FACULTEIT
BIO-INGENIEURSWETENSCHAPPEN

EVALUATING RNAI AS A CONTROL STRATEGY AGAINST *FUSARIUM* AND ITS MYCOTOXINS IN MAIZE

Anne Van Humbeeck
Stamnummer: 01401308

Promotoren: Prof. Dr. ir. Guy Smagghe , Prof. Dr. ir. Kris Audenaert

Tutoren: Dr. Nji Tizi Clauvis Taning, Dr. Minh Trang Tran

Masterproef voorgelegd voor het behalen van de graad in Master of Science in de industriële wetenschappen: biochemie

Academiejaar: 2020 - 2021

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Preface

The implementation of a thesis idea always comes with many challenges and would not be possible without the help and guidance of the many people involved. In the first place, I would like to thank Prof. Dr. ir. Guy Smagghe and Prof. Dr. ir. Kris Audenaert for the opportunity to conduct the practical thesis work in their research labs. I have been very lucky to have had access to such well-equipped and inspiring work environments. Besides, I am also very grateful for their advice and comments on my thesis manuscript and progress throughout the study. Of course, a big thank you also goes out to my supervisors Dr. Nji Tizi Clauvis Taning and Dr. Minh Trang Tran who have helped me to become familiar with a wide variety of theory and lab techniques. Whenever I had questions or needed some help with the lab problems I faced, they were always available for advice.

In addition, I would like to thank Prof. Dr. Sarah De Saeger and the lab members of the Centre of Excellence in Mycotoxicology and Public Health for allowing me to analyze my samples for mycotoxin contamination with their LC-MS/MS device and expertise. Overall, I received a lot of spontaneous help in the lab of the Department of Plants and Crops, especially from Larissa De Troyer, Waldo Deroo and Mitchel De Cock with whom I worked together to tackle the problems we faced with contamination of the used fungi. Also, Noémie De Zutter, Boris Bekaert and Maarten Ameye were very kind to provide me with guidance and help regarding the use of the phenotyping robot and other technical practicalities. In general, working in this lab has been wonderful thanks to the kindness and welcoming atmosphere of everyone present. It didn't take long for me to feel like I was a member of the group and I appreciate this greatly.

At last, I would also like to acknowledge my family and loved ones for their unconditional support. Without them, studying for my second master degree at UGent would not have been a possibility in the first place, or would have been much less of a pleasant experience. Studying during the Covid pandemic can be very challenging and mentally draining at times. Therefore, I am immensely lucky to have such warm and loving people around to encourage me when times get difficult and stressful. Thank you.

Abstract in Dutch

Wereldwijd zorgen *Fusarium* schimmels voor grote problemen in de landbouwindustrie. Niet enkel zijn ze gerelateerd aan allerhande gewasziekten en verliezen, maar ook vormen de mycotoxines die door deze organismes worden uitgescheiden een gevaar voor mens en dier. De vraag naar milieuvriendelijke alternatieven voor hedendaagse fungiciden wordt alsmaar groter vanwege strengere overheidsmaatregelen en allerhande vormen van resistentie ontwikkeling. Spray-induced gene silencing (SIGS) is een relatief nieuwe vorm van gewasbescherming waarbij dubbelstrengig RNA (dsRNA) via een spray methode op de plant wordt aangebracht. Hierna worden de RNA moleculen opgenomen door plant en pathogeen en verwerkt via de aanwezige RNA interference (RNAi) pathway om zo gene silencing van het gewenste target gen te bekomen.

In deze studie werd SIGS toegepast op de pathogeen *F. verticillioides,* een schimmel wiens fumonisin productie voor bezorgdheid zorgt vanwege de mogelijks carcinogene aard. Het chitine synthase 3 gen (*Chs3b*) van de schimmel werd gekozen als target en doorheen de studie bestudeerd met behulp van SIGS. Het effect van dit dsRNA op de groei en infectie van *F. verticillioides* werd getest op drie niveaus: microscopische schaal, *in-vitro* 6-well schaal en detached-leaf assay op mais bladeren. *F. graminearum* (GFP getagged) fungeerde als controle organisme in de studie.

Repliceerbare protocollen werden opgebouwd om het effect van *Chs3b* dsRNA op *F. verticillioides* te testen. Subtiele verschillen tussen *Chs3b* dsRNA behandeling en controle werden opgemerkt tijdens de 6-well experimenten en mycotoxine analyse. Echter, over het algemeen leek de behandeling weinig tot geen effect te hebben. Mogelijkse verklaringen zijn het kiezen van een inefficiënt target gen of een ongeschikte applicatiemethode.

Kernwoorden: gene silencing, dubbelstrengig RNA, *Fusarium verticillioides, Fusarium graminearum,* detached leaf-assay, mycotoxines

Abstract in English

Fusarium fungi are a source of worldwide problems in the agricultural industry. Not only are they associated with different kinds of crop diseases and losses, but also the mycotoxins emitted by these organisms pose a threat to animals and humans. The demand for more environmentally friendly fungicide products is only increasing due to stricter government measurements and the rise of fungal resistance. Spray-induced gene silencing (SIGS) is a relatively new kind of crop protection in which double-stranded RNA (dsRNA) is applied by a spraying method onto the plant leaves. Afterwards, the RNA molecules are taken up by the plant and pathogen and processed by the present RNA interference (RNAi) pathway in order to become silencing of the desired target gene.

In this study, the SIGS method was applied on the pathogen *F. verticillioides*, a fungus which fumonisin production causes concern because of its potential carcinogenic nature. The chitin synthase 3 gene (*Chs3*) of the fungus was chosen as target and investigated throughout the study by means of SIGS. The effect of this dsRNA on the growth and infection of *F. verticillioides* was tested on three levels: microscopic scale, *in-vitro* 6-well scale and maize detached leaf assay. *F. graminearum* (GFP tagged) was used as a control organism throughout the study.

Replicable protocols were built de-novo to investigate the effect of *Chs3b* dsRNA on *F. verticillioides.* Subtle differences between the *Chs3b* dsRNA treatment and control were witnessed during the 6-well experiments and mycotoxin analysis. Nevertheless, the treatment had in general little to no effect. Possible explanations can be found in the inefficient selection of target gene or unsuitable application method.

Keywords: gene silencing, double-stranded RNA, *Fusarium verticillioides, Fusarium graminearum,* detached-leaf assay, mycotoxins

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List of Abbreviations

AGO	Argonaute protein
bp	Base pairs
cDNA	Complementary DNA
cGFP	Corrected green fluorescent protein
CHS	Chalcone synthase
Chs	Chitin synthase
CRISPR/Cas	Clustered regularly interspaced short palindromic repeats/Cas9
dai	Days after infection
DCL	Dicer-like protein
DON	Deoxynivalenol
dsRNA	Double-stranded RNA
FB	Fumonisin B series
FER	*Fusarium* ear rot
FHB	*Fusarium* head blight
Fump	*Fum* protein
GFP	Green fluorescent protein
eGFP	Enhanced green fluorescent protein
GMO	Genetically modified organism
GUS	B-glucuronidase
HIGS	Host-induced gene silencing
HPLC	High-performance liquid chromatography
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
miRNA	MicroRNA
nt	nucleotides
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
piRNA	Piwi-interacting RNA
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference
SIGS	Spray-induced gene silencing
siRNA	Short interfering RNA

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1. Introduction

Fungal species belonging to the *Fusarium* family are known worldwide for causing severe crop diseases and related problems. The most notorious ones are *Fusarium* head blight (FHB) in wheat and *Fusarium* ear rot (FER) in maize. Besides causing spoilage in the crops, the fungi are also capable of producing mycotoxins, which are secondary metabolites. These molecules can cause threats to animal and human health and can accumulate during plant growth. Thus, it is no wonder that in the last decades many efforts were directed towards the creation of successful fungicides to combat these problems. However, the overreliance on fungicides is harmful for the environment or prone to the development of fungal resistance. Recent governmental restrictions considering the use of these dangerous components have caused a high need for the development of alternative biofungicides that are save for humans and environment.

In the last years, numerous studies have turned towards the use of the RNA interference (RNAi) pathways present in most types of organisms to silence genes that are vital to dangerous pests or pathogens. At first, the focus lied upon the creation of transgenic plants that can create small interference RNAs able to silence target genes of pests, a technique that was called 'host-induced gene silencing'. Given that a lot of (European) countries are non-inviting when it comes to the implementation of GMOs on the fields, the focus shifted towards another technique: spray-induced gene silencing (SIGS). In this case, the small interfering RNA (siRNA) or double-stranded RNA (dsRNA) is applied straight onto the plant by means of a spray application. After uptake of the RNA molecules by the plant or pathogens, they are processed by the RNAi machinery and used to silence the target gene for which they were designed. Thus, hereby creating a highly species-specific pesticide or fungicide with less direct harmful effects on the environment.

In this study, *F. verticillioides* was chosen as target organism. This fungal species is best known for causing pink ear rot in maize in (semi-) tropical and temperate regions. Not only is this pathogen responsible for great agricultural losses, but it also produces fumonisins. These mycotoxins cause a lot of concern worldwide since they have been associated with human oesophagael cancer in Italy, Southern Africa, Southeastern U.S.A. and China. During this investigation, the use of SIGS as a way to target *F. verticillioides* and reduce growth and infection of the fungus was investigated. To do this, dsRNA targeting the chitin synthase 3b gene (*Chs3b*) of *F. verticillioides* was designed. This same gene was found to be of vital importance to the fungus *F. graminearum,* which is why this organism was chosen as control throughout the study. The used *F. graminearum* strain (PH-1) was also GFP-tagged and thus dsRNA was also designed to target the *eGFP* gene present, as another form of method control.

In order to investigate the effect of the dsRNA on multiple levels, three protocols were established. The dsRNA treatment was first applied on a microscopic scale in order to look for effects early on in spore germination. Afterwards, *in-vitro* 6-well plates were used to allow for a longer observation of the fungal growth under control and dsRNA treated conditions. At the end of this protocol, samples were also collected and analyzed for mycotoxin contamination. At last, a detached-leaf assay method was used in order to investigate the necessity for the interaction between host-plant and *F. verticillioides* to facilitate successful gene silencing.

This thesis comprises of five chapters of which the first one is the Literature study. Here, a wide overview about relevant literature concerning the topics of the study is provided. This part is by itself divided into four main sections. First of all, *Fusarium* fungal pathogens and the problems they cause in crop production are described, in particular the main organism of this study, *F. verticillioides* and *F. graminearum*. The second section goes more into dept about fumonisins, the main mycotoxins produced by *F. verticillioides*, and their gene cluster and biosynthesis. Afterwards, the study focuses on the RNAi machinery in plants and fungi. At last, the final section deals with RNAi-based control of fungal pathogens and gives a detailed overview about the advances in this field so far and the challenges that are still being present.

The second main chapter is the methodology. Here, a detailed description is given concerning all the protocols that were used during the investigation of this study. In the third chapter, the results of the practical work that was conducted in light of this thesis (and was described in the methodology) are introduced and visualized with figures and statistical analyses. In chapter four, these results are critically discussed and compared to the expectations that were set at the beginning of the investigation. Also, possible explanations are given for unexpected results and prospects for future work on the subject are proposed. At last, chapter five is an overview of the work that has been conducted and summarizes in short the main findings and message to take home from the overall study.

2. Literature study

2.1 *Fusarium* spp.

Fusarium species are distributed worldwide and are amongst the most prominent plant pathogens (Babadoost, 2018). Depending on the species, *Fusarium* can infect most plant parts at different phenological growth stages such as roots, stems, fruits, and seeds (Askun, 2018). The host range within the genus is very broad and comprises both monocots and eudicots. In the last years, the *Fusarium* taxonomy has been revisited due to the highly complex nature of the species in this genus. It is now generally accepted that this genus is divided into species complexes such as the *Fusarium fujikuroi* species complex (containing *F. verticillioides)*, the *Fusarium sambucinum* species complex (containing *F. graminearum, F. pseudograminearum* and *F. langsethiae*), the *Fusarium oxysporum* species complex (containing *F. oxysporum*) and many others (O'Donnell *et al*, 2013; Summerell, 2019).

Although many *Fusarium* spp. have been studied as pathogens, research on this genus is primarily fueled by the production of toxic secondary metabolites (e.g., fumonisins, deoxynivalenol and zearalenone) by several *Fusarium* species. Under stress or during plant invasion, pathogenic fungi often produce these secondary metabolites that act as a defense mechanism or help to facilitate the infection process. The so-called mycotoxins can accumulate during plant growth and can persist during storage (Wakuliński, 1989; Askun, 2018; Babadoost, 2018; Jimenez-Garcia *et al*, 2018). Mycotoxins also pose a threat to the health of animals and humans ranging from acute toxicity causing for example vomiting to chronic diseases such as cancer (Nelson *et al*, 1993; Krcmery *et al*, 1997; Weidenbörner, 2017). Often, mycotoxins are produced by the *Fusarium, Aspergillus* and *Penicillium* fungal genera (Majumdar *et al*, 2017). There is a wide variety of known *Fusarium* toxins and they range from fumonisins to trichothecenes, zearalenones, beauvericin and more (Desjardins & Proctor, 2007). The different types of mycotoxins usually have their own gene cluster like the *TRI* clusters (trichothecenes), the *ZEN* cluster (zearalenone) and the *FUM* cluster (fumonisins) (Perincherry *et al*, 2019).

The two most important diseases linked to *Fusarium* species are *Fusarium* head blight (FHB) in wheat and *Fusarium* ear rot (FER) in maize. Species that are known to cause FHB include, but are not limited to, *F. graminearum, F. culmorum* and *F. avenaceum* belonging to the *F. sambucinum* and *F. tricinctum* species complex (Muthomi *et al*, 2008; Machado *et al*, 2018). In maize, on the other hand, ear rot is the most common disease. There are two kinds of FER present in maize. Red ear rot is mostly caused by infections of *F. graminearum, F. avenaceum* and *F. culmorum.* The other variant, pink ear rot, is associated with *F. verticillioides, F. proliferatum, F. subglutinans* and *F. fujikuroi* (Logrieco *et al*, 2002). Many mycotoxins comprising fumonisins, trichothecenes, zearalenones, moniliformin, beauvericin and fusaproliferin can be found on maize crops because of these kinds of infections (Jimenez-Garcia *et al*, 2018). The high incidence of *Fusarium* species and associated mycotoxins in maize products destined for human consumption in African regions is one of the major threats to human health on the African continent (Fandohan *et al*, 2005; Muthomi *et al*, 2008; Ekwomadu *et al*, 2018). Worldwide losses in maize yield due to *Fusarium* species are

numerous and are situated in both pre-harvest due to infected plants in the field as well as during post-harvest chain (Bottalico *et al*, 1989; Burgess & Bryden, 2012).

In Europa, red ear rot is most often caused by *F. graminearum* and the reddish mould is regularly found in northern European areas (Bottalico & Perrone, 2002). Pink ear rot in maize, on the other hand, is mostly due to infections of *F. verticillioides* (Munkvold *et al*, 1997). It is more common in central to southern European regions. Both types of plant diseases are found worldwide, however recently shifts in the population have been observed in which *F. verticillioides* migrates northwards (Munkvold *et al*, 2018).

2.1.1 *Fusarium graminearum*

Fusarium graminearum (also known by its older teleomorphic name *Gibberella zea*) is one of the main participants in several crop diseases of which FHB is among the most common and severe. This disease causes crop damage and losses in China, the United States, South America and many other regions due to the fungus' wide distribution. It mainly targets cereal crops and grows especially well in warmer regions, although *F. graminearum* can withstand a wide range of temperatures up to 30°C (Osborne & Stein, 2007; Xu & Nicholson, 2009). Symptoms of FHB include necrosis and spikelets in cereal heads, kernel damage and *Fusarium* toxins accumulation (Góral *et al*, 2019). Often, pink fungal sporodochia can also be seen on the infected heads (Fig. 1B) (Osborne & Stein, 2007). *F. graminearum* produces several harmful mycotoxins including zearalenone (ZEN) and trichothecenes such as deoxynivalenol (DON). DON is the mycotoxin that is worldwide the most common in cereal grains (Lee & Ryu, 2017; Chen *et al*, 2019). In mammals, it causes a wide range of symptoms going from growth and reproductive effects to immune dysregulation, mainly due to its binding to the ribosome and following protein inhibition (Pestka, 2010).

F. graminearum can infect crops via dispersed asexual conidia or sexual ascospores (Gilbert & Fernando, 2004). The *F. graminearum* conidia are macroconidia with an elongated, slender shape consisting of three to five cells divided by septa (Fig. 1A) (Harris, 2005). During germination, hyphae are formed on the exterior surfaces of glumes and florets after which access to penetration susceptible tissues is gained through the stomata (Seong *et al*, 2008).

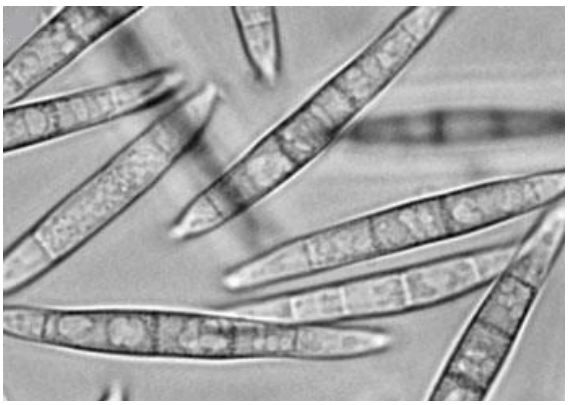
A  B 

Figure 1: A) macroconidia from Fusarium graminearum and B) Fusarium head blight in the field (Kikot et al, 2011; Smith, 2013).

2.1.2 *Fusarium verticillioides*

Maize is grown in a wide geographical range from North America to Europe, Russia and Oceania. It is a crop of very high importance with applications in food, animal feed and industrial purposes (Muimba-Kankolongo, 2018). *F. verticillioides* is the major *Fusarium* species causing pink ear rot in maize in (semi)-tropical and temperate regions including Europe (Logrieco *et al*, 2002), the USA (McMullen *et al*, 2012) and several parts of Africa (Fandohan *et al*, 2005; Muimba-Kankolongo, 2018; Ekwomadu *et al*, 2018). *F. verticillioides* infections can be asymptomatic but may also be very severe and result in wilting and rotting (Oren *et al*, 2003).

During warmer and drier years, there is a higher occurrence of *F. verticillioides* in maize crops (Reid *et al*, 1999). The fungus produces both micro – and macroconidia, although this last one to a much smaller extent. Macroconidia are curved, contain two to four septa and measure approximately 20 by 5 µm. Microconidia are fusiform to clavate and measure approximately 9 by 2 µm (Fig. 2A) (Young *et al*, 1978). Most frequently, the conidia first infect the silks after which the invasion spreads to the kernels (Munkvold *et al*, 1997; Oren *et al*, 2003). Often, damage caused by insects forms easy access for the fungus to infiltrate the plant tissues. Infection of the maize is characterized by white-colored molds scattered on the ear (Fig. 2B) (Reid *et al*, 1999). In infected soil, the mesocotyl tissue and lateral roots are the first to be infected, followed by rotting in the mesocotyl and main roots 25 to 30 days after (Oren *et al*, 2003). In case of infected seeds by mycelia or conidia, the fungus spreads from the roots to the stalk and kernels onwards (Murillo-Williams & Munkvold, 2008). Thus, systemic infection of the maize plant starting from the seed and stalk is possible, but more common and important is the invasion via the silk (Munkvold *et al*, 1997). The main mycotoxins produced by *F. verticillioides* are fumonisins (Logrieco *et al*, 2002).

