (JOOSTEN and DE WIT 1999). *Avr9* was followed by the identification *C. fulvum Avr2*, *Avr4*, *Avr4E*. The *C. fulvum* avirulence proteins are all small, cysteine-rich and are recognized by the Cf proteins Cf-9 (THOMMA *et al.* 2005), Cf-2 (DE WIT *et al.* 2009), Cf-4 (JOOSTEN and DE WIT 1999), and Cf-4E respectively. Furthermore, extracellular proteins (Ecps) Ecp1, Ecp2 (DE KOCK *et al.* 2005), Ecp4 (LAUGE *et al.* 2000), Ecp5, Ecp6 (BOLTON *et al.* 2008), and Ecp7 were identified in *C. fulvum*. It is believed that all Avrs and Ecps play a role in virulence (BOLTON *et al.* 2008; THOMMA *et al.* 2005; VAN ESSE *et al.* 2008).

Recently, the virulence factors Czk3 and Crp1 were identified in *C. zeae-maydis*. *C. zeae-maydis* Czk3 is highly similar to mitogen-activated protein (MAP) kinases found in other fungal species. Czk3 regulates the genes involved in cercosporin production and is also involved in conidiation and virulence of *C. zeae-maydis* (SHIM and DUNKLE 2003). Crp1 also regulates pathogenesis processes such as stomatal tropism, the formation of appressoria, and cercosporin production (KIM *et al.* 2011). Crp1 also plays a key role in photoreactivation after extensive UV light exposure (KIM *et al.* 2011). The Cereal Foliar Pathogen research group (CFPR) at the University of Pretoria is currently investigating the role of Czk3 and Crp1 in *C. zeina* pathogenesis.

Cercosporin is a perylenequinone toxin that is produced by *Cercospora* species and an important pathogenicity factor (DAUB and EHRENSHAFT 2000). In severe disease conditions, cercosporin is thought to cause extensive blighting of the upper maize leaves which results in major yield losses (LIPPS 1989). Cercosporin requires light for activation and is able to

cause damage to host cell membranes, through reactive oxygen species (ROS), in order to release nutrients from host cells for the survival of the pathogen (DAUB and EHRENSHAFT 2000). When grown *in vitro* (0.2 x Potato Dextrose Agar) in the presence of light, *C. zeae-maydis* is able to produce cercosporin which can be visualised as a dark red pigment that accumulates in the culture medium after approximately three days (Figure 2B) (BLUHM *et al.* 2008). No *in vitro* production of cercosporin by *C. zeina* has been shown (USDA 2009) which may suggest that cercosporin production is not needed for *C. zeina* pathogenicity. A current study in our research group focuses on the expression of the *C. zeina* cercosporin biosynthesis gene cluster in different *in vitro* and *in planta* conditions to determine if these genes are expressed under specific conditions. This research will shed some light on the molecular mechanism(s) underlying cercosporin production in *C. zeina*.

Avr genes have also been cloned from the casual agent of blackleg disease in Brassica crops (*Leptoshaeria maculans*) (FUDAL *et al.* 2007; GOUT *et al.* 2006; PARLANGE *et al.* 2009), and is also a Dothideomycete fungal pathogen. These *Avr* genes showed over expression during primary leaf infection stages and have been shown to be involved in virulence through complementation studies. Genome-wide searches for putative effector candidates in many Dothideomycete species such as *L. maculans*, *C. fulvum*, *Mycosphaerella graminicola*, *M. fijien*sis and *Dothistroma septosporum* have been initiated (ROUXEL et al. 2011). In future, these whole-genome searches for effectors will be expanded to the *C. zeina* genome sequence.

3.4.2 *Cladosporium fulvum* effectors

In 1994, Joosten and colleagues successfully isolated the *C. fulvum* Avr4 race-specific protein elicitor from the apoplastic fluid of infected tomato leaves (JOOSTEN *et al.* 1994). The Avr4 protein elicitor specifically induces a hypersensitive response (HR) when the complementary resistance gene *Cf-4* is present in the host. However, it has been shown that some natural isoforms of Avr4 are able to bind chitin without being detected by the host *Cf-4* resistance gene (JOOSTEN *et al.* 1997; STERGIOPOULOS *et al.* 2007; VAN DEN BURG *et al.* 2003).