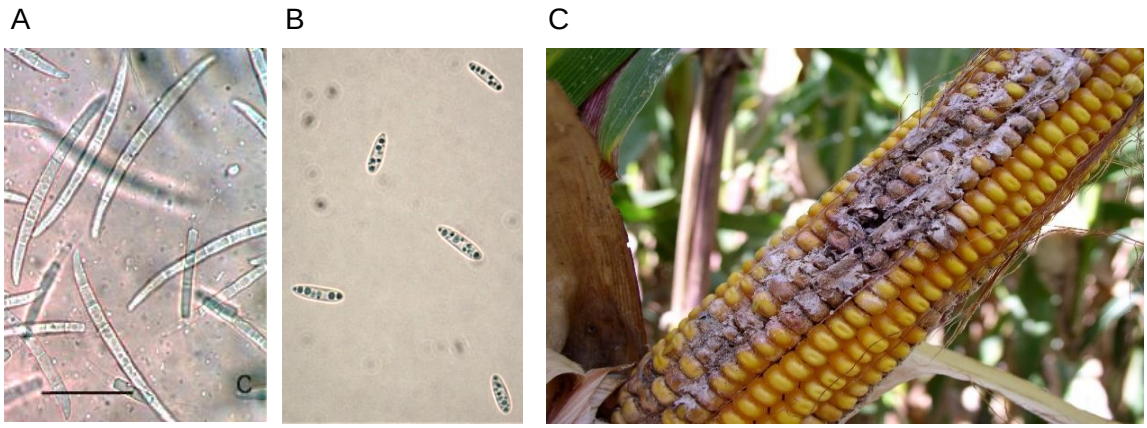


Figure 2: macro (A) – and microconidia (B) from Fusarium verticillioides and C) maize pink ear rot (Pavlovic et al, 2016; Crop Protection Network, 2020)

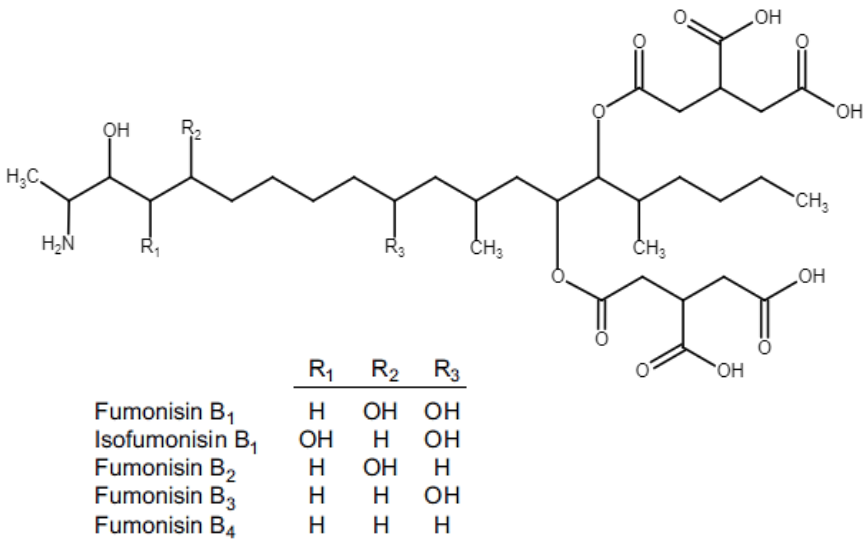
2.2 Fumonisins

The target organism in this study is *F. verticillioides.* Its main mycotoxins are fumonisins which cause a lot of concern worldwide as will be discussed in this section. The first discovery of fumonisins dates back to 1988 when a long investigation revealed their possible role in reportedly high numbers of esophageal cancer in the Southern Africa Transkei region (Bezuidenhout *et al*, 1988; Gelderblom *et al*, 1988; Nelson *et al*, 1993). The first isolation of this kind of polyketide mycotoxins came from *F. verticillioides* (Nelson *et al*, 1992). Together with *F. proliferatum, F. verticillioides* is still considered as the main producer of fumonisins (Askun, 2018). Many crops have already shown fumonisin contamination in association with *Fusarium,* such as wheat, rice, beans and maize (Chehri *et al*, 2010; Hsuan *et al*, 2011; Gomes *et al*, 2015). They aid in the infection process by the disruption of the plant plasma membrane, accumulation of toxic sphingolipid intermediates and the resulting inhibition of the ceramide synthase and following disruption of cell signaling and apoptosis (Perincherry *et al*, 2019). At present, there are four described fumonisin series: A, B, C and P. The four toxins of the B-series are in general the most common, where fumonisin B1 (FB1) is the fumonisin with the highest mycotoxicological risk and abundance (Abbas *et al*, 1998; Logrieco *et al*, 2002; Alexander *et al*, 2009). This mycotoxin is associated with human oesophageal cancer (HEC) in China, Southeastern U.S.A., southern Africa and Italy (Franceschi *et al*, 1990; Marasas, 1995). When present in feed, FB1 pose a risk for animals as it can cause pulmonary oedema and hepatic syndrome in swine, altered hepatic and immune function in cattle, leukoencephalomalacia in horses and liver cancer in rats (Ross *et al*, 1990; Gelderblom *et al*, 1994). Therefore, the mycotoxins produced by *F. verticillioides* have been classified as carcinogenic to animals and possibly carcinogenic to humans (Logrieco *et al*, 2002; International Agency for Research on Cancer, 2021).

2.2.1 Fumonisin genes

In general, B-series fumonisins are long-chain amino polyalcohols with a 20-carbon backbone including an amine function at carbon atom 2 (C-2), tricarballylic esters at C-14 and C-15 and methyl functions at C-12 and C-16 (Alexander *et al*, 2009). Differences between the four fumonisins in the B-series lie in the presence or absence of hydroxyl functions at C-4, C-5 and C-10 (Fig. 3). The one most often encountered, FB1, is a propane-1,2,3-tricarboxylic diester of 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxycosane (Nelson *et al*, 1993; Rheeder *et al*, 2002). The genes responsible for fumonisin biosynthesis are for *F. verticillioides* located in one locus of 42-kb (FUM cluster, Fig. 4) (Desjardins & Proctor, 2007; Proctor *et al*, 2008). Thus far, it is predicted that only 11 out of 17 genes from this cluster are required for fumonisin synthesis (Desjardins & Proctor, 2007). *FUM1* encodes a polyketide synthase gene and is the first one of the cluster. Furthermore, *FUM8* is a gene that encodes an aminotransferase while *FUM13* of the cluster is most likely encoding C-3 carbonyl reductase (Bojja *et al*, 2004). Necessary oxygenation enzymes are represented by *FUM2* (C-10), *FUM3* (C-5), and *FUM6* (undetermined site) (Proctor *et al*, 2006). Genes necessary for esterification are *FUM14, FUM10, FUM7* and *FUM11*. Other genes of the cluster that are not vital for fumonisin

production are *FUM17, FUM18* and *FUM19* (Desjardins & Proctor, 2007). The function of *FUM20* is not yet understood (Alexander *et al*, 2009).



	R_1	R_2	R_3
Fumonisin B_1	H	OH	OH
Isofumonisin B_1	OH	H	OH
Fumonisin B_2	H	OH	H
Fumonisin B_3	H	H	OH
Fumonisin B_4	H	H	H

Figure 3: chemical structure of fumonisins from the B-series.

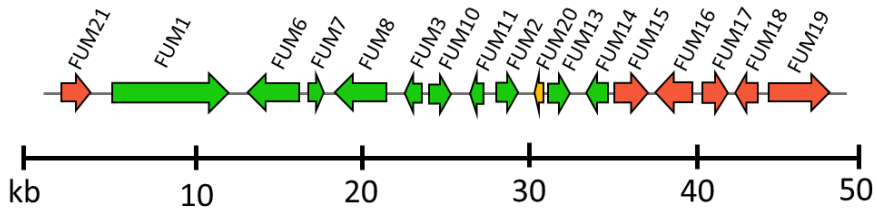


Figure 4: F. verticillioides FUM gene cluster coding for fumonisin mycotoxins. Arrows indicate the orientation of gene transcription, where green arrows are essential for fumonisin production and red arrows have indirect roles or are not essential. Information on the function of FUM 20 (in orange) is not yet available. Adapted from Alexander et al (2009).

2.2.2 Fumonisin biosynthesis

Even though the responsible gene cluster is well investigated by now, it is still a challenge to determine the exact regulatory mechanism behind fumonisin synthesis (Sagaram *et al*, 2006). Here, the biosynthesis of FB1 by *F. verticillioides* will be explained in detail. The starting components of the fumonisin biosynthesis pathway have not yet been isolated, thus they are proposed based on the subsequent intermediates of the pathway that have been characterized. The first compound to be formed by *FUM1* protein (Fum1p) out of nine acetates and two methyl units is 10,14-dimethyloctadecanoic acid. Afterwards, it undergoes decarboxylative condensation with alanine by Fum8p (Fig. 5) (Alexander *et al*, 2009; Uhlig *et al*, 2012). This, most likely, results in 2-amino-12,16-dimethylicosane-3-one (Bojja *et al*, 2004). As a third step, this component will undergo hydroxylation by *FUM6* protein (Fum6p) at C-14

and C-15 (Alexander *et al*, 2009). The formed fumonisin analogue, at last, undergoes a C-3 carbonyl reduction, hydroxylation at C-10 and/or C-5 and C-14/C-15 esterification (Uhlig *et al*, 2012). Although these steps (respectively mediated by Fum13p, Fum2p and Fum14p/Fum10p) are visualized in Figure 5 to follow one another, their positions in the biosynthesis pathway are most likely not fixed and they can each occur in the absence of the other reactions. In addition, Fum7p and Fum11p presumably also play a role in the esterification (Alexander *et al*, 2009). In order to become FB1, the last step is a hydroxylation at C-5 and is mediated by Fum3p which is predicted to be a dioxygenase (Alexander *et al*, 2009).

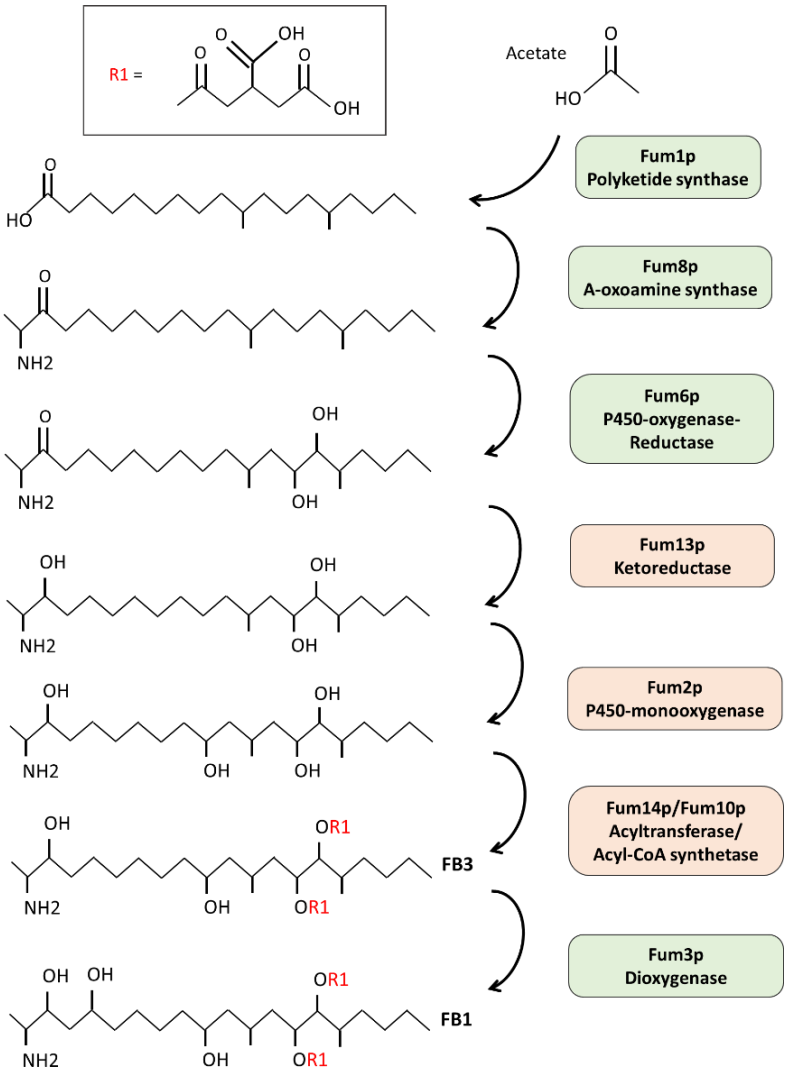


Figure 5: biosynthesis of fumonisin B1 in Fusarium verticillioides starting from acetate. For enzymes in orange, the position in the biosynthesis pathway is not fixed. Adapted from Du et al. *(2008)* and *Alexander* et al. *(2009).*

2.3 RNA interference

A broad variety of eukaryotic organisms possess a post-transcriptional gene silencing machinery, also known as RNA silencing or RNA interference (RNAi) (Pyott & Molnar, 2015; Li *et al*, 2018). RNAi was first observed in plants: while aiming to increase pigmentation of petunia florals by insertion of chalcone synthase (CHS) sequences, an adverse albino effect was witnessed (Napoli *et al*, 1990). At the time being, the discovery was called cosuppression. Later, the molecular mechanism behind this RNAi pathway was uncovered in 1998 by Fire *et al*. Since then, multiple types of naturally occurring small RNA molecules were identified, of which the most elaborately described ones are microRNAs (miRNAs), short interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) (Meister & Tuschl, 2004). MicroRNAs and siRNAs have a lot in common. Both are small RNA molecules with a length of around 21-25 nucleotides generated by a similar molecular machinery (Dang *et al*, 2014). They are abundant in plants, animals and viruses where they play a key role in gene expression regulation as post-transcriptional regulators (Bushati & Cohen, 2007). The biggest difference between the two is their origin: miRNAs are synthesized out of hairpin structured transcripts whereas siRNAs are produced from dsRNA precursors. Both however, can also have an exogenous origin (Dang *et al*, 2014). PiRNAs are only found in animals and are very different. Their size ranges from 26-31 nucleotides and they mostly function in germline cells where they cause post-transcriptional gene silencing of genetic elements like retrotransposons (Luteijn & Ketting, 2013).

At first, the RNAi pathway was believed to be mainly a defense system against transposons, viruses and other exogenous sequences (Nicolás & Garre, 2017). Later, more interest was given to the endogenous functions of RNAi in development, growth, differentiation and cell death (Alvarez-Fernandez *et al*, 2011; O'Brien *et al*, 2018). In the following sections the focus lies on the siRNA pathway in fungi and plants which is most relevant for the research conducted in this thesis.

2.3.1 SiRNA pathway in fungi

The first fungus that has been investigated for its active RNAi mechanisms is *Neurospora crassa*. After transformation of *N. crassa* with extra copies of a gene responsible for carotenoid production (*al-1*), an albino phenotype was observed. This, at the time unknown, active pathway found in the vegetative stage of this fungus was called 'quelling', a natural process to eliminate and control transposons (Romano & Macino, 1992). Later, it became clear that the RNAi mechanism in fungi is highly conserved and it is most likely that the common ancestor of fungi was in possession of a functional RNAi pathway (Nakayashiki *et al*, 2006; Choi *et al*, 2014).

Although the RNAi machinery can differ within as well as between organisms, some main defining components usually reoccur. In fungi, the first component of the pathway is RNA-dependent RNA polymerase (RdRP) which is responsible for the production of double-stranded RNA (dsRNA) molecules out of single-stranded RNA precursors (Cogoni & Macino, 1999; Xie *et al*, 2004). In the following step, the produced dsRNA is cleaved into smaller pieces:

siRNAs. This role is usually carried out by Dicer enzymes that are RNase III family nucleases (MacRae & Doudna, 2007). Dicer-like enzymes are only found in eukaryotes and it is common to find more than one Dicer enzyme coding gene in fungi that possess an active RNAi pathway. In the case of *N. crassa*, two Dicer-like proteins are present: DCL-1 and DCL-2 (Dang *et al*, 2014). Out of the dsRNA, guide and passenger siRNA strands are formed and the passenger strand is usually degraded. After cleavage by Dicer, the Argonaute protein (AGO) comes into play. AGO proteins can bind the guide siRNA strand in a specific binding pocket (Dalakouras *et al*, 2020). Together with the siRNA, AGO will form an RNA-induced silencing complex (RISC) which will guide the siRNA to the mRNA target complementary sequence (Pratt & MacRae, 2009). When the siRNA and target sequence form a perfect complement, the mRNA is usually sliced and degraded. In case of a partial complement, the binding results in a translation blockage (Jackson & Standart, 2007) (Fig. 6). Again, the architecture of the AGO proteins in fungi is well-conserved but some differences between fungal species are possible. For example, the AGO protein of *Mucor circinelloides* (a popular species in RNAi research) is named AGO-1 whereas QDE-2 is the main AGO protein of *N. crassa* (Dang *et al*, 2011; Nicolás & Garre, 2017).

The diverse nature of RNAi pathways leads to its involvement in many functions in fungal species. As already mentioned before, the mechanism plays a crucial role in the defense against sequences inserted via transposons, viruses and other sources (Nicolás & Garre, 2017). Even more so, the RNAi machinery is often necessary for proper fungal pathogenesis since the mechanism makes it possible to silence the host defense and immunity genes by hijacking its RNAi machinery with fungal siRNAs (Weiberg *et al*, 2013). Additionally, it is proposed that the transient nature of the RNAi machinery opens the way to phenotypic plasticity and the possibility for rapid adaptation (Calo *et al*, 2014). Due to its high variability, the RNAi machinery proves very suitable for the investigation of gene function or the deliberate silencing of genes essential for fungal growth or mycotoxin production (Nakayashiki *et al*, 2005).

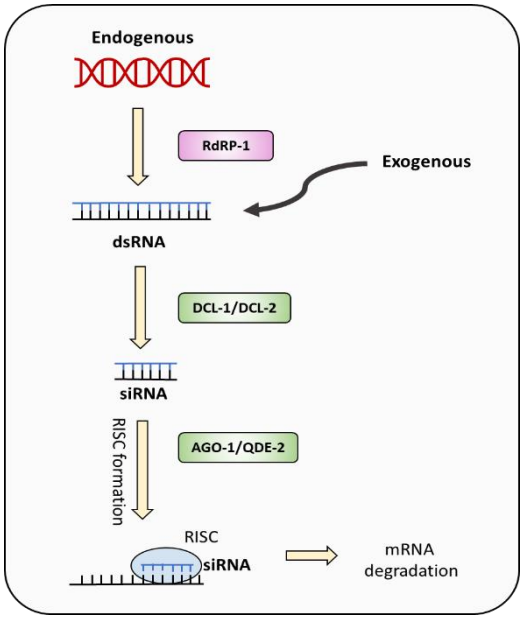


Figure 6: siRNA RNAi pathway in fungi starting from endogenous RNA precursors or exogenous dsRNA. Differences in use of DCL enzymes and AGO proteins are found between species.

2.3.2 Interaction between fungal pathogens and host plant siRNA pathways

In plants, the RNAi machinery has often been used to combat pests and pathogens by deliberately silencing organism-specific gene sequences (Chen *et al*, 2016). This can lead to an increased plant resistance or tolerance against the pathogen (Muhammad *et al*, 2019). Additionally, the RNAi technology has proven very useful in the improvement of plant stress tolerance, crop yield and determination of gene functions (Kamthan *et al*, 2015).

In general, the RNAi machinery in plants is very similar to the one in fungi (Fig. 7) (Kamthan *et al*, 2015). It is again Dicer-like nucleases that will cleave dsRNA into functional siRNAs (Tang *et al*, 2003; Bouché *et al*, 2006). In plants, four clades of DCL proteins are found and they are responsible for the production of siRNAs with different lengths. In model organism *Arabidopsis thaliana,* siRNAs with a length of 22, 24 or 21 nt are produced by DCL2, DCL3 and DCL4 respectively while DCL1 produces microRNAs with a length of 21 or 22 nt (Borges & Martienssen, 2015; Muhammad *et al*, 2019). The AGO proteins also show a lot of variety. There are four plant Ago protein groups where AGO1, 2, 3, 5, 7 and 10 are involved in posttranscriptional gene silencing and AGO4, 6 and 9 in transcriptional gene silencing (Liu *et al*, 2018; Muhammad *et al*, 2019). In *Arabidopsis thaliana,* both siRNAs with 21 and 22 nt of length are loaded on AGO1. However, only together with the 22 nt variant is AGO 1 able to recruit RDR6 for transcribing the target sequence into dsRNA, which will then lead to synthesis of more siRNAs (Chen *et al*, 2010; Dalakouras *et al*, 2020). The 21 nt long siRNAs on the other hand will together with AGO1 find complementary target sequences and cleave the strands resulting in degradation (Mi *et al*, 2008). In addition, the 24 nt siRNAs are loaded onto AGO4 and the complex will be responsible for methylation of target sequences (Chan *et al*, 2004).

The organism of interest in this study, maize plants (*Zea mays*), has 5 different DCL proteins, 18 AGOs and 5 RDR proteins (Qian *et al*, 2011).

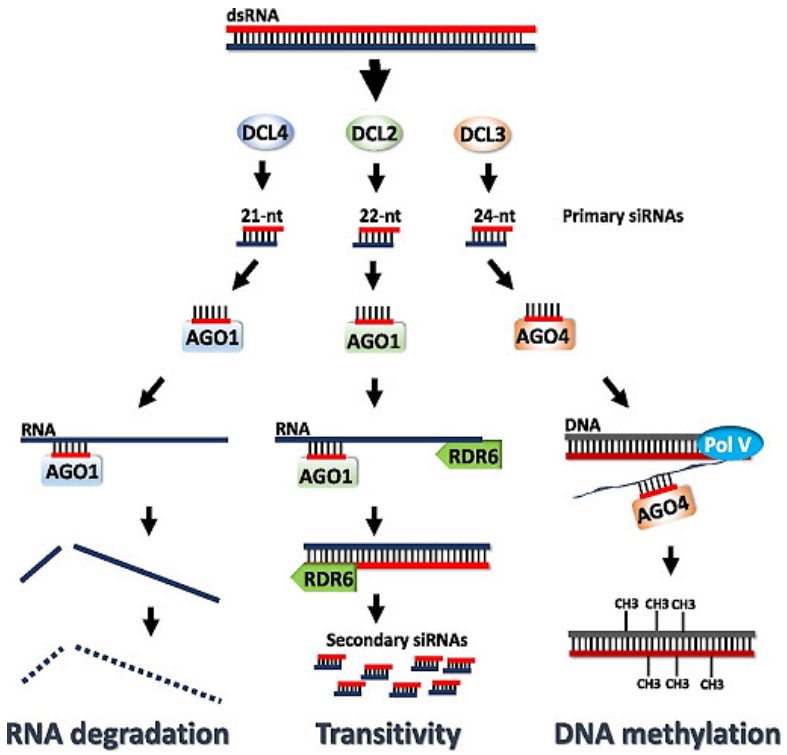


Figure 7: dsRNA pathway in plants. DCL4 processes the dsRNA into 21-nt long siRNAs. After loading on AGO1 this complex will cleave target sequences. DCL3 synthesizes 24-nt siRNAs and forms a complex with AGO4 to methylate the target sequence. DCL2 produces 22-nt siRNAs that form a complex with AGO1. The complex binds to the target and recruits RDR6 which is responsible for the creation of (secondary) siRNAs (Dalakouras et al, 2020).

RNAi molecules can move between organisms of different species. This communication can even cross the border of different kingdoms, as it is often witnessed between filamentous fungi or protists and host plants and animals. In these cases, the small RNAs are a form of pathogen-host communication (Tinoco *et al*, 2010a; Nowara *et al*, 2010). Proposed forms of transport that might facilitate the movement of small RNAs to different organisms are exosomes, membrane transporters, receptors and passive diffusion (Pyott & Molnar, 2015; Rutter & Innes, 2017). Pathogenic fungi can take advantage of the host plant RNAi machinery. When Fungal siRNAs are transported inside of the plant by means of vesicles, they can down-regulate host defense genes and thus enhance pathogenicity (Fig. 8) (Majumdar *et al*, 2017). For example, the fungus *Botrytis cinerea* secretes siRNAs that use the tomato plant AGO-1 to silence host genes important for defense pathways (Weiberg *et al*, 2013). Even more so, the same scientists discovered that a mismatch of the siRNA to the target sequence of 3-5 bp was still enough to successfully silence the target host defense gene. The other way around is also possible, host plants are able to use the RNAi machinery to their advantage in the defense

against pathogenic fungi. In this direction, the plant produced siRNAs can cross the border to the fungal hyphae in order to silence genes involved in virulence an pathogenicity (Fig. 8) (Wang *et al*, 2016). Since the 22 nt plant siRNAs are systemic and can be amplified by RdRp proteins, they will be most relevant in the interaction between fungi and host plant by means of extracellular vesicle transport.

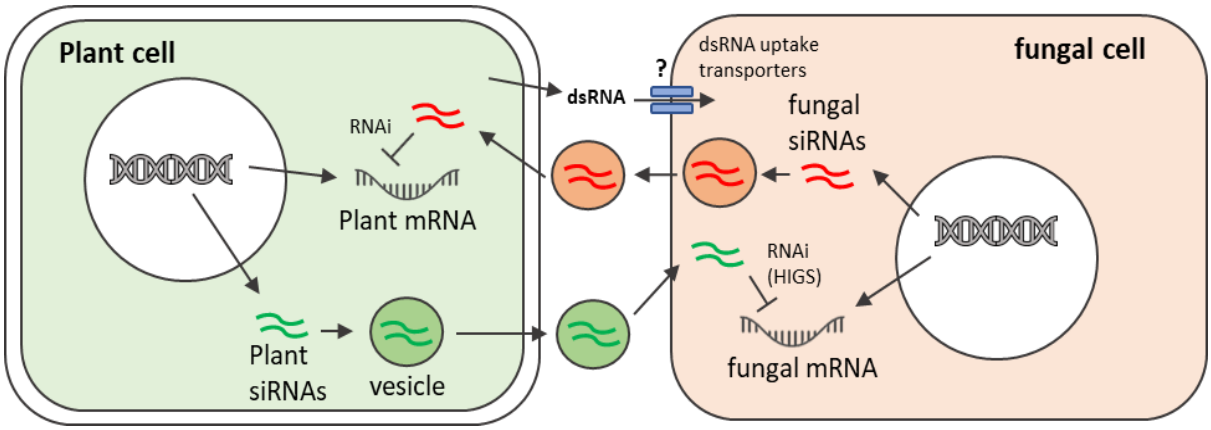


Figure 8: interaction between a host plant and pathogenic fungi at the siRNA level. Fungal siRNAs can be transported by vesicles to the host plant cells. Here it can cause silencing of host defense genes. Likewise, the plant produces siRNAs (either naturally or from transgenic hairpin RNA cassettes) that can enter the fungal cells via vesicles or possibly RNA uptake transporters and passive diffusion. Adapted from Majumdar et al. (2017).

2.4 RNAi-based control of fungal pathogens

2.4.1 Host-induced gene silencing

Investigations in plants have shown that RNA silencing signals are not restricted to the producing cells but are able to move to adhering cells by both short-range transport (10 – 15 cells) and long-range transport (across the whole tissue). It is proposed that plasmodesmata are responsible for symplastic transport. The plant phloem on the other hand might play a role in the long-range transport by source to sink dynamics (Pyott & Molnar, 2015; Machado *et al*, 2018).

The discovery that siRNAs are mobile and can move between organisms, has led to the creation of transgene host plants especially suited for this purpose. The transgenic plants produce siRNAs that can infiltrate the fungal pathogens and via this way silence fungal genes (Machado *et al*, 2018). This process is now known as host-induced gene silencing (HIGS) (Fig. 7). The first application on *Fusarium* species was carried out in 2010. Here, *GUS* (*β-glucuronidase*) from *F. verticillioides* was targeted by the use of transgenic tobacco plants. A hairpin dsRNA *GUS* fragment of 627-bp was expressed by the plant and transformed into siRNAs. The consequence was a high reduction in *GUS* expression in the invasive fungi (Tinoco *et al*, 2010a).

The following studies on HIGS focused more on *F. oxysporum.* A study by Ghag et al. (2014) targeted the genes *velvet* and *Fusarium transcription factor 1* in an attempt to control Fusarium

wilt disease in transgenic banana plants. As a result, the fungi showcased a reduction in growth and virulence. Later, in 2020, the same disease and fungal species were targeted by transgenic banana plants expressing dsRNA of fungal *C-24 sterol methyltransferase* (*ERG6)* and *cytochrome p450 lanosterol C-14α-demethylase (ERG11)* (Dou *et al*, 2020). Likewise, this resulted in inhibition of fungal growth and development. In addition, HIGS methods were used for *F. oxysporum* inhibition in transgenic tomato plants. In two separate studies, the silencing of the fungal *peroxisomal biogenesis factor 6 (PEX6)* and *ornithine decarboxylase (ODC)* genes was investigated, leading to moderate to high resistance of the plant to *F. oxysporum* (Tetorya & Rajam, 2018; Singh *et al*, 2020). In Arabidopsis, the fungal genes *FOW2, FRP1* and *OPR* were also silenced using HIGS with varying results, but overall a reduction in symptoms and pathogenicity of the fungus (Hu *et al*, 2016).

Other fungal species that have been targeted by HIGS are *F. graminearum* and *F. culmorum.* The former was used to investigate the gene silencing of *chitin synthase 3b* and *2 (Chs3b* and *Chs2)* in transgenic wheat plants (Cheng *et al*, 2015). The method proved highly effective as the fungal growth and virulence were significantly reduced. The attempted silencing of *F. culmorum* (*Fgl1)* (*Fmk1*), *β-1,3-glucan synthase (Gls1)* and *chitin synthase V (ChsV)* genes by HIGS resulted in a moderate reduction of the fungal spreading (Chen *et al*, 2016).

2.4.2 Spray-induced gene silencing

Various studies on HIGS have proven that RNAi is a promising way of targeting plant pathogens. However, implementing this method in food production comes with challenges. Most of them are related to the fact that many countries are still non-acceptive about GMOs in crop production and to the difficulty of certain crop plants to be transformed (Machado *et al*, 2018). As a possible solution to these problems, another RNAi approach was put forward by Wang et al. (2016): spray-induced gene silencing (SIGS). The technique relies on the same mechanisms as conventional RNAi with HIGS, but instead exogenous siRNAs or dsRNAs are sprayed directly onto the plants or fruits (Machado *et al*, 2018).

In this application, there are two ways for the siRNAs to reach the fungal target gene. In the first case, the plant processes the externally applied dsRNA and the produced siRNAs are taken up by the fungi. Alternatively, the fungus can take up the dsRNA from the leaf and process it into functional siRNAs using its own RNAi machinery (Fig. 9). It has been shown with fluorescently labeled siRNAs that fungi can take up siRNAs from the environment, although the exact mechanism behind this is not yet completely understood (Jöchl *et al*, 2009). Uptake of extracellular vesicles through endocytosis or extracellular fusogenic proteins has been found to play a role in the transport of siRNAs from fungi to host plants and vice versa (Nowara *et al*, 2010; Knip *et al*, 2014; Haag *et al*, 2015; Han & Luan, 2015). If plant extracellular vesicles are the main mode of siRNA transport, it might be important to keep the lifestyle of the fungus in mind. In case of a necrotrophic mode of action, the host plant cells may encounter severe damage which could result in loss of vesicle integrity (Majumdar *et al*, 2017). Another mechanism for RNA uptake in animals is via transmembrane transporters (Shih & Hunter, 2011). It is still unknown whether fungi possess a similar transporter mechanism. In the fungus *Sclerotinia sclerotiorum,* GFP-labeled siRNAs have revealed the involvement of clathrin-

mediated endocytosis in dsRNA uptake. Moreover, the dsRNA uptake was not homogenous and was seen mostly in actively growing regions of the fungi like newly formed hyphal branches and septate regions (Wytinck *et al*, 2020).

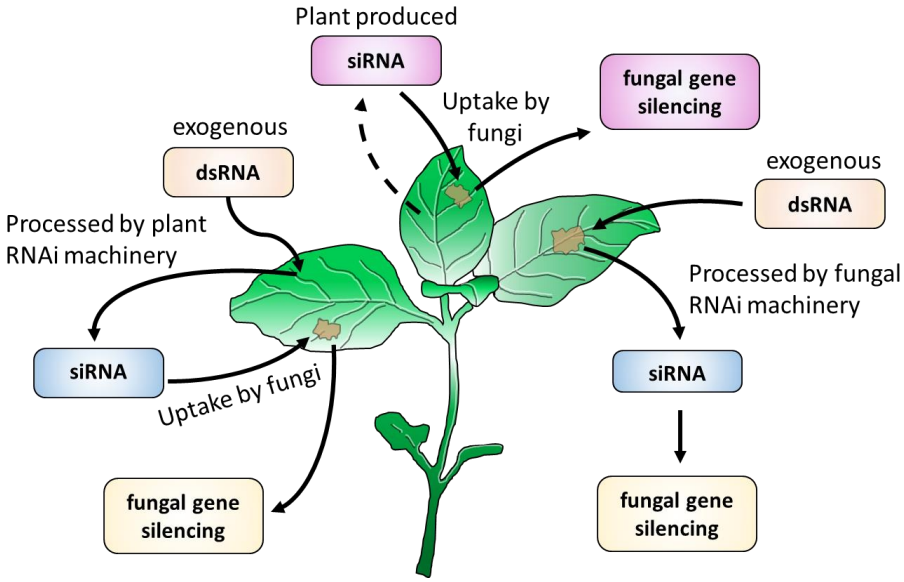


Figure 9: simplified mode of action of host-induced gene silencing (in pink) and spray-induced gene silencing (on the left and right) between a pathogenic fungi and its host plant.

SIGS was used the first time to silence the *Botrytis cinerea* genes *DCL1* and *DCL2* by spray application on a variety of fruits, vegetables and leaves. The application resulted in a reduction of grey mould disease formation compared to controls (Wang *et al*, 2016). After this discovery, it didn't take long before trials of SIGS on *F. graminearum* were conducted. The first dsRNA reported to successfully inhibit the fungus by SIGS is *CYP3* (792 nt) (Koch *et al*, 2016). This fragment targets three genes: *CYP51A, CYP51B* and *CYP51C,* all of which are cytochrome P450 *lanosterol C-14α-demethylases.* The treated barley leaves showed a substantial reduction of the size of the brown lesions as well as a reduction in gene expression of the *CYP51* genes ranging from 48 to 58%. This inhibition was not only seen on the directly sprayed parts of the leaves but also on the non-sprayed areas. A later study in 2020 by Werner et al. also investigated the possible application of SIGS to inhibit *F. graminearum* infections. In this case, the RNAi machinery genes *ARGONAUTE (AGO1* and *AGO2)* and *DICER (DCL1* and *DCL2)* were used to create dsRNA constructs. Detached barley leaves were sprayed with the dsRNA and drop-inoculated with fungal conidia 48 hours later. Especially the silencing of the *DICER* genes proved successful in increasing the plant resistance to the fungus (Werner *et al*, 2020).

Thus far, it doesn't seem like fungi have a preference for the uptake of short or long dsRNA (Majumdar *et al*, 2017). What does seem to be true is that the efficiency of dsRNA uptake can

differ greatly between fungal species, cell types and developmental stages (Qiao *et al*, 2021). It has been witnessed that fungal conidia harvested from a fungus treated with siRNA and sub-cultured *in vitro* still show signs of the applied siRNA. This suggests that the signal can perpetuate to subsequent generations after application (Masanga *et al*, 2015; Zhang *et al*, 2016). Another application yet to be investigated is whether siRNAs might also prove useful in the priming of seeds against pathogens and their mycotoxins (Majumdar *et al*, 2017).

In conclusion, SIGS is a relatively young RNAi application but proves to be remarkably efficient in silencing important pathogenic fungal genes. Lots of possible targets and applications are yet to be investigated. Hence, the motivation to focus in this dissertation on SIGS to reduce virulence and pathogenicity of *F. verticillioides* infections on maize crops.

2.4.3 Practical challenges

The future of SIGS as an application in fungal pest control looks promising. It becomes more and more apparent that resistance against conventional fungicides is a problem of high importance (Spolti *et al*, 2014). This is often the consequence of over application of the fungicidal component or the frequent targeting of the same chemical group. In field cases where most pathogens can be well-managed with conventional methods except for one species, it might be very helpful to use SIGS to target this one outlying species (Machado *et al*, 2018). Especially the high sequence specificity of SIGS will provide a great advantage in these cases. In practice, HIGS will not be very useful for post-harvest applications. SIGS, on the other hand, could be more effective to control pathogens post-harvest (Machado *et al*, 2018).

As with every new evolving pest-control technique, there are some challenges involved. For SIGS in particular, most of them relate to the degradation of the dsRNA in the environment and the possible risk of targeting non-target organisms, as will be discussed here.

2.4.3.1 DsRNA degradation and off-target effects

When put into practice, run-off products of the spray-applied dsRNA or dsRNA produced by GM plants may enter the neighboring soil or water. Thus, the application of RNAi in pest control raises some questions about environmental safety and uptake by off-target organisms. Only a few studies have actively looked into this matter and will be shortly discussed here.

A dsRNA used for pest control in the Western Corn Rootworm (DvSnf7) was investigated for its degradation rate in agricultural soil samples collected in the field in Ilinois, Missouri and North Dakota (USA) (Dubelman *et al*, 2014). DvSnf7 was added to the different soils at a concentration of 7.5 µg per gram of soil. The half-life (DT_{50}) of the dsRNA ranged from 18 to 29h based on the assay and soil sample. In all three soils, 90% of degradation (DT_{90}) occurred in less than 35h. After 2 days, the dsRNA was undetectable and thus fully degraded.

The same group later published another paper after investigating the same process in aquatic environments with different chemical and physical properties (Fischer *et al*, 2017). The added concentration of DvSnf7 ranged from 300-330 ng RNA/mL water. The DT_{50} was less than 3 days and DT_{90} less than 4 days. At 7 days, the dsRNA was no longer detectable in the water.

Albright *et al.* (2017) deliberated further on this subject and tested different aquatic microcosms combinations of sterilized/unsterilized sediment and sterilized/unsterilized water. In this study, the added concentration of dsRNA was 1.5 µg/mL water. This experiment confirmed the rapid degradation of dsRNA in aquatic environments since the molecules were undetectable after 96h.

Research by Parker *et al*. (2019) has further focused on the degradation of dsRNA molecules in agricultural soils. By the use of phosphorus-32 radiolabeled dsRNA, they were able to detect dsRNA with higher precision (~ ng/g soil) compared to previously used techniques. Their findings prove that the dsRNA reduction in the soil is not only due to degradation (with or without microorganisms). Alongside these processes, dsRNA is also adsorbed to particle surfaces, which causes losses that seemed to be underestimated in prior studies. The rate by which these processes happens is depending on soil-specific factors like texture, organic matter content and basal respiration rate. In conclusion, all of the studies conducted thus far showcased a rapid degradation of the dsRNA in the environment, which is most likely due to nuclease enzymes (Albright *et al*, 2017). This information will be very useful in future ecological risk assessments.

At the moment, possibilities of off-target transcript knockdown are under investigation. It was initially thought that even one mismatch of the siRNA to a target sequence would be enough to avoid gene silencing (Majumdar *et al*, 2017). However, it was later on shown that a partial complement (15 out of 19 base pairs) with the target mRNA sequence can already cause off-target effects (Jackson *et al*, 2003; Persengiev *et al*, 2004; Jackson *et al*, 2006). Nevertheless, this work was focused on the medical field and it is therefore not certain that the same kind of partial complement could also cause off-target effects in fungi. Therefore, it is crucial to keep in mind and detect any possible off-target genes before and during dsRNA design. Sequence complementarity-based approaches with computational tools could assist in this process (Taning *et al*, 2020). Even though it looks like RNAi is a promising and safe future pest control agent, more high-level studies are needed to provide general confidence in the safety of this fast-evolving technique.

2.4.3.2 DsRNA uptake through food

The possible uptake of applied dsRNA by animals and humans and the following possibility of altered gene expression is an emerging and important research topic. However, plants are natural producers of dsRNAs, microRNAs and siRNAs. Thus, humans take up exogenous dsRNAs and siRNAs from plants on a regular basis as a naturally occurring process (Machado *et al*, 2018). In the unlikely case where the dsRNA or siRNA is still intact after ingestion and going through the digestive tract, the molecules have to reach the target tissue in high enough quantity and with an almost perfect sequence complementarity (Roberts *et al*, 2015). Some studies concerning this topic have been conducted. A study by Zhang et al. (2012) has found evidence of the delivery of siRNA into mammalian systems. Most other studies don't show any proof of gene expression alteration after uptake of exogenous applied dsRNAs or siRNAs or failed to replicate the data of Zhang et al. (Witwer *et al*, 2013; Witwer & Hirschi, 2014). Some however, found data consistent with the original study (Wang *et al*, 2012; Beatty *et al*, 2014).

Additionally, another question that should be asked is whether the siRNAs that make it to the mammalian gut have the power to affect the present gut bacterial populations (Majumdar *et al*, 2017). Since bacteria don't possess the RNAi machinery like eukaryotes but rely mostly on CRISPR/Cas systems for gene silencing, this scenario seems rather unlikely (Horvath & Barrangou, 2010; Sherman *et al*, 2015).

There is still a lack of independent corroborating data concerning this topic and future studies will hopefully bring more insight for risk assessment purposes (Roberts *et al*, 2015; Machado *et al*, 2018).

2.3.4.3 Other challenges

The exogenous application of spraying dsRNA on plants might only work actively for approximately five to seven days (Gan *et al*, 2010). Therefore, it is useful to find ways of applying that guarantee a longer effect. Recently, clay nanosheets have been investigated for the purpose of applying dsRNA onto plants. When layered double hydroxide clay nanosheets were loaded with the dsRNA and sprayed onto leaves, the dsRNA did not wash off and was still detectable 30 days after application. Protection against the targeted virus was still present 20 days after spraying (Mitter *et al*, 2017). While trying another way of improving the adhesion of the dsRNA onto the leaves, the surfactant Silwett L-77 has been used during spray application experiments (McLoughlin *et al*, 2018). Other possibly helpful agents are peptide-based RNA delivery systems and cationic nanoparticles (Numata *et al*, 2014; Jiang *et al*, 2014; Dubrovina & Kiselev, 2019).

Another factor to take into account is the costs associated with dsRNA production. At the moment, kits used for dsRNA synthesis are relatively expensive (Das & Sherif, 2020). However, industrial-scale applications are in development in several agricultural businesses (Palli, 2014). This is also noticeable in the production price. Today, it is possible to produce dsRNA at $60 per gram wherein 2008 the cost would be $12 500 for the same amount (Zotti *et al*, 2018; Dalakouras *et al*, 2020; Das & Sherif, 2020). This is a beneficial evolution given that per hectare of agricultural soil, 2 to 10 gram of dsRNA would be needed (de Andrade & Hunter, 2016). Some high-scale production techniques in the making at the moment are microbial fermentation technology (RNAgri, 2020) and the use of bacterial minicells to produce encapsulated dsRNAs (AgroSpheres, 2020).

2.5 Genes of interest

2.5.1 *Chs3b*

The cell wall of most fungi is build out of β-1,3-glucans interwoven with chitin microfibrils which are embedded in a gel-like matrix consisting of glycoproteins and α-1,3-glucan polysaccharides (Riquelme *et al*, 2018). The chitin part of the cell wall is the most abundant component and is of major importance to the integrity and shape of the cells. The long microfibrils are synthesized at the cell surface at sites of cell wall expansion by chitin synthase (Chs) membrane proteins (Riquelme & Bartnicki-García, 2008). In fungi, the chitin synthases are placed into seven different classes based on their amino acid sequences. They play various roles in both virulence and development (Cheng *et al*, 2015). The Chs proteins are inactively transported by vesicles (Riquelme *et al*, 2018). The fact that the chitin synthases are not present in plants or mammals makes them promising antifungal targets. A study of Cheng *et al*. (2015) tried to identify the *Chs* genes that are essential for *F. graminearum* growth and infection. This species has eight *Chs* genes and among them *Chs3b* showed the highest level of expression throughout a seven day colonization of wheat. Thus, they chose to silence this chitin synthase gene by HIGS and found that it was essential for fungal growth and infection. Consequently, *Chs3b* was chosen as target gene for *F. verticillioides* treatment in this study. Eight chitin synthase genes were identified in this species but it is unclear whether knock-out of the *Chs3b* gene could have the same lethal effects as it has in *F. graminearum* (Larson *et al*, 2011).

2.5.2 *eGFP*

Green fluorescent protein (GFP) was first obtained from the jellyfish *Aequorea Victoria* and has since been used as a reporter for monitoring gene expression and localization (Chalfie *et al*, 1994). In eukaryotic cells, GFP expression leads to green fluorescence when excited by blue or ultraviolet light (Zhang *et al*, 1996). Since the sensitivity of the wild-type GFP is below that of standard reporter proteins, an enhanced GFP (eGFP) variant was created which is 35 times brighter and more easily expressed and folded (Cormack *et al*, 1996). The *F. graminearum* strain used in this study (PH-1) is marked with eGFP and will serve as a control for gene silencing.