The gene of the isolated Avr4 protein was cloned to obtain the sequence of the encoded avirulence gene. The Avr4 pre-pro-protein (a protein precursor containing a signal peptide sequence for secretion) consists of 135 amino acids (JOOSTEN *et al.* 1994). The 18 amino acid N-terminal signal peptide is cleaved after extracellular targeting and the remaining

amino acids is processed into the mature Avr4 effector protein that contains eight cysteine residues (JOOSTEN *et al.* 1994).

The *Avr4* gene expression is specifically induced *in planta* as *Avr4* transcripts have been detected from six days post inoculation during infection (JOOSTEN *et al.* 1994). Southern Blot analysis confirmed that *C. fulvum Avr4* was present as a single-copy gene in this pathogen (JOOSTEN *et al.* 1994). An important finding of this study was that even a single base-pair mutation in the *Avr4* gene can cause susceptibility in a previous resistant tomato plant as a result of the plant being unable to detect the slightly altered version of the protein. This point mutation replaced one of the eight cysteine residues with a tyrosine residue and is believed to have caused an altered secondary or tertiary protein structure which may affect the binding specificity of the protein, making it unrecognizable by the host.

Surprisingly, *C. fulvum* Avr4 and Avr9 are very similar effectors that are recognized by similar resistance genes. Therefore the possibility existed that these two effectors might have similar functions. Cai and colleagues shed some light on the molecular differences between Avr4 and Avr9 as well as between the resistance genes *Cf-4* and *Cf-9* (CAI *et al.* 2001). Some similarities between the protein sequences of Avr4 and Avr9 include an even number of cysteine residues, with Avr4 having eight cysteine residues and Avr9 having six cysteine residues. Both Avr4 and Avr9 contain genes that encode pre-proteins and contain a signal sequence. Other than the above mentioned similarities, there was no other form of sequence similarity. Interestingly, qualitative analysis *in planta,* showed that *C. fulvum* Avr4 is more active than *C. fulvum* Avr9 (CAI *et al.* 2001).

A comparison of the tomato *Cf-4* and *Cf-9* sequences showed that these genes encode very homologous (more than 91% identity), extracellular membrane-anchored glycoproteins with leucine rich repeat (LRR) domains (JONES *et al.* 1994; THOMAS *et al.* 1997). It was suggested that recognitional specificity of the two Cf proteins lies in the LRRs at the amino-terminus of both proteins, as amino acid variation exists at this region in both Cf proteins (CAI *et al.* 2001).

Differences in the HR caused by recognition of *C. fulvum* Avr4 and Avr9 effectors were also investigated (CAI *et al.* 2001). Differences included distinct necrotic patterns and different developmental stages of necrosis. Although differences were seen in the HR caused by Cf-4/Avr4 and Cf-9/Avr9 interactions, these differences were not due to differences in gene expression, as similar defence-related gene expression patterns were observed for both the *Avr* genes (CAI *et al.* 2001). Differences between Cf-4/Avr4 and Cf-9/Avr9 necrotic patterns

could be due to differences in stability between Avr and/or Cf proteins in different tissues (CAI *et al.* 2001). Therefore, although Avr4 and Avr9 are similar effectors, they have different functions.