3. Methodology

3.1 Species and Sampling

Isolates of the fungi *F. graminearum* and *F. verticillioides* were used in this investigation. The F. *verticillioides* isolate was isolated in Vietnam and kindly provided by Minh Trang Tran. For more information on this strain I kindly refer to Tran et al. (2021a+b). For *F. graminearum* was the reference strain PH1 which was GFP-tagged. PH-1 (Michigan State University, USA) is a *F. graminearum* isolate that produces type B mycotoxins and has been widely studied around the world (Trail & Common, 2000). It is full-genome sequenced and always used as *Fusarium* genome reference which makes it more convenient for molecular studies (King *et al*, 2017). They were grown on potato dextrose agar (PDA) (40 g/L) and in liquid medium containing 20 g/L of malt extract broth. In order to induce spore formation, inoculated plates were placed under near-UV light for at least one week. Afterwards, conidia were harvested in sterile water, counted under the microscope and diluted to a final concentration of 1×10^7 conidia/mL for *F. verticillioides* and 1×10^6 conidia/mL for *F. graminearum.*

3.2 RNA extraction and cDNA synthesis

Fungal samples for RNA extraction were grown in liquid malt extract medium (20 g/L) for one week before harvesting. Afterwards the fungal biomass was centrifuged for 5 min at 10 000 rpm, freeze dried overnight and crushed. RNA was extracted by means of TRIzol™ reagent and the protocol provided by the producer (Invitrogen, Carlsbad, U.S.A). For the following cDNA synthesis, the iScript™ cDNA synthesis Kit (Bio-Rad, Hercules, U.S.A.) was used according to the provided protocol.

3.3 Construction of Templates and dsRNA synthesis

3.3.1 *Chs3b* and *eGFP*

The target gene sequences for *Chs3b (F. verticillioides), Chs3b (F. graminearum)* and *eGFP* were amplified with a temperature gradient PCR ranging from 50.0 - 58.1 °C and *F. verticillioides* and *F. graminearum* cDNA using self-designed primers (see supplement I). Afterwards, the fragments were loaded on 1.5% agarose gel at 100V and stained with ethidium-bromide. Another PCR reaction was then started at the temperature with the clearest band on gel for each of the genes (52.7 °C). When the PCR fragments showed a clear band at the desired length, the PCR product was used as template for another PCR amplification in order to increase the fragment concentration. A clean-up of the resulting PCR products was carried out by use of the Wizard SV gel and PCR clean-up kit® (Promega, Madison, US). The resulting concentrations of the PCR products are listed in supplement I.

3.3.2 DsRNA synthesis

The PCR products were used as template for dsRNA synthesis with the MEGAscript™ RNAi Kit (Thermo Fisher Scientific, Waltham, U.S.A.). The transcription reaction assembly, RNA annealing reaction, nuclease digestion and purification of the dsRNA were carried out by following the instructions of the producer. A 10X diluted sample was taken after transcription reaction, RNA annealing and final clean-up to load on 1.5% agarose gel. This allowed for a

visual confirmation of the correct fragment size. The final dsRNA concentrations agarose gel visualizations are listed in supplement I.

3.4 Microscopic assay

Microscopic slides were prepared by using a modified method described by Riddell (1950). In short, microscopic slides and glass cover slides were heat treated with a Bunsen burner and placed in a sterilized petri dish to cool. From a prepared PDA plate (40 g/L), 1 cm^2 shapes of around 2 mm thick were cut and one square was placed in the middle of each microscopic slide. On each agar square, 2.5 µL conidia suspension (1.0×10^7 conidia/mL for *F. verticillioides* and 1×10^6 conidia/mL for *F. graminearum)* was placed on two opposing sides along the edge. For the control slides, 2.5 µL of water was added on top of the conidia suspension and for the dsRNA treatment slides, 2.5 µL of dsRNA (100 ng/µL) was added. The agar squares were covered with the glass cover slides and the petri dishes were closed and sealed. Three slides were prepared for each treatment (*F. verticillioides chs3b, F. graminearum chs3b* and *F. graminearum eGFP*) along with three control water slides for each. After 24h of incubation on room temperature, pictures were taken with Olympus IX81 inverted microscope equipped with an Olympus XC50 camera. An X-Cite Fluorescence LED illuminator was used to visualize the GFP signals present in the *F. graminearum* conidia (Excelitas, Waltham, U.S.A). The germinated conidia lengths were measured with the Cell^F imaging software (Olympus, Tokyo, Japan)

3.5 *In-vitro* dsRNA assay

3.5.1 Experimental set-up

A droplet of 5 µL conidia suspension (1×10^7 conidia/mL for *F. verticillioides* or 1×10^6 conidia/mL for *F. graminearum*) was put in the middle of each well of a 6 well plate. In the case of *F. verticillioides* application, the 6-well plates were filled with solid PDA (40g/L). The plates with *F. graminearum* were filled with a mycotoxin production inducing mineral medium containing 30 g sucrose, 30 g agar, 2 g L-arginine, 1 g KH2PO4, 0.5 g MgSO4·7H2O, 0.5 g KCl, 10 mg FeSO4·7H2O and 200 µL of trace element solution (per 100 mL: 5 g citric acid, 5 g ZnSO4·7H2O, 0.25 g CuSO4·5H2O, 50 mg MnSO4·H2O, 50 mg H3BO3, 50 mg NaMoO4·2H2O) in 1 L. The medium pH was set at 6.5 with addition of NaOH (Gardiner *et al*, 2009).

In the first trial on the 6-well plates, 5 µL of *Chs3b* dsRNA (100 ng/µL) was added either only at the beginning of the inoculation to the conidia or applied daily on the growing fungi. The control plate consisted of the same starting concentration of conidia but 5 µL of nuclease-free water was added instead at the beginning of the experiment. Separate plates were made for *F. verticillioides* and *F. graminearum*, where for the latter extra plates were made to test the *eGFP* dsRNA (100 ng/µL). Later, in the second trial on the 6-well plates, and extra control plate was added for each fungi and dsRNA combination where the water control was not only added at the beginning but also daily. Pictures were taken each 24h with a Wiwam automated phenotyping robot (Wiwam, Eeklo, Belgium) for 4 days in case of *F. verticillioides* and 3 days in case of *F. graminearum*. The surface area covered by the fungi on the agar plate was

measured with ImageJ open source image processing program (Schneider *et al*, 2012) (Supplement II).

A final 6-well trial was carried out with the same experimental set-up. The only difference being that extra medium types were added. For *F. verticillioides,* the first medium in the trial is the solid PDA as described before. In addition, the same set-up was carried out on a medium containing a 10X dilution of the PDA medium (replacing the needed amount of PDA with normal white agar). The last medium contained normal white agar (40 g/L) and tap water to create an environment with very minimal nutrient sources for the fungi. For the *eGFP* and *chs3b F. graminearum* plates, extra set-ups were added of the previously described mineral medium in a 10X dilution and the same water medium as for *F. verticillioides* (Supplement II).

3.5.2 Mycotoxin analysis by LC-MS/MS

At the end of the last *in-vitro* 6-well trial, the daily control and daily application plates of *F. verticillioides* were selected to be used for fumonisin analyses. For each treatment, (one daily control plate and one daily application plate for each of the three different media) three wells were randomly selected and crushed by using liquid nitrogen. In addition, six wells containing only solid PDA medium were also crushed and used as blank samples. An amount of 1 gram was weighted for each of the samples and used for further analyses.

The following protocol was modified from De Boevre *et al* (2012) and Tran *et al* (2021). All the samples were spiked with 20 µL of an internal de-epoxy-deoxynivalenol (DOM) standard (50 ng/µL, 1000 µg/kg end concentration). In addition, the blanks were also spiked with fumonisin (FB1, FB2 and FB3) standard for the creation of a standard curve (0 to 4000 µg/kg). Afterwards, the samples were extracted in 5 mL of extraction solvent containing ethyl acetate and 1 % formic acid and vortexed well. Later they were agitated for 15 min on an overhead shaker and centrifuged for 15 min at 3000 g. The supernatant was passed through a folded paper filter into a new extraction tube and the solution was vaporized under a nitrogen flow at 1 atm in a water bath at 40 °C. Afterwards, the remaining residue was resolved in 200 µL injection solvent containing mobile phase A [water/methanol/acetic acid (94/5/1, v/v/v) + 5 mM ammonium acetate] and mobile phase B [methanol/water/acetic acid (97/2/1, v/v/v) + 5 mM ammonium acetate] with a 60/40 ratio of A/B. To continue, the samples were vortexed well and transferred onto an Ultrafree® centrifuge filter (Millipore Bedford, MA, U.S.A.) followed by ultracentrifugation at 10 000 g for 10 min. At last, the filtrate was transferred to a HPLC micro-injection vial prior to injection into the LC-MS/MS.

Analysis with LC-MS/MS was done by a previously described protocol by Ediage *et al* (2011). A Waters Acquity HPLC-Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) in positive electrospray ioni- zation (ESI+) mode was used for the detection and quantification of the three targets FB1, FB2 and FB3 in each sample. The chromatic separation was obtained by use of a Symmetry C18 (150 mm × 2.1 mm, i.d. 5 µm) column with a guard column (10 mm × 2.1 mm i.d.) of the same material (Waters, Zellik, Belgium) (De Boevre *et al*, 2012; Tran *et al*, 2021a).

3.6 Infection of seedlings

Maize seeds (NK7328; Syngenta, Basel, Switzerland) were disinfected in 1.4% sodium hypochlorite, washed with sterile water and dried to the air for about 2 hours. Afterwards, the seeds were planted in moist vermiculite until root and shoot just appeared. They were then transferred to a sterile petri dish where seedlings with roughly the same root and shoot length were inoculated in conidia suspension of *F. graminearum* and *F. verticillioides* for one hour. Afterwards, the seedlings were planted in glass tubes containing 200 gram of quartz sand mixed with 1.5 gram polymer per kg of sand and placed under growing lights. Ten mL of nutrient solution containing KNO_3 (2M), $Ca(NO_3)_2*4H_2O$ (2M), iron (Sprint 138) (15 g/L), $MgSO_4*7H_2O$ (2M), $CuSO_4*5H_2O$ (0.08 g/L), $Na_2MoO_4*2H_2O$ (0.12 g/L), $MnCl_2*H_2O$ (1.81 g/L), H_3BO_3 (2.86 g/L) and KH_2PO_4 (1M) was added to each tube. The plants were watered every 2 days with 5 mL of tap water. After 16 days of growing in a standard lab environment, the plants were harvested and the length and weight of the plant, leaves and roots was measured with an analytical balance. The biomass was afterwards dried for 7 days at 60°C after which the dry biomass was again weighted on an analytical balance.

3.7 Detached leaf assay

Maize seeds (NK7328; Syngenta, Basel, Switzerland) were disinfected in 1.4% sodium hypochlorite, washed with sterile water and dried to the air for about 2 hours. Afterwards, the seeds were planted in moist vermiculite and grown for two weeks or until the desired plant height was reached. The first leaf was cut from the plant with a sterile scalpel and washed in 1.4% sodium hypochlorite followed by sterile water. The leaves were either wounded by a small diagonal scratch with a scalpel on the upwards facing side of the leaf or left intact.

3.7.1 Infection trial

The first detached leaf experiment was an infection trial to form a solid representation of the infection symptoms of *F. graminearum* and *F. verticillioides.* Six unwounded and six wounded leaves were placed on plant agar plates (40 g/L) followed by infection with 10 µL *F. verticillioides* conidia (~0.5 × 10^7/mL) and the same amount of leaves with 10 µL *F. graminearum* conidia (~0.5 × 10^6 /mL). As a control, six unwounded and six wounded leaves were inoculated with 10µL sterile water. Every 24h, multispectral pictures were taken with a Wiwam automated phenotyping robot (Wiwam, Eeklo, Belgium) for five days (Supplement III).

3.7.2 *Chs3b* and *eGFP*

The effect of the dsRNA on the fungal infection was first tested with *Chs3b* and *eGFP* constructs. Seven plant agar plates were used for the first trial with each containing five wounded maize leaves (Supplement III). For this trial the third leaf of each plant was used to see whether this would increase the consistency of the experiment. One control plate for each fungi was inoculated with 5 µL of approximately 1.0 × 10^7 conidia/mL of *F. verticillioides* or *F. graminearum* of 1.0 × 10^6 conidia/mL and 5 µL of sterile water. The third plate was inoculated with 5 µL of *F. verticillioides* conidia like the previous plates together with 5 µL of *F. verticillioides* specific *Chs3b* dsRNA in a concentration of 200 ng/µL. The fourth plate is the same as the third but instead *F. graminearum* conidia (1.0 X 10^6 conidia/mL) and *F.*

graminearum specific *Chs3b* dsRNA was applied. The fifth plate consisted of 5 µL *F. graminearum* conidia like described before and 5 µL *F. graminearum* strain specific *eGFP* dsRNA. The next plate is the same with the only difference that *F. verticillioides* conidia were used. Here, the *eGFP* dsRNA should not have an effect and this is thus a control plate. At last, the final plate also contained five leaves where the first two were inoculated with 10 µL sterile water, the next one with 10 µL of *F. verticillioides* specific *Chs3b* (200 ng/µL) and the last with 10 µL of *F. graminearum* specific *Chs3b* (200 ng/µL). Pictures were taken with a Wiwam automated phenotyping robot (Wiwam, Eeklo, Belgium) every 24h for five days (Supplement III).

A second trial was carried out the same way as the infection trial with the first leaves of the plants (Supplement III). As in the infection trial, six leaves were used per plate. The experiment is the same as the first *Chs3b* and *eGFP* trial except that 5 µL of dsRNA is initially applied in a concentration of 100 ng/µL. During this trial, again 1.0×10^7 conidia/mL of *F. verticillioides* were used and 1.0×10^6 conidia/mL of *F. graminearum.* This in order to get a final concentration on the leaves at time of infection of respectively 0.5×10^7 conidia/mL and 0.5×10^6 conidia/mL, as was the case in the first infection trial. During the five days lasting trial, the dsRNA is applied daily right after taking pictures with the a Wiwam automated phenotyping robot.

4. Results

The colors used for the visual representation of the data in the result section are allocated according to treatment. Negative controls (water) are visualized in green, positive controls (conidia suspension without dsRNA treatment) are orange to red and the dsRNA treated samples are always depicted in blue colors.

4.1 Microscopic Assay

DsRNA was created for the chitin synthase 3b gene (*chs3b*) of *F. verticillioides* and *F. graminearum* specifically, along with dsRNA designed to target the eGFP gene which is incorporated into the used *F. graminearum* strain (Supplement I). Microscopic slides were made in triplicates containing 2.5 µL of conidia suspension (1×10^7 conidia/mL for *F. verticillioides* and 1×10^6 conidia/mL for *F. graminearum*) and 2.5 µL of water (control) or 2.5 µL of conidia suspension and 2.5 µL of dsRNA (100 ng/µL). Pictures of germinated conidia were taken approximately 24 hours after preparation of the slides (five pictures per slide).

After germination, the microconidia of *F. verticillioides* were hard to distinguish from the formed mycelium (Fig. 10 B, C). Therefore, the length of the total germinated spore was measured for all the spores on each of the five pictures that were taken per microscopic slide. In total, 239 spores were measured for the control group and 272 for the dsRNA treated slides. Based on a Mann-Whitney U test, no significant difference in length between the two treatments was witnessed (Fig. 10 A, p = 0.16). Upon further inspection at a higher magnification, no visible differences were observed in the cell membrane or overall mycelium structure between the two groups.

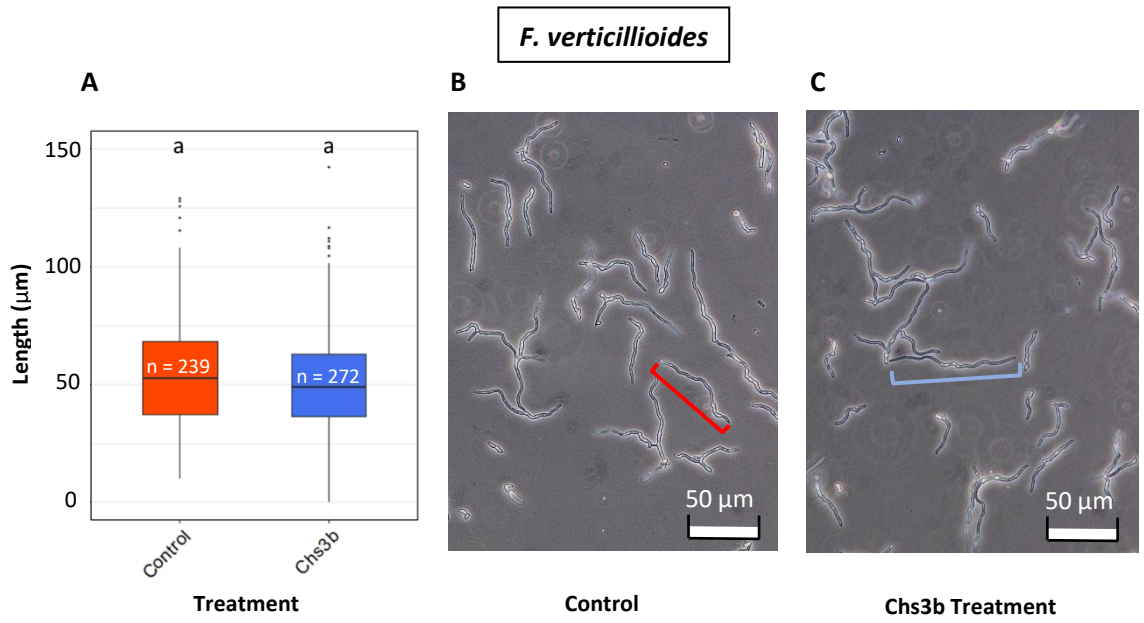


Figure 10: A) germinated Fusarium verticillioides spore length according to control or chs3b dsRNA treatment of the spore suspension carried out on 15 microscopic pictures for each. Statistical comparison based on Mann-Whitney U test. B) microscopic picture of conidia treated with water (control) C) microscopic picture of conidia treated with chs3b dsRNA (100 ng/µL). Red and blue indicators visualize the measurement method.

The macroconidia of *F. graminearum* typically germinate at both ends of the spore. In addition, side branches can also be formed at other septa (Fig. 11 B and C). First of all, the new mycelium at both the ends of the conidia was measured on the microscopic pictures. A total of 320 measurements were made for the control group and 298 for the dsRNA treated samples. Based on a Mann-Whitney U test, no significant difference in length between the two treatments was witnessed (Fig. 11 A, p = 0.51). Upon further inspection at a higher magnification, again no visible differences were observed in the cell membrane or overall mycelium structure between the two groups.

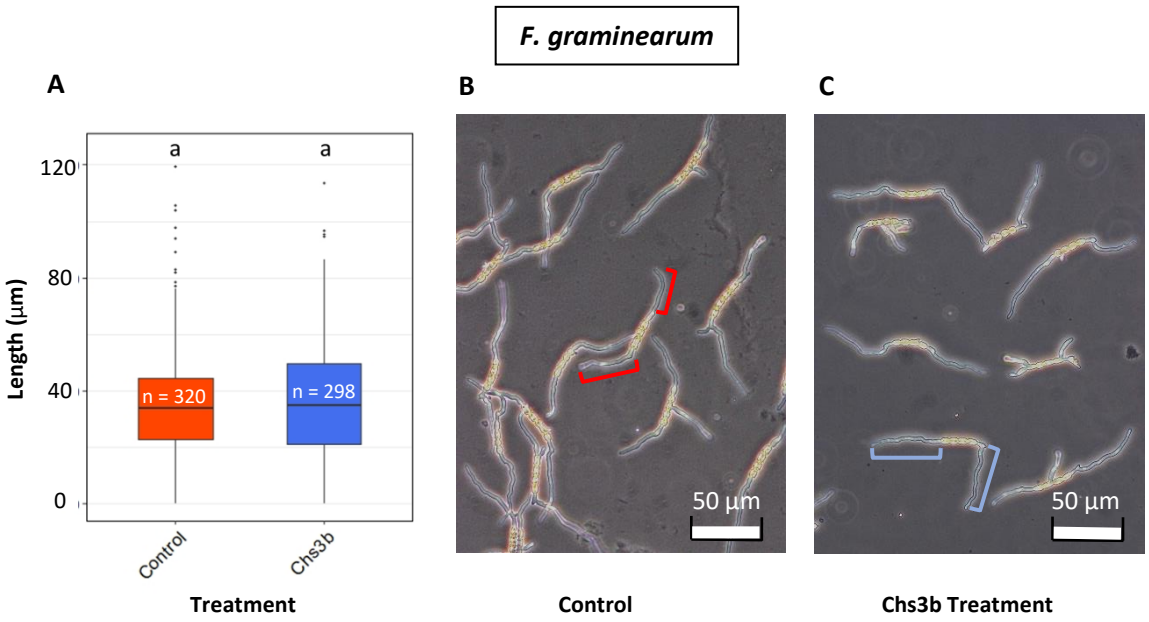


Figure 11: A) germinated Fusarium graminearum spore length according to control or chs3b dsRNA treatment of the spore suspension carried out on 15 microscopic pictures for each. Lengths measured at both sides of the germinating spore. Statistical comparison based on Mann-Whitney U test. B) microscopic picture of conidia treated with water (control) C) microscopic picture of conidia treated with chs3b dsRNA (100 ng/µL). Red and blue indicators visualize the measurement method.

On the same microscopic pictures, the number and length of the side branches were obtained. A total of 54 side branches were present in the control group pictures and 105 were counted in the dsRNA treated ones. Visually, it seemed that the side branches of the dsRNA treated slides were shorter when compared to the control. However, based on a Mann-Whitney U test, no significant difference in length of the side branches between the two treatments was witnessed (Fig. 12 A, p = 0.14). In addition, the number of side branches for each spore was noted and the percentage of spores with either 0, 1, 2, or 3 side branches calculated (Fig 12 B). After visualization, it looked like the dsRNA treated samples overall produced more side branches than the control treatment. According to a Mann-Whitney U, no significant difference in amount of side branches was present (p = 0.686).

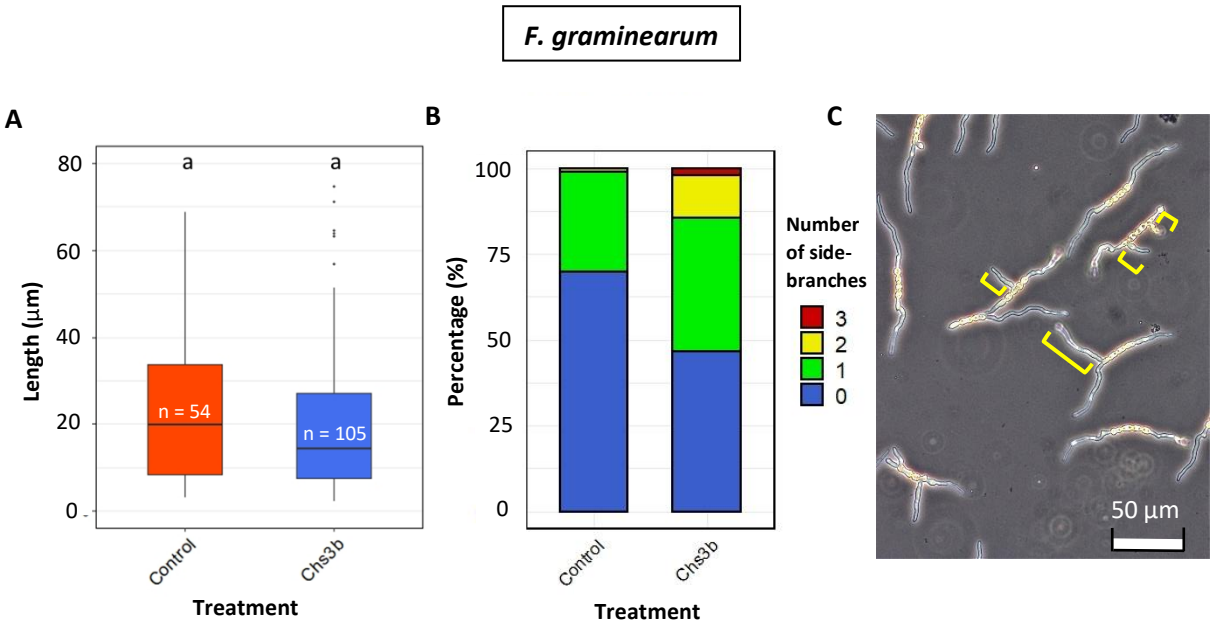


Figure 12: A) germinated Fusarium graminearum conidia side branch length according to control or chs3b dsRNA treated conidia suspension carried out on 15 microscopic pictures for each. Statistical comparison based on Mann-Whitney U test. B) proportional number of side branches according to control or chs3b dsRNA treated spore suspension C) microscopic picture of conidia treated with chs3b dsRNA (100 ng/µL). Yellow indicator visualizes the measurement method.

At last, pictures were taken of the *eGFP* dsRNA treated *F. graminearum* slides and the *F. graminearum* control slides treated with water. No visual difference was evident between the treatments in the GFP signals transmitted by conidia or mycelium (Fig 13). Nevertheless, it is important to bear in mind that this is only a visual constatation and not based on quantitative data.

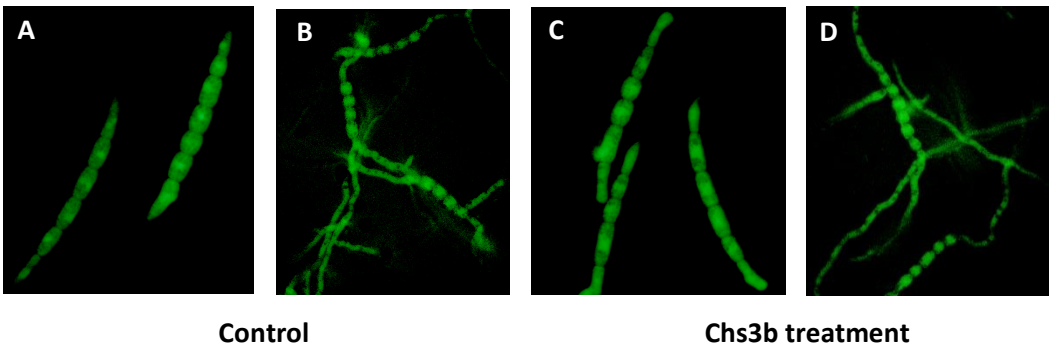


Figure 13: microscopic pictures of GFP signal transmitted by Fusarium graminearum conidia and mycelium for the control treatment with water (A and B) and conidia treated with chs3b dsRNA (100 ng/µL, C and D).

4.2 *In-vitro* assay

4.2.1 Six-well plate assay

In order to examine the effects of the dsRNA treatment on a larger scale and longer timeframe, an *in-vitro* assay with 6-well plates was developed. Four plates were used for each dsRNA and pathogen combination (*F. verticillioides chs3b, F. graminearum chs3b* and *F. graminearum eGFP*): a control plate inoculated with 5 µL conidia suspension (1.0×10^7 conidia/mL conidia/mL for *F. verticillioides* and 1×10^6 conidia/mL for *F. graminearum)* and 5 µL sterile water, a treatment plate with 5 µL conidia suspension and 5 µL dsRNA (100 ng/µL), a second control plate where the 5 µL sterile water is added daily and a fourth plate where the 5 µL dsRNA treatment is added daily (Supplement II). The plates for *F. verticillioides* contained PDA medium whereas *F. graminearum* plates were filled with mineral medium. Pictures were taken each 24h with a Wiwam custom-build multispectral phenotyping platform. The assay lasted for four days for *F. verticillioides* but only three days for *F. graminearum*, since the faster growth rate of this fungus enabled it to fill the entire well more rapidly. After validating the normality of the data with a Shapiro-Wilk test, an ANOVA test was conducted followed (when significant) by a Dunnett's post-hoc test. In the latter, the three treatments (daily control, daily dsRNA application and dsRNA application at the beginning) were each compared to the water control.

On the second trial day, the area covered by *F. verticillioides* treated with *chs3b* dsRNA (either applied at the trial start or daily) showed a significant difference compared to the control (Dunnett's test $p < 0.05$, Fig. 14). Three days after the start of the trial this was only the case for the daily applied dsRNA treatment. At the last trial day, there was no longer a significant difference between the four treatments. Pictures of the control plate during the four days of trial are added to Supplement II for reference.

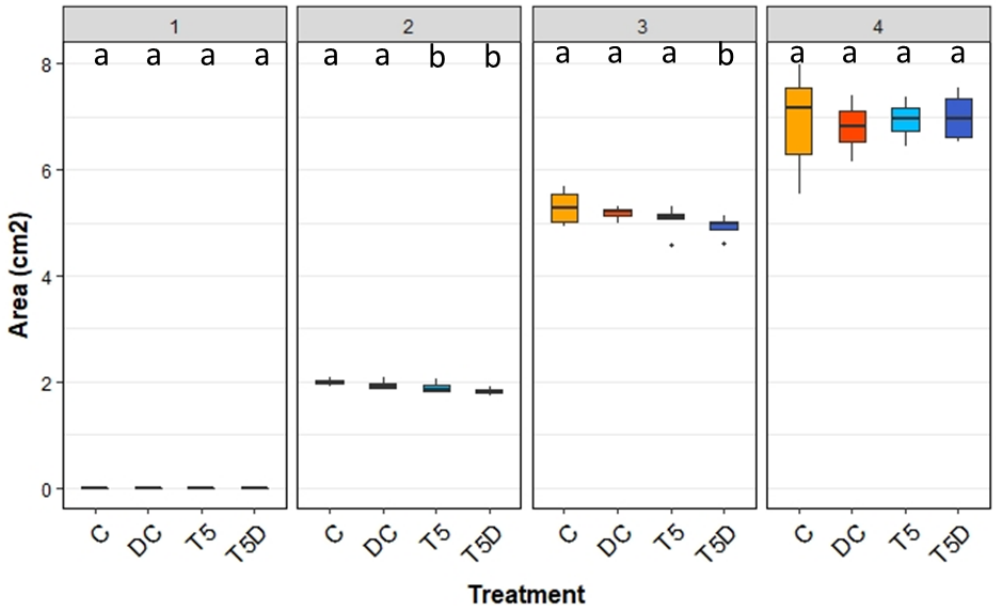


Figure 14: graphical representation of the area covered on 6 well-plate during 4 days of trial according to treatment by Fusarium verticillioides starting from 5 µL conidia suspension (1 x 10^7 conidia/mL). C = control with conidia suspension and water (5 µL), DC = control with daily application of water (5 µL), T5 = conidia suspension with chs3b dsRNA treatment (5 µL, 100ng/µL) and T5D = chs3b dsRNA treatment (5 µL) applied daily. Statistical analyses based on ANOVA test and Dunnett's test, all statistical analyses are performed in R.

During the three days lasting trial for *F. graminearum,* there was no significant difference between the treatments and control on any given day (Fig. 15 A). In addition, there was also no effect witnessed on the corrected GFP (cGFP) values on the plates with added *eGFP* dsRNA (Fig. 15 B). The cGFP values are measured by the multispectral phenotyping platform and used to analyze the GFP signal emitted by the fungal pathogen. In this case, there is a background cGFP signal of around 1000 present. However, this background noise can differ greatly between trials depending on the used media, application method and/or camera settings. Since it takes a while for the conidia to germinate and the fungus to start the GFP protein production, the first day of trial is a good indication of the background noise present. Pictures of the control plate during the four days of trial are added to the Supplement II for reference.

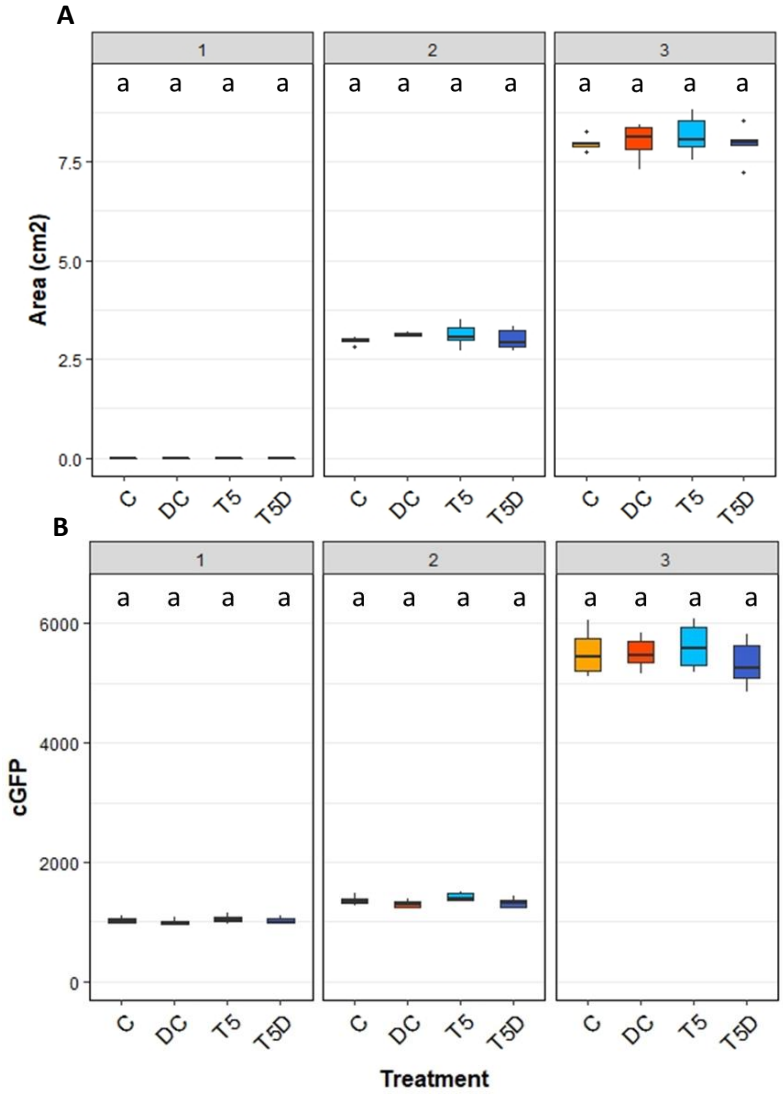


Figure 15: A) graphical representation of the area covered on 6 well-plate during 3 days of trial according to treatment by Fusarium graminearum starting from 5 µL conidia suspension (1 x 10^6 conidia/mL). B) graphical representation of cGFP signal measured during the trial. C = control with conidia suspension and water (5 µL), DC = control with daily application of water (5 µL), T5 = conidia suspension with chs3b dsRNA treatment (5 µL , 100ng/µL) and T5D = chs3b dsRNA treatment (5 µL) applied daily. Statistical analyses based on ANOVA test and Dunnett's test, all statistical analyses are performed in R.

The dsRNA treatment did not seem to have a major effect on the fungus growth or emitted cGFP values. It is to be noted however, that the PDA and mineral medium used for the trial contained a large amount and variety of nutrients. In a real-life infection scenario, the available nutrients on the maize plant would be rather limited until the fungus manages to enter the plant tissues. For this reason, the 6-well plate trial was repeated as before but some extra plates were added containing different media. In the case of *F. verticillioides,* four PDA plates were used for each treatment as before. In addition, four treatment plates were added with ten times diluted PDA medium and four with a medium containing poor nutrient agar and tap water, giving a total of twelve plates (Supplement II). The set-up for *F. graminearum* was similar, only here the first four plates are the normal mineral medium, the next series a ten times dilution of this mineral medium and the last ones the same tap water based medium as used for *F. verticillioides*. For both *F. graminearum* and *F. verticillioides* it was evident that the fungus had more difficulties when growing on the poorer nutrient media. Both the surface area covered as the density of the mycelium showed a reduction when comparing to the ten times diluted media to the undiluted ones and the water based medium to the others.

In contradiction to the first time when the trial was conducted, no significant difference in covered fungal area could be found between the *F. verticillioides* treatments compared to the control on normal PDA medium (Fig. 16 A). The samples grown on ten times diluted PDA however did show a significant difference between the daily applied *chs3b* and the control on the second day of trial (Fig. 16 B, Dunnett's test $p < 0.05$). From day three onwards, this effect was no longer noticeable. The same goes for the water based medium where both the daily application treatment and application at the start of the trial differed from the control on the second trial day, but did not show the same result later on (Fig. 16 C).

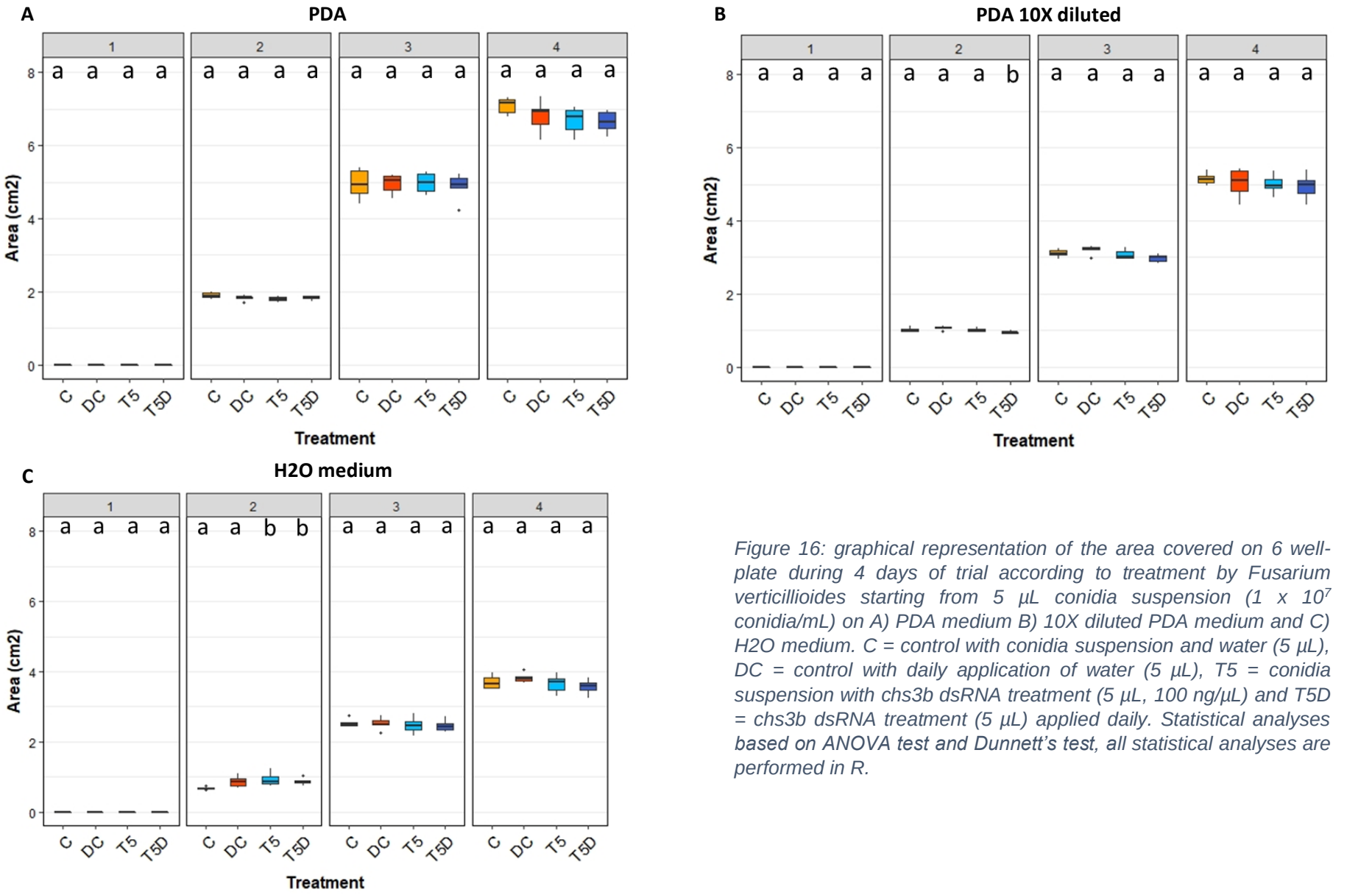


Figure 16: graphical representation of the area covered on 6 well-plate during 4 days of trial according to treatment by Fusarium verticillioides starting from 5 µL conidia suspension (1 x 10^7 conidia/mL) on A) PDA medium B) 10X diluted PDA medium and C) H2O medium. C = control with conidia suspension and water (5 µL), DC = control with daily application of water (5 µL), T5 = conidia suspension with chs3b dsRNA treatment (5 µL, 100 ng/µL) and T5D = chs3b dsRNA treatment (5 µL) applied daily. Statistical analyses based on ANOVA test and Dunnett's test, all statistical analyses are performed in R.

For the *F. graminearum* assays, the set-up was constructed for *chs3b* dsRNA treatment on the one hand and *eGFP* dsRNA treatment on the other. A significant difference between the daily applicated *chs3b* dsRNA plate fungal area and the control was noticeable on the second trial day when *F. graminearum* was grown on normal mineral medium (Fig. 17 A, Dunnett's test $p < 0.05$). When the medium was a ten times dilution, both the daily dsRNA application and application at the start resulted in a significantly smaller area coverage by the fungus in comparison to the control. Also, the daily water control showed a slight reduction in covered surface area when compared in the same way (Fig. 17 B). During the three days of trial on water medium, no difference in covered surface area compared to the control was present for any of the treatments (Fig. 17 C).

The same experimental set-up was created for the combination of *F. graminearum* and *eGFP* dsRNA. The background noise cGFP signal was different for each of the three used media. After three days of trial, there was no significant difference in cGFP signal emitted by the fungus in the treated samples compared to the control for either the normal mineral medium or the ten times diluted version (Fig. 18 A and B). Only in the samples grown on water based medium, a difference between the daily control and daily dsRNA application compared to the control was witnessed on the last day of trial (Fig. 18 C). Nevertheless, it needs to be noted that the growth of the fungus on the water based medium was rather limited and therefore it is no surprise that the corresponding cGFP values for this medium type are very low throughout the trial.

The effects of dsRNA treatment on the two *Fusarium* species are rather moderate to non-existent, both on microscopic scale as on 6-well plate set-up. This leads to the question whether the interaction of the plant RNAi machinery with the fungus is necessary during infection to succeed in successful silencing of the gene of interest. The following detached-leaf assays conducted in this study were created to elaborate further on this possibility.