Ecp2 was first identified, isolated and purified from apoplastic fluid of *C. fulvum*-infected tomato leaves (WUBBEN *et al.* 1994). The *C. fulvum* Ecp2 protein is 165 amino acids in size. After N-terminal cleavage of 22 amino acids, the remaining protein is processed into a mature protein of 143 amino acids (17 kDa) (VAN DEN ACKERVEKEN *et al.* 1993). The *Ecp2* gene contains a single intron. Immunogold localization was used to determine that Ecp2 can be found in the apoplastic space near the fungal and host cell walls (WUBBEN *et al.* 1994). The expression levels of *C. fulvum* Ecp2 was determined to be very high *in planta* during the colonization of *C. fulvum* in the apoplasic space (VAN DEN ACKERVEKEN *et al.* 1993; WUBBEN *et al.* 1994). Expression of Ecp2 was very low to undetectable *in vitro* (VAN DEN ACKERVEKEN *et al.* 1993; WUBBEN *et al.* 1994).

Ecp6 was discovered through two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis of *C. fulvum* secreted proteins during plant-pathogen interactions (BOLTON *et al.* 2008). *C. fulvum Ecp6* has an open reading frame of 669 bp which is interrupted by two intron regions that are 68 bp and 111 bp, respectively. The *C. fulvum* Ecp6 mature protein consists of 222 amino acids and it was predicted to be 21 kDa in size. The Ecp6 protein has three lysine motifs (LysM domains) that has been shown to function in carbohydrate binding (BOLTON *et al.* 2008). *C. fulvum* Ecp6 is a crucial virulence factor as heterologous expression of Ecp6 in *Fusarium oxysporum* showed an increase in pathogen virulence (BOLTON *et al.* 2008).

3.4.3 Homologs of *Cladosporium fulvum* Avr4, Ecp2 and Ecp6

Although it is been generally accepted that most fungal virulence factors only facilitate virulence on specific host species, this concept was challenged by showing the *C. fulvum* effectors Avr4, Ecp2 and Ecp6 (from here on referred to as CfAvr4, CfEcp2, CfEcp6) have homologs in other fungal species of the Dothideomycete class (DE WIT *et al.* 2012; STERGIOPOULOS and DE WIT 2009; STERGIOPOULOS *et al.* 2010). CfAvr4, CfEcp2, and CfEcp6 homologs were found in *M. fijiensis*, *M. graminicola*, and *D. septosporum* genomes through BLASTp searches (DE WIT *et al.* 2012; STERGIOPOULOS and DE WIT 2009), whereas CfAvr4 homologs in *Cercospora* genomes could be obtained through a PCR-based approach (STERGIOPOULOS *et al.* 2010).

A functional homolog for the CfAvr4 was found in *M. fijiensis* and even though it shared low sequence identity with its *C. fulvum* homolog, MfAvr4 was still able to trigger an HR in

tomato. However, the response triggered was weaker and less rapid when compared to the response triggered by CfAvr4 (STERGIOPOULOS *et al.* 2010). The MfAvr4 protein consists of 121 amino acids (aa) of which 21 aa contain the signal peptide, has a functional chitin-binding Peritrophin-A domain, and 10 cysteine residues compared to the eight conserved cysteine residues in CfAvr4. Homologs of CfAvr4 were also found in *Cercospora* species (*C. beticola*, *C. zeina*, *C. apii*, and *C. nicotianae*). All four *Cercospora* Avr4 homologs identified (CbAvr4, CzAvr4, CaAvr4, and CnAvr4) have high sequence similarity in common. The *Cercospora* protein homologs each consist of a signal protein of 19 aa followed by 116 aa (*C. beticola, C. apii*) or 115 aa (*C. nicotianae, C. zeina*) with a conserved chitin-binding Peritrophin-A domain and nine conserved cysteine residues (STERGIOPOULOS *et al.* 2010). It has not been shown that CnAvr4 (*C. nicotianae* Avr4) or CbAvr4 (*Cercospora beticola*) are detected by Cf-4, as no HR was observed in MMCf-4 and MMCf-0 (Money Maker - MM) tomato lines inoculated with CnAvr4 or CbAvr4 (STERGIOPOULOS *et al.* 2010). A functional homolog of CfAvr4 has also been identified in *D. septosporum* and is also detected by Cf-4 causing an HR (DE WIT *et al.* 2012).

Three homologs of the CfEcp2 effector have been found in *M. fijiensis* of which one showed the induction of different levels of necrosis or HR in the presence of the putative Cf-Ecp2 resistance gene (STERGIOPOULOS *et al.* 2010). The three MfEcp2 effector proteins each contains a signal protein of approximately 19 aa, followed by 161 aa (MfEcp2), 174 aa (MfEcp2-2), and 236 aa (MfEcp2-3) respectively. All three MfEcp2 proteins and the CfEcp2 protein have four conserved cysteine residues and a conserved intron spanning the third cysteine residue. The independently conserved intron regions in all three MfEcp2 proteins and in CfEcp2 is statistically very unlikely to occur by chance, and therefore suggests a common evolutionary origin (STERGIOPOULOS *et al.* 2010). These authors also identified three CfEcp2 homologs in *M. graminicola*, namely MgEcp2, MgEcp2-2 and MEcp2-3. The three MgEcp2 homologs each contain 179 aa, 170 aa, and 159 aa respectively, followed by a signal peptide of approximately 20 aa. All three *M. graminicola* homologs contain four conserved cysteine residues, but only MgEcp2 has the conserved intron as MgEcp2-2 and MgEcp2-3 lack intron sequences. Three homologs of CfEcp2 were also identified in *D. septosporum.* It was shown that the Ecp2-1 homolog in *D. septosporum* is a functional homolog of CfEcp2 as it causes an HR in the presence of the Cf-Ecp2 resistance gene (DE WIT *et al.* 2012).

Even though homologs of effector genes outside the Dothideomycete class have not been reported previously, homologs of CfEcp6 and CfEcp2 (termed Hce2 effectors) have been found across many other fungal species (BOLTON *et al.* 2008; STERGIOPOULOS *et al.* 2012;

STERGIOPOULOS *et al.* 2010). Homologs of *C. fulvum* Ecp2 (Hce2) all contain an Hce2 domain, which is a putative necrosis-inducing domain (STERGIOPOULOS *et al.* 2010). Ecp6 homologs are referred to as LysM effectors as these effectors contain different amounts of LysM domains and no other recognizable protein domains (DE JONGE and THOMMA 2009). LysM effectors share low sequence identity, are predicted to be secreted, and are involved in the direct binding of chitin (DE JONGE and THOMMA 2009; OHNUMA *et al.* 2008).

3.4.4 Functions of Avr4, Ecp2 and Ecp6

Fungal cell walls are mainly consists of three main carbohydrate polymers (β-glucans, chitin, and mannans) as well as glycoproteins. It has been shown that the dispersal of these three carbohydrates in the cell wall differs to a great extent and can even differ between species or morphological structures of the same strain (LATGE 2007; LATGE 2009). Although these three carbohydrate molecules can be dispersed throughout the cell wall, mannans are dispersed more towards the outer cell wall area, whereas chitin is mainly found near the plasma membrane as seen in Figure 9 (BOWMAN and FREE 2006; LENARDON *et al.* 2010). Chitin is an N-acetyl-D-glucosamine (GlcNac) homopolymer that can be found in fungal cell walls, but not in plant cell walls. Therefore chitin is recognized as a non-self molecule or a pathogen-associated molecular pattern (PAMP) and triggers plant immune responses (FELIX 1993; SHIBUYA *et al.* 1993).