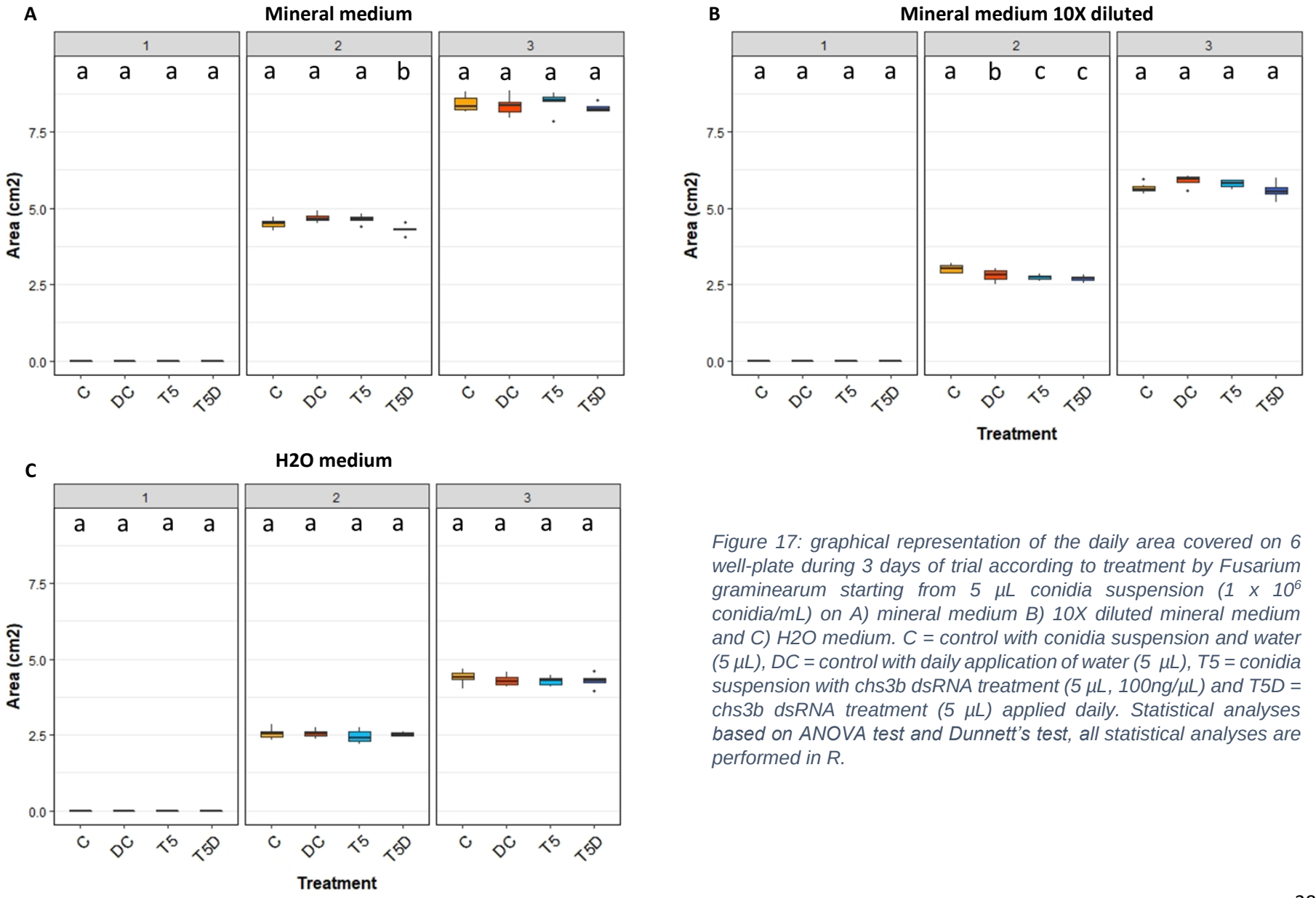


Figure 17: graphical representation of the daily area covered on 6 well-plate during 3 days of trial according to treatment by Fusarium graminearum starting from 5 µL conidia suspension (1 x 10^6 conidia/mL) on A) mineral medium B) 10X diluted mineral medium and C) H2O medium. C = control with conidia suspension and water (5 µL), DC = control with daily application of water (5 µL), T5 = conidia suspension with chs3b dsRNA treatment (5 µL, 100ng/µL) and T5D = chs3b dsRNA treatment (5 µL) applied daily. Statistical analyses based on ANOVA test and Dunnett's test, all statistical analyses are performed in R.

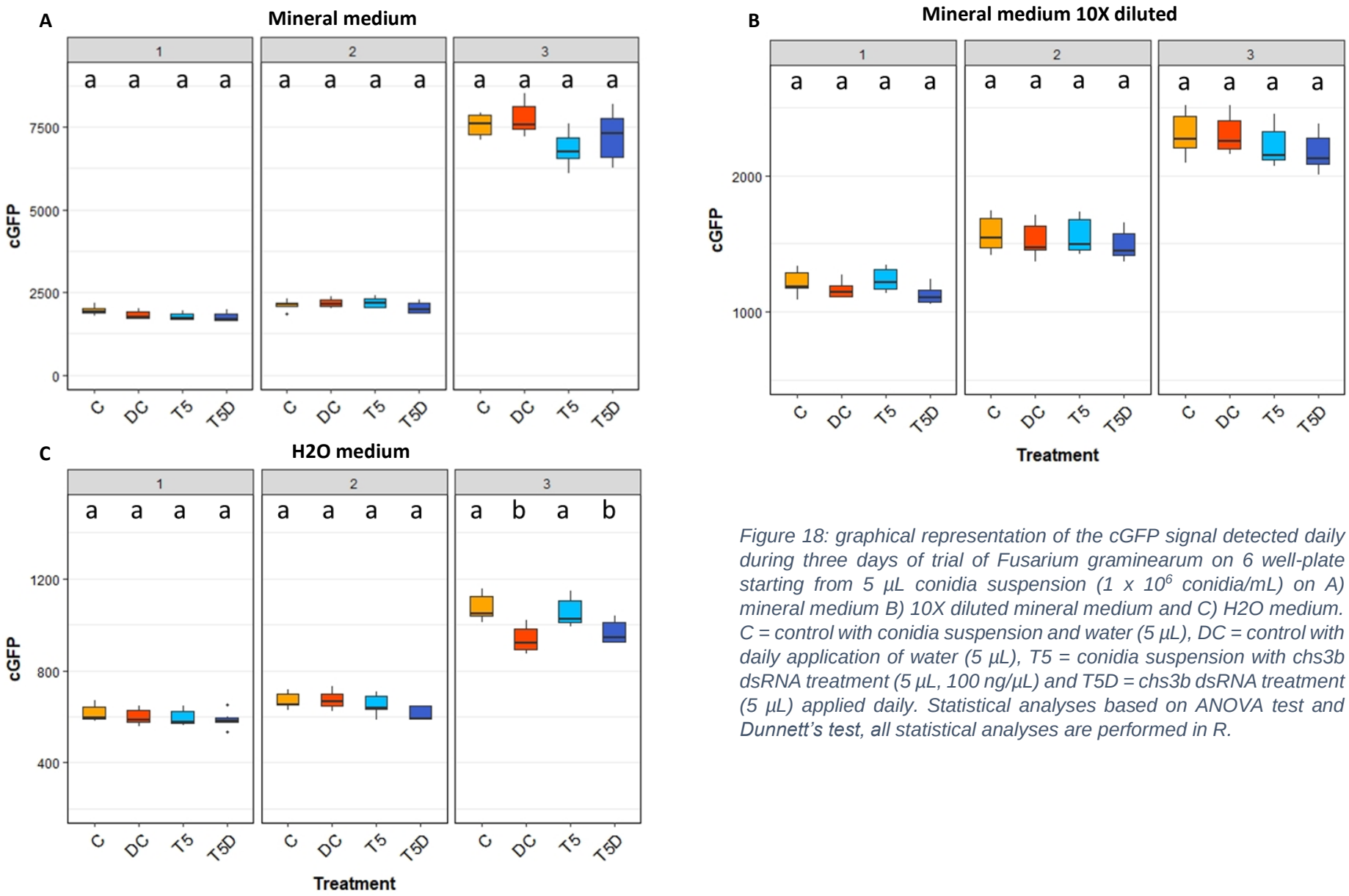


Figure 18: graphical representation of the cGFP signal detected daily during three days of trial of Fusarium graminearum on 6 well-plate starting from 5 µL conidia suspension (1 x 10^6 conidia/mL) on A) mineral medium B) 10X diluted mineral medium and C) H2O medium. C = control with conidia suspension and water (5 µL), DC = control with daily application of water (5 µL), T5 = conidia suspension with chs3b dsRNA treatment (5 µL, 100 ng/µL) and T5D = chs3b dsRNA treatment (5 µL) applied daily. Statistical analyses based on ANOVA test and Dunnett's test, all statistical analyses are performed in R.

4.2.2 Effect of dsRNA on fumonisin production

After the completion of the *in-vitro* trial, fumonisin production by *F. verticillioides* was investigated. For the three media types used, three samples were taken at the end of the experiment from the daily control and daily *Chs3b* dsRNA application plate. After sample clean-up and preparation they were analyzed by LC-MS/MS. With the used method, FB1, FB2 and FB3 concentrations could be quantified. No fumonisins were detected in the samples grown on the water based medium.

The samples grown on normal PDA medium showed the fastest fungal growth and mycelium density. In line with this observation, the highest fumonisin concentrations where witnessed in the samples grown on this medium. The FB1 concentration ranged from approximately 4000 to 6500 µg/kg and there was a visually smaller amount of this component present in the samples treated with *Chs3b* dsRNA (Fig. 19 A). However, statistical analysis did not show a significant difference between the two groups ($p = 0.063$). The same effect was seen in FB2 and FB3 concentrations, ranging respectively from around 800 to 1400 µg/kg and 230 to 400 µg/kg (Fig. 19 B and C). For both components there was a visually lower concentration present in the dsRNA treated samples but this difference was not statistically significant ($p = 0.243$ and $p = 0.299$ respectively).

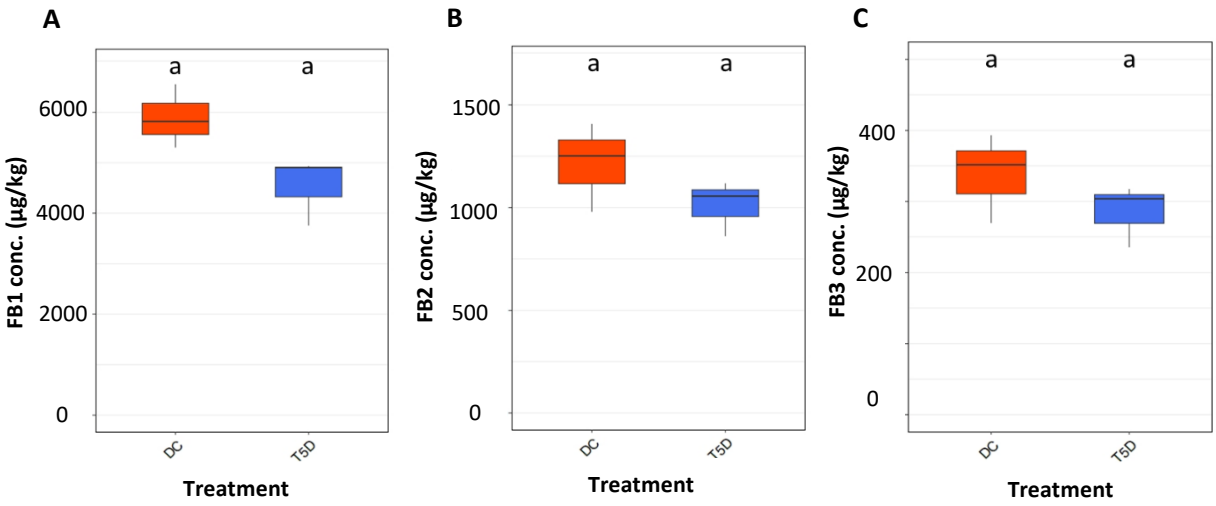


Figure 19: fumonisin B1 (A), B2 (B) and B3 (C) concentration in daily control and daily Chs3b dsRNA application samples (three for each) from the 6-well in-vitro trial growing on normal PDA medium. Statistical analyses based on two-sample t-test for normally distributed data, all statistical analyses are performed in R. DC = daily control, T5D = daily Chs3b dsRNA treatment.

For the *F. verticillioides* samples grown on ten times diluted PDA, the fumonisin concentrations were overall lower in comparison to the ones grown on normal PDA. They fell in the range of 600 to 750 µg/kg, 500 to 650 µg/kg and 55 to 80 µg/kg for FB1, FB2 and FB3 respectively (Fig. 20 A, B and C). This was not unexpected, since the fungi had a harder time growing on this diluted medium. Again, there was a visually lower fumonisin concentration in the dsRNA treated samples for each of the three components that were measured. However, the differentiation is more subtle in comparison to the samples grown on normal PDA and again there is no significant statistical difference ($p = 0.242$, $p = 0.594$ and $p = 0.695$ respectively).

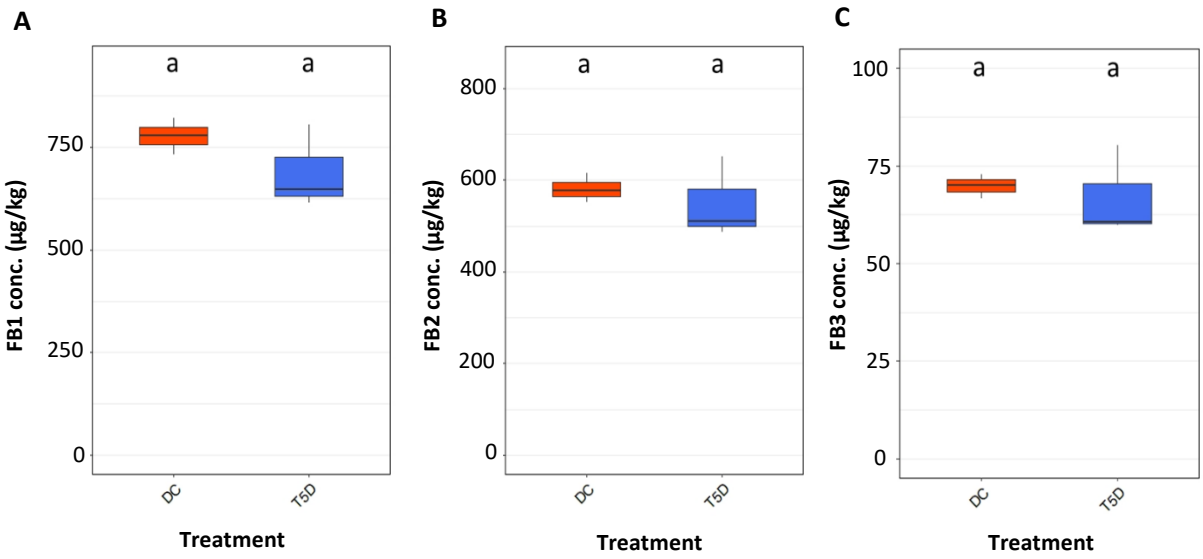


Figure 20: fumonisin B1 (A), B2 (B) and B3 (C) concentration in daily control and daily chs3b dsRNA application samples (three for each) from the 6-well in-vitro trial growing on 10 times diluted PDA medium. Statistical analyses based on two-sample t-test for normally distributed data, all statistical analyses are performed in R. DC = daily control, T5D = daily Chs3b dsRNA treatment.

4.3 Infection of seedlings

In order to get a better understanding of the infection process of *F. graminearum* and *F. verticillioides* in young maize plants, an infection trial was established. Maize seeds were inoculated in conidia suspensions for two hours after which the seedlings were allowed to grow for 16 days. The control group were seeds inoculated in sterile water. The infected plants visually looked shorter than the control group, this difference was more pronounced for the plants infected with *F. graminearum* (Fig. 21). Moreover, the roots of infected plants were shorter and less dense.

After harvesting, measurements were taken of the leaf length, root length, dry leaves biomass and dry root biomass (Fig. 22). There is a significant difference in the length and mass of the leaves between the plants infected with *F. graminearum* in comparison with the control and *F. verticillioides* infected seedlings (Tukey test $p < 0.05$). Plants infected with *F. verticillioides* were also visually shorter but the difference with the control is not significant. The roots on the other hand, showed similar differences in dry biomass of the plants infected with the two fungi in comparison to the control (Dunn's test $p < 0.05$). The root length showed a more modest divergence from the control group. In this case the difference compared to the control group is not significant for either of the fungal infections.

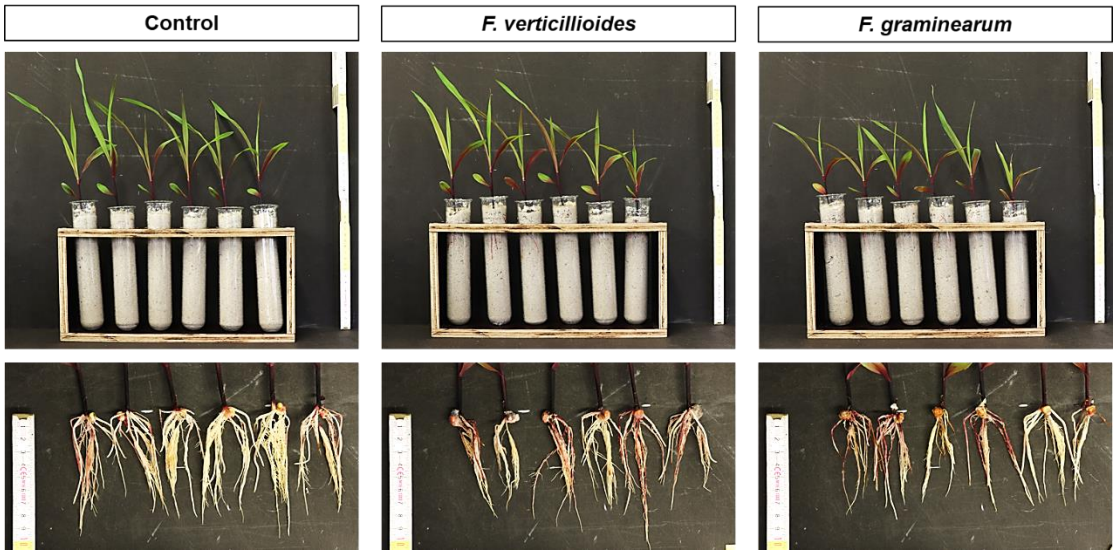


Figure 21: 16 days old maize plants grown from seedlings infected with Fusarium verticillioides, Fusarium graminearum or uninfected seeds (top). On the bottom are close-ups of the roots belonging to the full grown plants above.

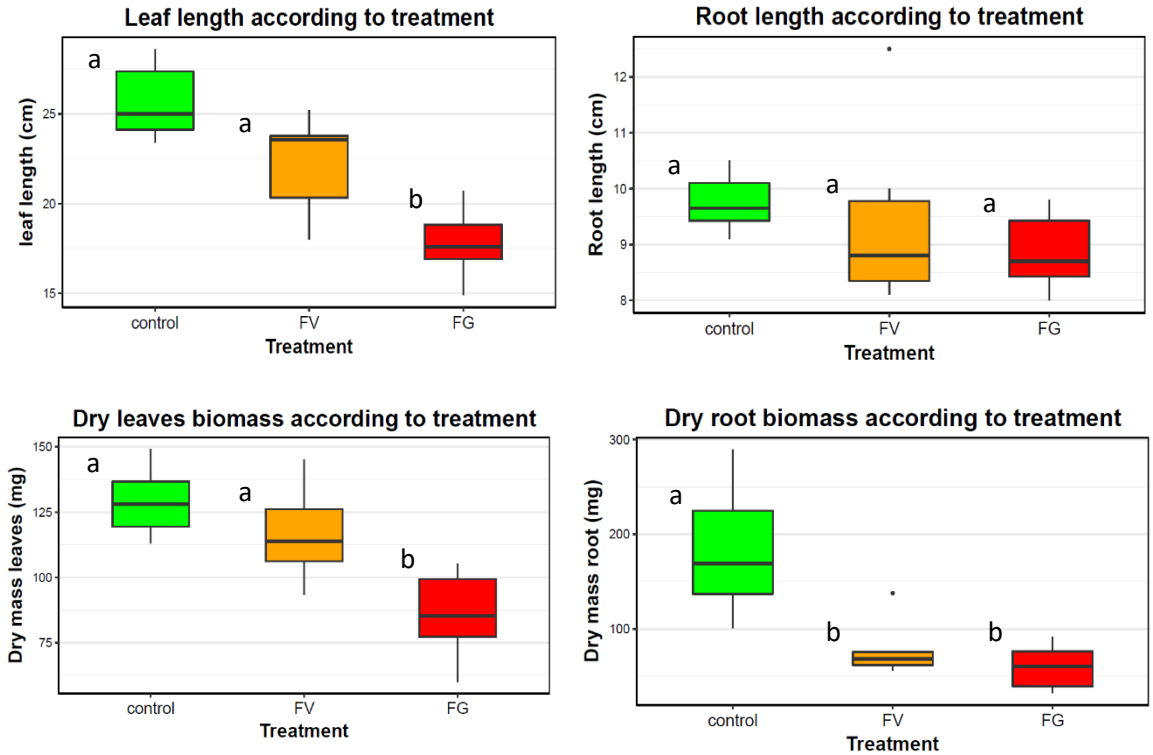


Figure 22: graphical representation of the leaf length, root length, dry leaves biomass and dry root biomass of the plants according to their treatment. Grouping of the data is based on an ANOVA and Tukey test for normally distributed data (leaf length and biomass) or a Kruskal-Wallis and Dunn's test when the data was not normally distributed (root length and biomass). All statistical analyses are performed in R. FV = Fusarium verticillioides treatment FG = Fusarium graminearum treatment.

4.4 Detached leaf assay – infection trial

In the following infection trials, the focus lied on the infection of maize leaves. In order to establish the right protocol for the upcoming experiments with dsRNA, a detached leaf assay was created for both *F. verticillioides* and *F. graminearum*. Six leaves (the first leaves on the plant) were put on a plate filled with plant agar and inoculated by a 10 µL droplet of either *F. verticillioides* (5 x 10^6 conidia/mL), *F. graminearum* (0.5 x 10^6 conidia/mL) or sterile water (control). In parallel, the experiment ran according to the same protocol with wounded leaves. Pictures were taken every 24h with the Wiwam automated phenotyping robot. The results of the trial are shown in Figure 23. The Fv/Fm value is an indicator of plant stress. It's a parameter measuring chlorophyll fluorescence, where lower values correspond with more stress. The chlorophyll index on the other hand quantifies the total chlorophyll content of the leaves. The higher this value is, the more chlorophyll is present in the leaf. At last, the necrosis area is calculated as the total amount of pixels in Fv/Fm class I, which means that they are no longer recognized as living tissue by the Cropreporter software. In order to obtain this data, a region of interest was set around the leaves of which the necrosis area had to be calculated.

During the five days of the unwounded leaf assay, the data did not show signs of a severe infection of *F. graminearum* nor *F. verticillioides.* Only at 4 dai, the Fv/Fm value was significantly lower for both fungal infections compared to the control (Fig. 23, A). At 5 dai however, this difference was not present anymore. As a result, the necrosis area also did not show a significant increase for *F. graminearum* or *F. verticillioides* during the 5 trial days (Fig. 23, B). The values of the chlorophyll index were similar for the control and leaves infected with *F. verticillioides,* however, *F. graminearum* infected leaves had a significantly higher chlorophyll index which was unexpected (Fig. 23, C).

The assay with wounded leaves showed a clearer picture of the infection process of both fungal species. The Fv/Fm values of the infected leaves decreased during the 5 days of trial where in the end *F. graminearum* caused more severe damage to the leaves. *F. verticillioides* infection also significantly decreased the Fv/Fm values of the leaves but to a lesser extent (Fig. 23, D). The same pattern was witnessed for the necrosis area values. Here, it is again *F. graminearum* that causes a significantly larger necrotic area on the leaves compared to *F. verticillioides* and the control. As before, *F. verticillioides* infected leaves also showed a larger necrotic area compared to the control at 5 dai but in a lesser extent than *F. graminearum* (Fig. 23, E). At last, the chlorophyll index changed in line with these observations. It is again *F. graminearum* infection that caused the biggest difference in chlorophyll index. *F. verticillioides* infection also reduced the chlorophyll index of the leaves but in a more moderate way (Fig. 23, F).

The data from the infection trials showed that *F. graminearum* is a more virulent pathogen than *F. verticillioides* when used in maize detached leaf assays. In addition, it became evident that the best way to create clear infection symptoms with these pathogens, in a short time range, is by use of wounded leaves. Thus, wounded leaves were used for the following detached leaf assays involving *F. graminearum* and *F. verticillioides*.

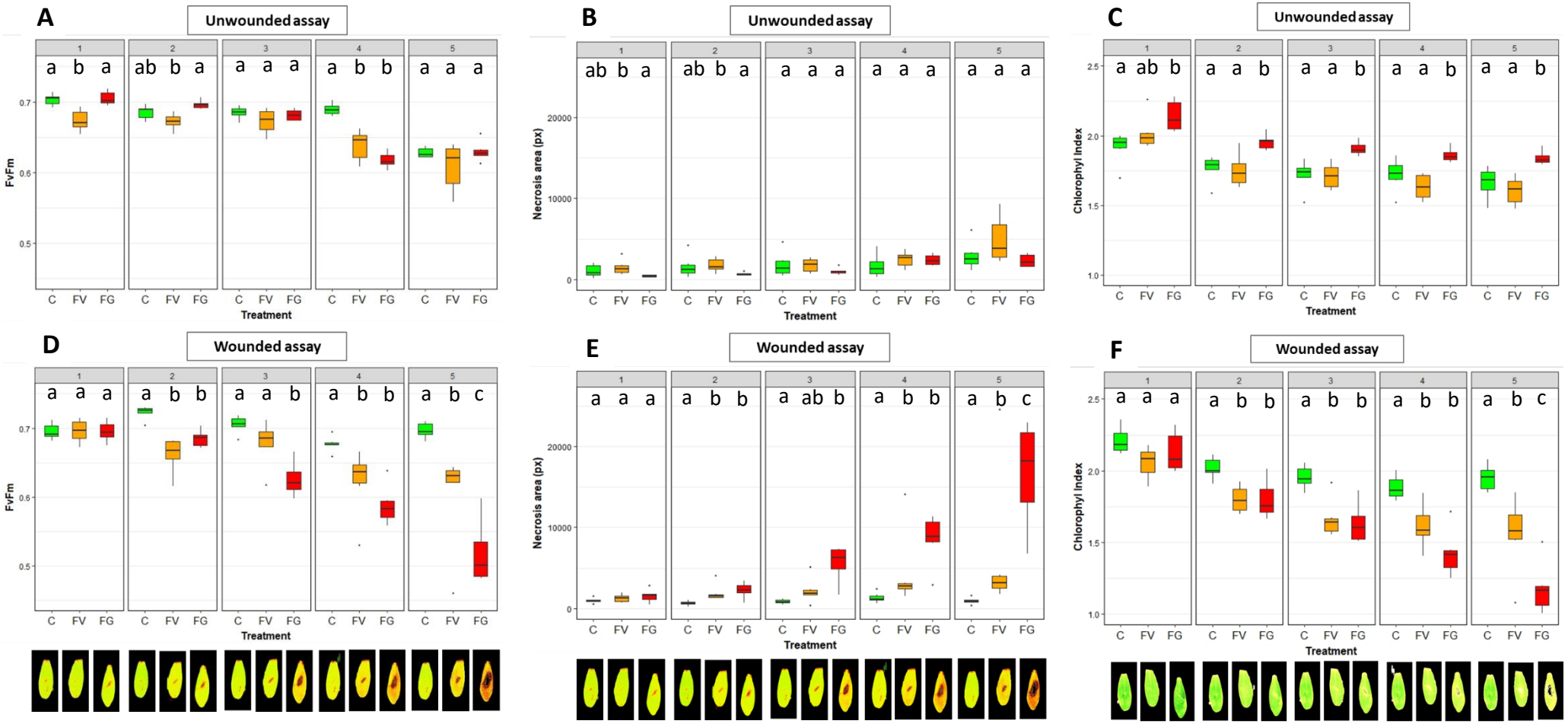


Figure 23: graphical representation of Fv/Fm value, chlorophyll index and necrosis area of detached leaf assays performed with conidia of Fusarium graminearum (0.5 x 10^6 conidia/mL) or Fusarium verticillioides (0.5 x 10^7 conidia/mL) for 5 days. Either unwounded (A, B and C) or wounded leaves (D, E and F) were used. Pictures underneath the figures are added as visual representation of Fv/Fm value. Grouping of the data is based on an ANOVA and Tukey test for normally distributed data or a Kruskal-Wallis and Dunn's test when the data was not normally distributed. All statistical analyses are performed in R. FV = F. verticillioides treatment, FG = F. graminearum treatment and C = control treatment with sterilized water.

4.5 Detached leaf assay – chs3b and eGFP dsRNA

DsRNA was created for the chitin synthase 3b gene (*chs3b*) of *F. verticillioides* and *F. graminearum* along with dsRNA designed to target the *eGFP* gene which is incorporated in the *F. graminearum* strain. In the first detached leaf trial, the plates always consisted of five leaves placed on plant agar (Supplement III). The third leaf of the young maize plants was used in order to increase the consistency in leaf size and shape compared to the infection trials, where the first leaf was used. The negative control consisted of 10 µL sterile water only. For *F. verticillioides,* two plates were made where the first one was the positive control to which 5 µL conidia suspension and 5 µL sterile water was added. The second *F. verticillioides* plate's leaves were inoculated with 5 µL conidia suspension and 5 µL *chs3b* dsRNA (100 ng/µL). For *F. graminearum,* the same plates were prepared and an extra plate was added with 5 µL conidia suspension and 5 µL *eGFP* dsRNA. In addition, an extra control plate with *F. verticillioides* conidia and *eGFP* dsRNA was added together with two control leaves containing only the two *chs3b* dsRNAs without conidia. The purpose was to see if the non *F. verticillioides eGFP* dsRNA would cause an effect on the *F. verticillioides* infection or the dsRNA on its own would result in a different effect on the leaves compared to the water control. This was not the case for any of the two extra controls.

The data of the *F. verticillioides* detached leaf assay are shown in Figure 24. The Fv/Fm values showed some inconsistencies in the negative control data, which seemed to fluctuate, especially at 3 dai. For the infected and treated leaves, the Fv/Fm measurement decreased during the trial and resulted in a lower value compared to the control group at 5dai (Fig. 24, A). However, no significant difference between the two treatments was observed. The chlorophyll index also showed inconsistencies in the negative control and (apart from 3dai) there is no significant difference between the negative control, positive control and treated leaves (Fig. 24, C). At last, the necrosis area data looked more cohesive (Fig. 24, B). Although there was no significant difference between the negative control, positive control and dsRNA treated leaves, it did seem that the treated leaves showed less severe necrosis symptoms compared to the positive control.

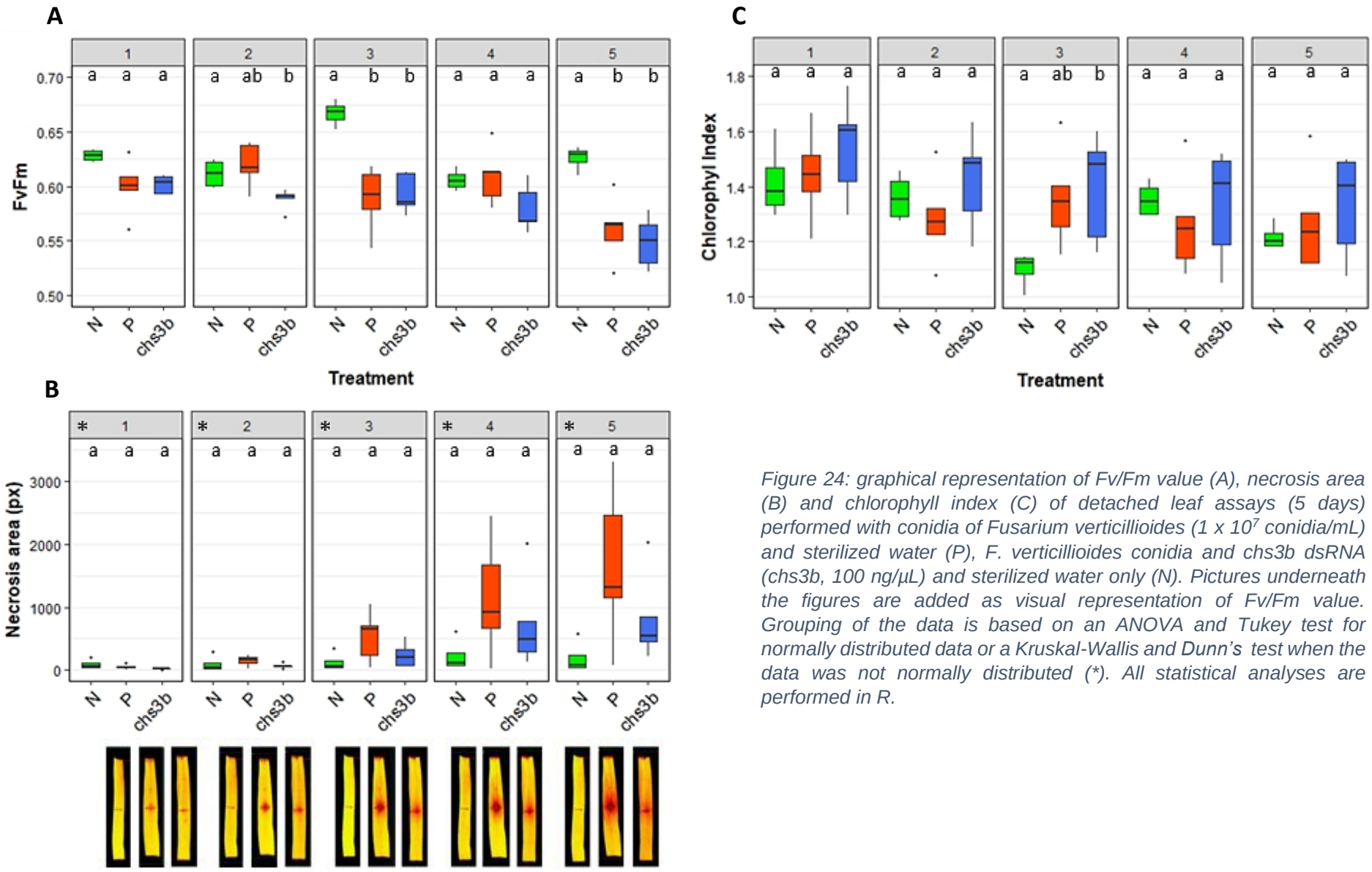
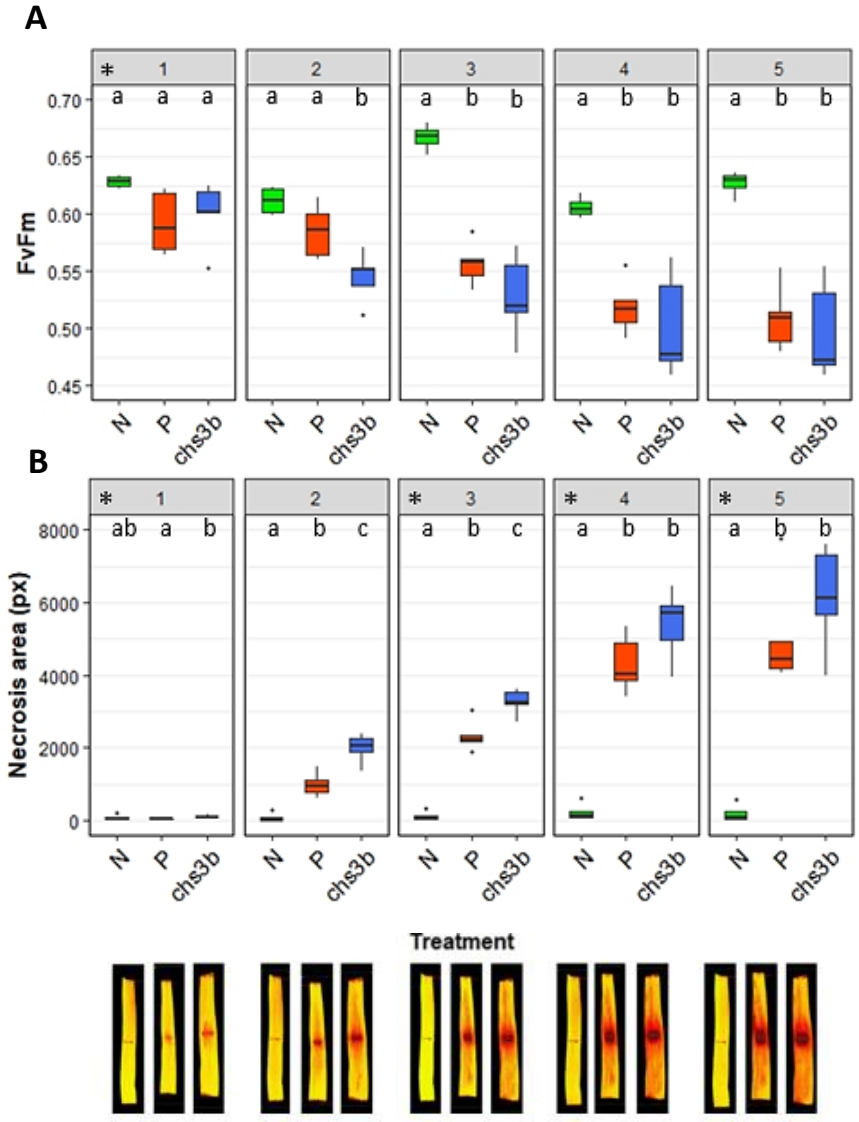


Figure 24: graphical representation of Fv/Fm value (A), necrosis area (B) and chlorophyll index (C) of detached leaf assays (5 days) performed with conidia of Fusarium verticillioides (1 x 10^7 conidia/mL) and sterilized water (P), F. verticillioides conidia and chs3b dsRNA (chs3b, 100 ng/µL) and sterilized water only (N). Pictures underneath the figures are added as visual representation of Fv/Fm value. Grouping of the data is based on an ANOVA and Tukey test for normally distributed data or a Kruskal-Wallis and Dunn's test when the data was not normally distributed (*). All statistical analyses are performed in R.

The results of the detached leaf assay with *F. graminearum* infection are shown in Figure 25. Although the negative controls' Fv/Fm values looked inconsistent at 3dai (as was also the case for the *F. verticillioides* treatment), the positive control and dsRNA treated leaves overall had Fv/Fm values lower than the negative control (Fig. 25, A). However, no significant difference between the positive control and dsRNA treated leaves was present. The same kind of inconsistency is present in the chlorophyll index data at 3 dai, but overall no significant difference between the three treatments was found concerning this parameter (Fig. 25, C). This was not the case for the necrosis area data where the negative control proved consistent over the course of the trial. The positive control and dsRNA treated leaves showed a significantly higher necrosis area but there was no difference between the two (Fig. 25, B). At last, the *F. graminearum* infected leaves were also used for a treatment with *eGFP* dsRNA. The corrected GFP value (cGFP) is used to analyze the GFP signal emitted from the fungal pathogen and a value around 200 is realistic as background noise here. Since it is expected for the cGFP signal to go up when the fungus is growing, the data obtained from this treatment did not look the way that was predicted (Fig. 25, D). The inconsistency in the cGFP data was most likely caused by the plates not being in the dark long enough before taking pictures. Thus, in the next detached leaf assays the plates were always stored in the dark at least half an hour before taking pictures.

In general, it did not seem like the dsRNA treatment was successful for neither the established infection of *F. graminearum* nor *F. verticillioides.* In addition, the leaves had a more noticeable pinkish color during this trial compared to the earlier detached leaf infection trial. The plates were stored next to a room that was heavily lit by bright growing lights and separated by a glass wall. It is likely that the bright light had an effect on the assay and that the pinkish color was caused by anthocyanin production in the leaves. Thus, in the next trials the plates were stored in an environment similar to the initial infection trials.



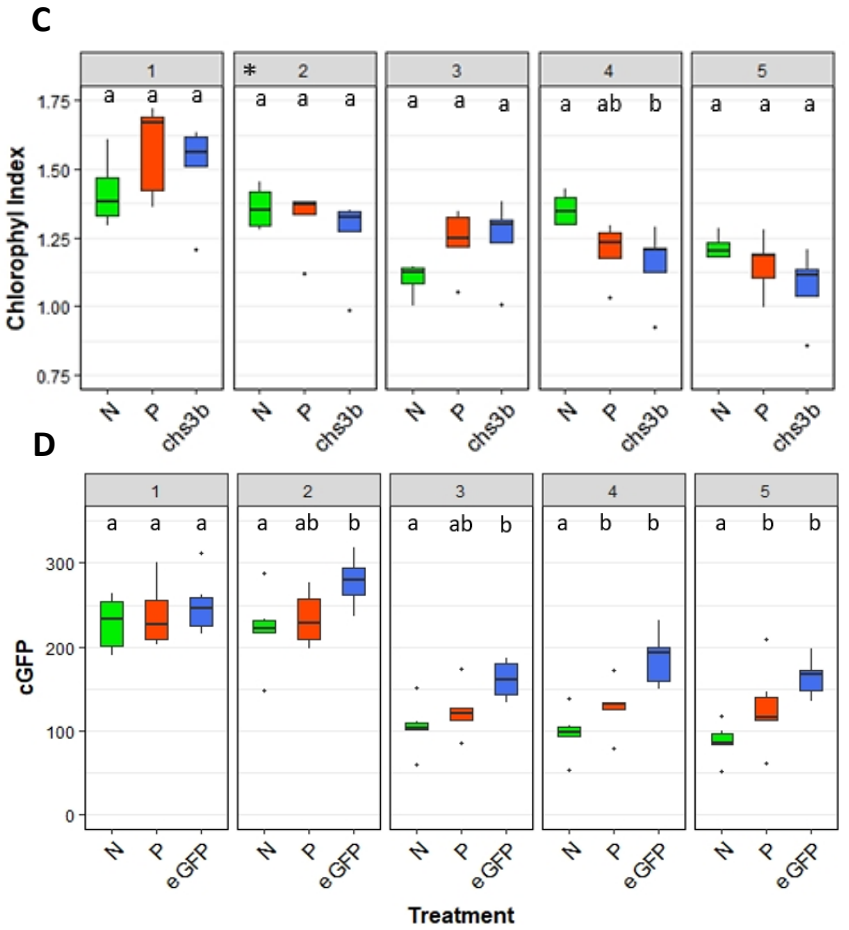


Figure 25: graphical representation of Fv/Fm value (A), necrosis area (B), chlorophyll index (C) and eGFP values (D) of detached leaf assays (5 days) performed with conidia of Fusarium graminearum and sterilized water (P), F. graminearum conidia and chs3b dsRNA (chs3b) or eGFP dsRNA (eGFP) and sterilized water only (N). Pictures underneath the figures are added as visual representation of Fv/Fm value. Grouping of the data is based on an ANOVA and Tukey test for normally distributed data or a Kruskal-Wallis and Dunn's test when the data was not normally distributed (*). All statistical analyses are performed in R.

In order to increase the reliability of the data, the detached leaf experiment with dsRNA treatment was repeated. The problems faced in the previous trial (surrounding light and placement in the dark half an hour before taking pictures) were taken into account and it was also opted to use the first leaves of the maize plant again. The first leaves had a more inconsistent shape compared to the very straight third leaves but made it easier to inoculate and visualize the infection symptoms. It seemed that the incorporated measures were successful and led to a more consistent negative control group and treatment data (Fig. 25 and 26).

The Fv/Fm values in the samples with *F. verticillioides* infection decreased gradually over the course of the assay (Fig. 26 A). From the third day of trial onwards, the positive control and *chs3b* dsRNA treated groups' Fv/Fm values diverged from the control group. Nonetheless, there was no significant difference between these two groups at the end of the trial. The same effect was observed in the chlorophyll index and necrosis area data (Fig. 26 B and C). Here again, no significant difference between the positive control and dsRNA treated samples was present at the end of the trial. However, visually it did seem like there was a slight difference between the Fv/Fm and necrosis area values of the two, where the dsRNA treated group had a positive effect on necrosis area and Fv/Fm.