Plants have evolved to produce chitinases in order to defend themselves against fungal attack. Two types of chitinases exist, namely endochitinases and exochitinases. Exochitinases can be found in the plant apoplastic space and although they are not detrimental to fungal growth, exochitinases break chitin down into short-chain chitin oligosaccharides that are released and can therefore be detected by host receptors and cause a PTI (FELIX 1993; SHIBUYA *et al.* 1993). It is upon host defence activation that endochitinases, which are detrimental to fungal growth, are accumulated in the vacuole and released upon programmed cell death. These endochitinases degrade fungal cell walls and act as effective antifungal enzymes (GRISON *et al.* 1996; NISHIZAWA *et al.* 1999; SCHLUMBAUM *et al.* 1986). In order to overcome this plant defence response, fungi have evolved effector molecules to bypass detection and subsequent host defence activation. Even though the molecular targets of the majority of effectors is still unclear (DE JONGE *et al.* 2011), it has been shown that some effectors directly target and destabilize host receptors (GIMENEZ-IBANEZ *et al.* 2009; SHAN *et al.* 2008). CfAvr4, cloned from *C. fulvum*, has been shown to be a cysteine-rich effector protein that is secreted upon tomato infection (JOOSTEN *et al.* 1994). It was proposed that CfAvr4 is directly detected by Cf-4 and causes an HR in

the host which halts fungal growth (Figure 9) (STERGIOPOULOS *et al.* 2010; VAN DEN BURG *et al.* 2006). The chitin-binding function of Avr4 was determined through affinity precipitation assays (VAN DEN BURG *et al.* 2006). Avr4 binds directly and specifically to chitin present in the fungal cell walls and protects it against hydrolysis by plant chitinases (VAN DEN BURG *et al.* 2006). By binding to chitin in fungal cell walls, Avr4 may also reduce the release of short-chain chitin oligosaccharides, forming a secondary protection method against fungal detection. Interestingly, it was shown that although MfAvr4 shares low sequence homology (42% identity at protein level) with CfAvr4, it was still able to bind to chitin present in fungal cell walls and therefore still able to protect fungal hyphae against plant chitinases as seen in Figure 11 (STERGIOPOULOS *et al.* 2010).

In contrast to the Avr4 defensive virulence factor, it is proposed that Ecp2 promotes virulence by causing host cell necrosis (STERGIOPOULOS *et al.* 2010). CfEcp2 is detected by the *Cf-Ecp2* resistance gene and causes an HR in its host, tomato (HAANSTRA *et al.* 1999). It was shown that one of the three CfEcp2 homologs identified in *M. fijiensis* (MfEcp2) is able to cause different levels of necrosis by interacting with an unknown plant target (STERGIOPOULOS *et al.* 2010). It could not be shown that MfEcp2-2 and MfEcp2-3 trigger an HR leading to necrosis. The different levels of necrosis caused by the detection of CfEcp2 and MfEcp2 can be understood in terms of the respective fungal lifestyles. *C. fulvum* is a biotrophic fungus; whereas *M. fijiensis* is a hemibiotroph. It is believed that the onset of necrosis favours the necrotrophic growth stage of a hemibiotrophic fungus (BEVERAGGI *et al.* 1995). It is therefore proposed that MfEcp2 causes a higher level of virulence when compared to CfEcp2 (STERGIOPOULOS *et al.* 2010). In Figure 11, it can be seen that in the presence of the putative Cf-Ecp2 resistance protein, it is proposed that Ecp2 is indirectly detected through Cf-Ecp2 that monitors the Ecp2 target virulence protein, causing an HR in the host (STERGIOPOULOS *et al.* 2010). Even though the exact function of Ecp2 still needs to be determined, it can be accepted that Ecp2 is a virulence factor as it has been shown that disruption of Ecp2 causes a decrease in fungal virulence (LAUGÉ 1997).

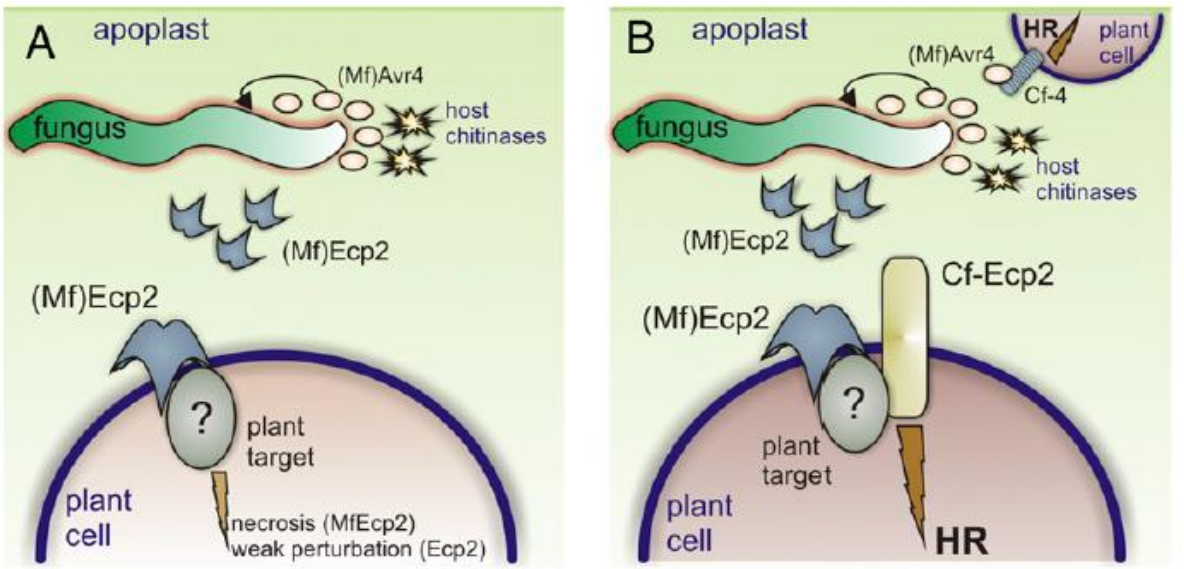

Figure 11. Proposed functions for Avr4 and Ecp2 (STERGIOPOULOS *et al.* 2010). (A) A scenario where the host is susceptible (absence of resistance genes) and Avr4 and Ecp2 remain undetected. Ecp2 interacts with an unknown plant protein and causes host cell necrosis which facilitates the necrotrophic fungal growth stage of hemibiotrophs (*M. fijiensis*). In the case of biotrophic fungi, such as *C. fulvum*, Ecp2 will cause a weaker host cell perturbation without causing host cell necrosis. This scenario can be a result of pathogen and host co-evolution. On the other hand, Avr4 can be seen as a defensive virulence factor as it binds to chitin in fungal cell walls forming a defensive barrier, protecting fungal hyphae against hydrolysis by host chitinases (B). When the Cf-Ecp2 resistance protein is present, Ecp2 can be detected through an indirect interaction, where Cf-Ecp2 monitors an unknown virulence target of Ecp2. When a change in the unknown virulence target is detected by Cf-Ecp2, an HR is induced. The detection of Avr4 by Cf-4 resistance protein can be seen as a direct interaction, also leading to an HR in the host.

Recently, it has been shown that Ecp6 plays an important role in virulence of *C. fulvum* (BOLTON *et al.* 2008). *C. fulvum* Ecp6 only binds to chitin oligosaccharides and is therefore able to suppress an immune response as a result of chitin detection by the plant as seen in Figure 12 (DE JONGE *et al.* 2010). However, CfEcp6 is only able to bind to chitin oligosaccharides, and is therefore unable to protect fungal hyphae from plant chitinases (DE JONGE *et al.* 2010). Three putative LysM effectors have been identified in *M. graminicola,* but only two of these effectors were expressed during infection (MARSHALL *et al.* 2011). Mg3LysM contains three LysM domains as seen in CfEcp6, whereas Mg1LysM contains only a single LysM domain (MARSHALL *et al.* 2011). Although both these LysM effectors are able to bind chitin, only Mg3LysM was able to prevent detection of short-chain chitin oligosaccharides by the plant and therefore suppress the subsequent host immune response in a similar manner as seen with CfEcp6 (MARSHALL *et al.* 2011). Mg3LysM was also shown to have a second virulence function in that it is able to protect fungal hyphae against plant chitinases as seen with *C. fulvum* Avr4 (MARSHALL *et al.* 2011). Even though Mg1LysM was not able to suppress a host immune response, it was shown to be a defensive virulence

factor that is functionally similar to CfAvr4 (MARSHALL *et al.* 2011). This finding might explain why no Avr4 homolog could be found in the *M. graminicola* genome (KOMBRINK *et al.* 2011). LysM effectors might have evolved to be able to perform both functions in *M. graminicola* (MARSHALL *et al.* 2011).

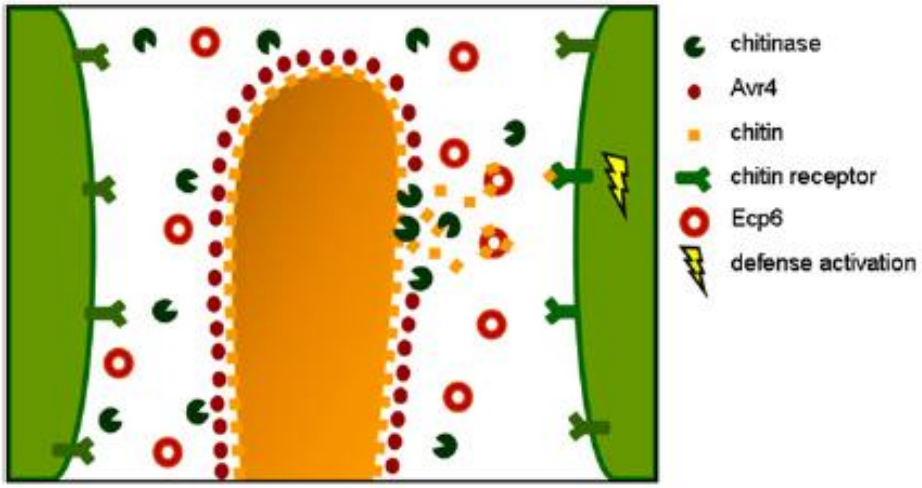

Figure 12. The proposed functional role of Ecp6 (KOMBRINK *et al.* 2011). Avr4 is able to bind to fungal chitin (yellow) and can therefore prevent plant chitinases (green) to metabolise fungal cell walls. When plant chitinases are able to hydrolyse chitin into chitin oligosaccharide fragments, these fragments are released and are able to cause plant defence activation. Ecp6 (red) is able to prevent defence activation by sequestering these chitin oligosaccharides.

Since the discovery of the first fungal effector in *C. fulvum*, many other fungal effectors in Dothideomycetes have been identified. Although many of these effectors have homologs in more than one fungal species, the functions of many of them still need to be elucidated. Research is required to determine if these conserved effectors have retained their function in related species. The effectors Avr4, Ecp2 and Ecp6 play an important role in fungal virulence in many fungal species of the Dothideomycete class. These effectors are widely dispersed across species in the Dothideomycete class and therefore provide interesting research opportunities to be able to understand plant-pathogen interactions. As new effectors are detected, it might provide a way to identify homologs in other fungal species if these effectors are conserved. An example of such an opportunity would be to investigate if the Avr4, Ecp2, and Ecp6 effectors are present in *C. zeina* and if they are expressed during infection. It would also be noteworthy to determine if these effectors are conserved in gene structure. Future studies will include functional analysis of these effector genes in *C. zeina* as well as the role they play in fungal virulence. This research will shed some light onto the

molecular mechanisms of infection of *C. zeina*, which will ultimately aid in sustainable GLS disease control that will in turn result in higher maize yields necessary for food security.

4. RESEARCH PROJECT

It is clear that insight is needed into the molecular mechanisms of *C. zeina* to be able to effectively manage GLS. The broader aim of this research project was therefore to gain insight into the molecular mechanisms underlying *C. zeina* infection by identifying effector Avr4, Ecp2, and Ecp6 homologs in *C. zeina*. By elucidating the *in planta* relative expression profiles of *C. zeina Avr4*, *Ecp2*, and *Ecp6* effectors, it can be determined if these effectors are important pathogenicity factors.

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