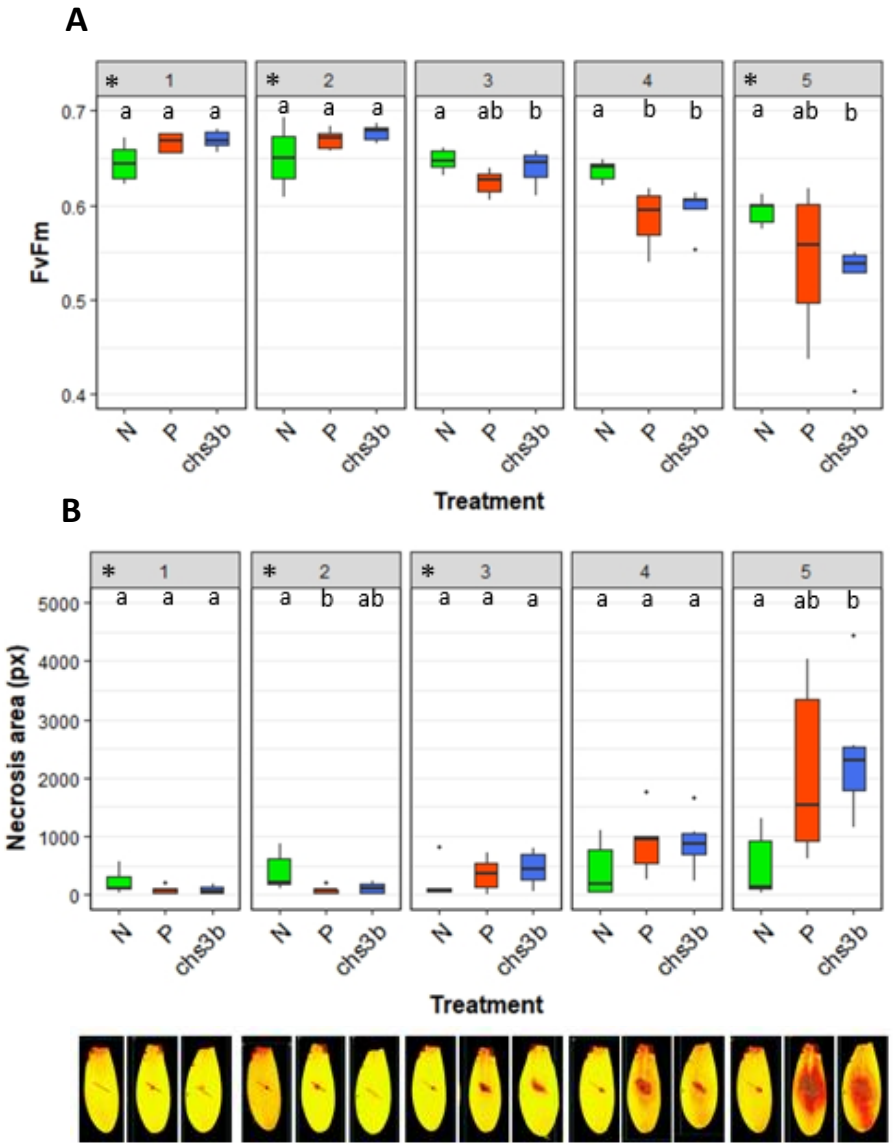


Figure 26: graphical representation of Fv/Fm value (A), necrosis area (B) and chlorophyll index (C) of detached leaf assays (5 days) performed with conidia of Fusarium verticillioides and sterilized water (P), F. verticillioides conidia and chs3b dsRNA (chs3b) and sterilized water only (N). Pictures underneath the figures are added as visual representation of Fv/Fm value. Grouping of the data is based on an ANOVA and Tukey test for normally distributed data or a Kruskal-Wallis and Dunn's test when the data was not normally distributed (*). All statistical analyses are performed in R.

At last, the *F. graminearum* data were examined. The Fv/Fm values of the positive control and the *chs3b* dsRNA treated group started differing from the negative control around three days after the start of the trial (Fig. 27 A). Nevertheless, no significant difference between the values of these groups was witnessed at the end. The necrosis area followed a similar pattern and again, the positive control and dsRNA treated samples showed no divergence from each other (Fig. 27 B). A difference between the chlorophyll index of the two treatment groups and the negative control became only evident after 4 dai. In the end however, there was again no significant difference between these two groups and the control (Fig. 27 C). Finally, the cGFP values of the three groups were similar until 5 dai. The *eGFP* dsRNA treated group had a slightly higher overall cGFP value at this day, which is unexpected since the *eGFP* signal should have been blocked in this group (Fig. 27 D).

Overall, the conducted microscopic, *in-vitro* and detached leaf trials all showed moderate to insignificant effects of the dsRNA treatment.

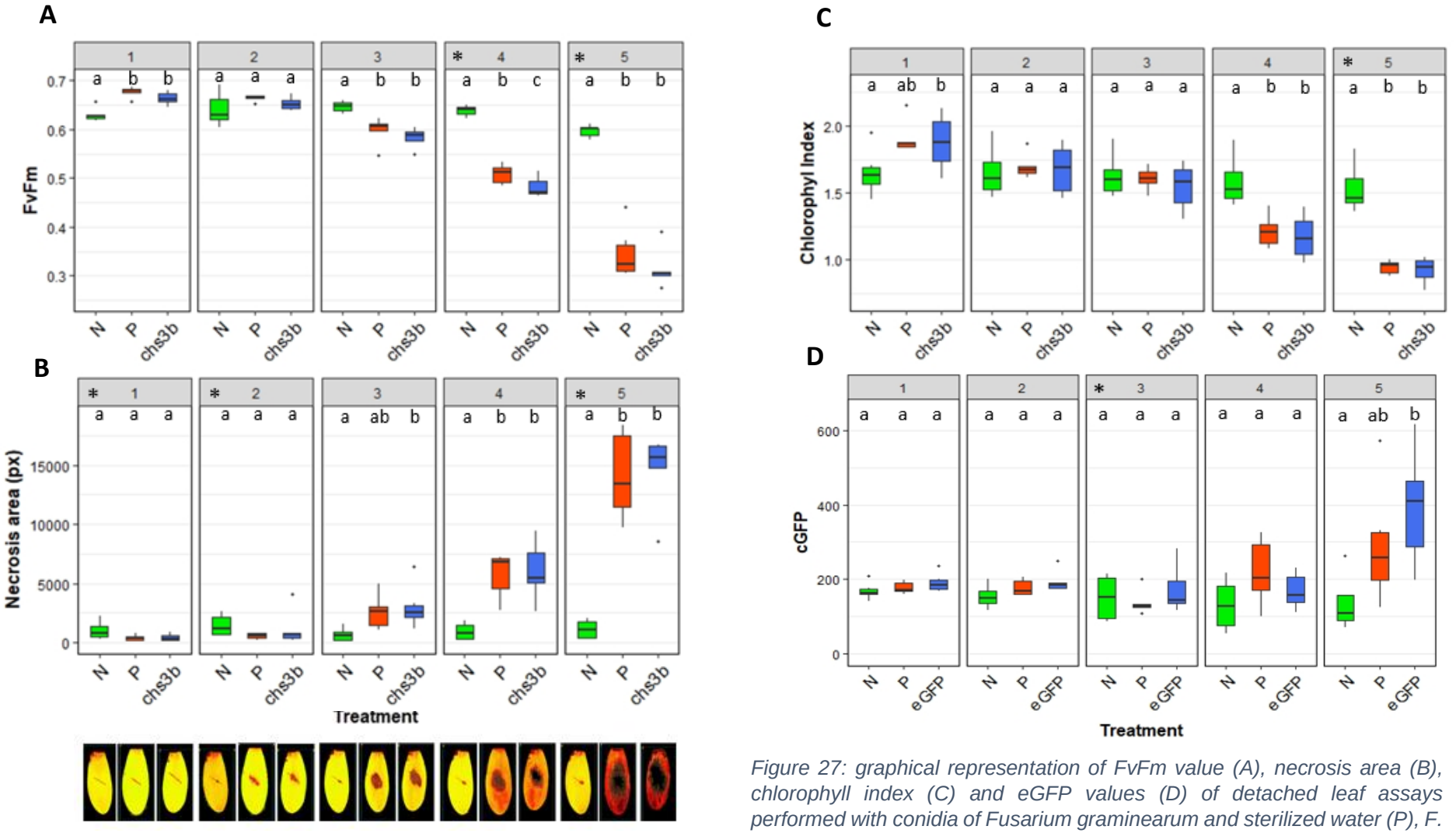


Figure 27: graphical representation of FvFm value (A), necrosis area (B), chlorophyll index (C) and eGFP values (D) of detached leaf assays performed with conidia of Fusarium graminearum and sterilized water (P), F. graminearum conidia and chs3b dsRNA (chs3b) or eGFP dsRNA (eGFP) and sterilized water only (N). Pictures underneath the figures are added as visual representation of Fv/Fm value. Grouping of the data is based on an ANOVA and Tukey test for normally distributed data or a Kruskal-Wallis and Dunn's test when the data was not normally distributed (*). All statistical analyses are performed in R.

5. Discussion

5.1 Result analysis

In this study, the effect of externally applied *Chs3b* dsRNA treatment on *F. verticillioides* and *F. graminearum* was investigated. *F. graminearum* was used in this investigation as a control species because of its previous success in SIGS trials and the GFP tagged nature of the strain. The dsRNA was tested on three levels by microscopic, *in-vitro* 6-well and detached leaf assays. This allowed for a thorough screening of the pathogen and its susceptibility to RNAi mediated gene silencing by external application.

During the microscopic experiments, no significant effects were observed of the *Chs3b* dsRNA (100 ng/µL) treatment on the germination length of *F. verticillioides* and *F. graminearum* conidia. However, the treated *F. graminearum* conidia did seem to have a higher number of side branches and a bit shorter length compared to the control group. This effect of developing more side branches had previously been witnessed in the lab when the conidia were put under stress during germination. In addition, *F. graminearum* conidia were treated with *eGFP* dsRNA. No visual effect of the dsRNA on the fluorescence of the conidia was observed, although there is no quantitative data to support this. The germinated conidia were also investigated on a higher magnification, but no indication of disturbed cell walls or mycelium was observed. To summarize, it might be possible that the difference in number of side branches during germination was due to a stress reaction caused by treatment with *Chs3b* dsRNA. Overall, the treatment did not have an effect on germinated spore length or cell wall structure when applied to *F. verticillioides* or *F. graminearum.*

The timeframe from spore suspension application to germination is quite short (less than 24h). In order for the dsRNA to work, it needs to have enough time to be transported into the cells, modified to siRNAs and act on the target sequences for gene silencing. Thus, *in-vitro* 6-well trials were used in order to observe the effect of dsRNA treatment on larger scale and timeframe. The first conducted assay only showed very moderate results of the *Chs3b* and *eGFP* dsRNA treatments. Small differences in fungal area coverage between the control and *Chs3b* dsRNA treatment were sometimes seen around the second day of trial but overall these effects were not noticeable anymore at the end of the assay. In real-life scenarios, the pathogens grow in a poor nutrient environment during the infection process of maize leaves until the fungus manages to get into the plant tissues. For this reason, the second *in-vitro* trial tested three different media types for each fungal species with a varying degree of available nutrients. There was overall no real difference between the media type used and fungal area coverage after dsRNA treatment in comparison with the control. However, again at around two days after the start of the trial some small differences in fungal area between the control and *Chs3b* treated samples were observed. The *eGFP* dsRNA application did not seem to have an effect on the emitted fluorescence of *F. graminearum* in any of the two *in-vitro* trials.

The microscopic trials showed that the fungi possibly underwent a stress reaction after application of *Chs3b* dsRNA but no visual effect on fungal growth was seen in the *in-vitro* trials. Consequently, a mycotoxin analysis was conducted to find out whether the dsRNA might have

some effect on mycotoxin production. Three samples from the daily control plate and daily application plate of *F. verticillioides* (second *in-vitro* trial) were collected for each of the three media. After cleaning-up they were analyzed with LC-MS/MS for the three main fumonisins: FB1, FB2 and FB3. Despite no statistical significance, the *Chs3b* dsRNA treated samples grown on normal PDA did show a visual reduction in the three fumonisin levels. Thus, it seemed that the treatment with *Chs3b* dsRNA had a small effect on the fumonisin synthesis. This was also the case for the samples grown on the ten times diluted PDA, although the visual difference was more subtle. The overall mycotoxin production was lower on the diluted medium, which was not surprising since the fungal area coverage and mycelium density were also lower in this case. The samples grown on the water based agar medium did not contain fumonisins in a concentration that was detectable. This was not unsuspected, as the fungal growth on this medium type was very slow and the resulting mycelium was not very dense.

Although subtle, there seemed to be a small effect of the applied *Chs3b* dsRNA. It needs to be taken into account that not only the fungus itself is responsible for the processing of the dsRNA. The maize plant is also able to take up the externally applied dsRNA and process it with its own RNAi machinery (Knip *et al*, 2014; Han & Luan, 2015). Consequently, the produced siRNAs can be transported to the fungi by means of extracellular vesicles. In normal infection situations, plants often use this kind of method as a host-defense mechanism (Tinoco *et al*, 2010b; Nowara *et al*, 2010). Thus, it was opted to add another experimental trial to this investigation. With detached maize leaves, the fungal infection process with and without dsRNA treatment was explored. Thereby, more information was gained about the possible necessity of the maize plant processing of the externally applied dsRNA in the effectiveness of the dsRNA fungal treatment.

First of all, an infection trial was created in order to gain a better understanding of the infection process of *F. verticillioides* and *F. graminearum* on maize leaves. Afterwards, wounded maize leaves were treated with either a water control, conidia suspension or conidia suspension treated with dsRNA (*Chs3b* for *F. verticillioides* and *Chs3b* and *eGFP* for *F. graminearum*). After analysis of the data, no difference was observed in any of the two detached leaf trials for the *F. verticillioides* and *F. graminearum* Fv/Fm values, necrosis area, chlorophyll index or cGFP signal. Thus, it seems that in this case the addition of the host plant system to the trials did not result in a better uptake or effect of the dsRNA in the fungal pathogen.

5.2 Target gene selection

The dsRNA designed to influence the expression of the *Chs3b* target gene did not cause the fungal growth and virulence decline we were expecting in the target species. Thus, the first thing that came to mind when reevaluating this investigation was whether this was a good target gene to begin with. The previous study of Cheng et al. (2015) identified the *F. graminearum Chs3b* gene as the *Chs* gene with the highest expression level throughout the colonization of wheat by this fungus. Besides, their work performed with the HIGS method showed that *Chs3b* was essential for fungal growth and survival. This study however, was focused on SIGS, which may mean that this method was perhaps not powerful enough to silence a profound amount of the present *Chs3b* genes to cause a real noticeable difference

in chitin synthesis and growth. Besides, the chitin synthases are a complex gene family and there is currently no guarantee that *Chs3b* plays an equally vital role in the development of the cell wall in *F. verticillioides* as it does in *F. graminearum.* It may very well be that in this species other chitin synthase gene(s) play a more crucial role in the synthesis of the chitin components. In addition, the interplay of the chitin synthase enzymes and their specific functions are not fully understood yet and can be very species specific. In this study, *F. graminearum* PH-1 was used and it may even be possible that a different strain can cause some differences in target gene expression. Furthermore, multiple chitin synthases can often be found at the same location in the fungal cells. This suggest that either there is a need for the enzymes to form multiunit complexes or genetic redundancy is present (Riquelme & Bartnicki-García, 2008). Hence, it is certainly possible that the down-regulation of *Chs3b* may cause a higher expression of other *Chs* family genes as a form of feedback regulation mechanism compensation (Cheng *et al*, 2015).

In this investigation, not only *Chs3b* treatment was investigated but also *eGFP* dsRNA was added to the *F. graminearum* strain following the same protocols. Thus, the *eGFP* dsRNA was a control for the choice of target gene. If the cGFP signal would have decreased but no effect was seen of the *Chs3b* treatment, this would indicate that the target gene was not efficient enough in blocking the fungal growth and virulence. However, no clear effect of the *eGFP* treatment was observed in this study. This indicates that the problem in the first place probably lies elsewhere in the application method.

5.3 DsRNA delivery method

In previous studies, *F. graminearum* was already the target of SIGS methods. The work of Koch et al. (2016) showed that the fungus can be successfully inhibited by SIGS application targeting cytochrome P450 lanosterol C-14α-demethylases. Later, Werner et al. (2020) targeted RNAi machinery genes of *F. graminearum* with SIGS and found that especially *DICER* genes were good targets for fungal inhibition. Thus, in this study we expected *F. graminearum* to be susceptible to the externally applied dsRNA. However, it needs to be noted that the previous investigations added the dsRNA onto the leaves by means of a spray application. This was not possible in this study given the high cost of dsRNA synthesis. The obtained volumes were not large enough to be able to spray the dsRNA onto the leaves and instead a more volume-friendly droplet application was chosen. Of course, it is possible that the droplet form is not as effective as the more widespread spray application of the entire leaf area.

Thus far, there is no evidence to state that fungi have a preference for the uptake of either long or short dsRNA molecules (Majumdar *et al*, 2017). In a study by Kogel (2021), fluorescent dsRNA with a length of 21 bp, 420 bp or 1451 bp was added to *Verticillium longisporum* conidia. Uptake of the dsRNA was successful for all of these used lengths. The same results were witnessed when the application was repeated on *Serendipita indica.* In addition, instead of applying long dsRNA that still requires processing, it is also possible to apply the desired siRNAs straight away. For example, *Botrytis cinerea* is capable of taking up both long dsRNAs and siRNAs targeting Dicer-like genes and both applications were successful in inhibiting the fungal growth on fruits, vegetables and flowers (Wang *et al*, 2016). These findings are different

from what can be witnessed when using insects as target organisms. Here, uptake is only achieved when dsRNAs longer than 50 bp are used and siRNA application is not successful (Ivashuta *et al*, 2015). It would be interesting to investigate whether the application of siRNA *Chs3b* instead of long dsRNA would make a difference in the successful silencing of the gene in our study organisms and set-up.

Furthermore, the concentration of the applied dsRNA may also play a crucial role in SIGS. There is no golden rule about which concentration works best since this is again very species and target specific. Currently, application dose is determined by trial an error. The previously described successful studies with SIGS on *F. graminearum* used concentrations on the leaves of 20 ng/µL dsRNA (Koch *et al*, 2016; Werner *et al*, 2020). Another study with *Slerotinia sclerotiorum* used 20 or 80 ng/µL on the surface of *Brassica* leaves and petals and 40 ng/µL when *B. cinerea* was applied to the *B. napus* (McLoughlin *et al*, 2018). Based on these previous studies, 100 ng/µL was applied in this investigation to have an abundance of dsRNA available for the fungus and plant and to try to compensate for the local nature of droplet application. Of course, it is possible that this concentration was not ideal and that a higher, or even lower, amount of dsRNA would have had a more beneficial effect on the treatment results.

At last, degradation of the dsRNA after application is an important factor to keep in mind. It has been estimated that after spraying onto the leaves, the dsRNA might actively work for five to seven days (Gan *et al*, 2010). A new innovation that addresses this problem is the use of nanosheets. DsRNA is loaded onto the double hydroxide clay nanosheets and subsequently sprayed onto the leaves, which in tests made the dsRNA active for up to twenty days post application (Mitter *et al*, 2017). Furthermore, cationic nanoparticles and peptide-based RNA delivery systems are also being investigated for this purpose (Numata *et al*, 2014; Jiang *et al*, 2014; Dubrovina & Kiselev, 2019). At last, it might be useful to improve the adhesion of the dsRNA onto the leaves. The surfactant Silwett L-77 has often been used in SIGS studies for this purpose (McLoughlin *et al*, 2018). It might be interesting to test whether the use of Silwett L-77 or clay nanosheets increases the effectiveness of the *Chs3b* treatment investigated in this study.

5.4 Host plant interaction

DsRNA molecules can move between different organisms of different species and can be processed by the RNAi machinery of both. It is believed that vesicles play an important role in the transport of the RNA molecules but also membrane transporters, receptors and passive diffusion are possible ways in which this process may occur (Majumdar *et al*, 2017). Hence, it might be crucial to have a host plant system present in the infection experiments to process the applied RNA molecules and to transfer them to the fungal pathogen. For this reason, many SIGS studies already apply the dsRNA onto the detached leaves or plants a couple of days before the actual infection with fungal pathogens (Koch *et al*, 2016; McLoughlin *et al*, 2018; Werner *et al*, 2020). In this investigation, the dsRNA treatment was always added at the beginning of the trial or daily. Perhaps, this timeframe was not ideal for this specific host plant and pathogen interaction and maybe the results would have been better if the dsRNA was already applied beforehand.

As already mentioned, uptake of dsRNA or siRNA differs between different types of organisms. In fungi, the uptake of the dsRNA does not happen evenly along the hyphae. In a study by Wytinck et al. (2020) in *S. sclerotiorum*, it was observed that the uptake happens mostly at the tip of actively growing hyphal branches. In addition, they also investigated the role of clathrin-mediated endocytosis, which is often used by insects to facilitate dsRNA uptake. Knock-down in *S. sclerotiorum* of genes related to this mechanism made it impossible for the dsRNA to enter the fungal cells. Thus, in this species clathrin-mediated endocytosis plays an essential role in the uptake of externally applied dsRNA or siRNA. It is suggested that afterwards the dsRNAs are incorporated into vesicles and subsequently released into the cell. Nevertheless, this does not necessarily mean that the same mechanisms are responsible for dsRNA uptake in other fungal species. More information regarding this topic is still needed and would be very helpful in the future design process of RNAi mediated fungal pest control.

5.5 Future prospects

This study attempted to address the effectiveness of *Chs3b* dsRNA application against *Fusarium* on three different levels. Nevertheless, a great amount of other approaches is yet to be explored. First of all, it would be a good idea to set-up trials in which the dsRNA is applied onto the leaves a couple of days before infection. In addition, the application method itself can also be further finetuned by exploring different media and concentrations. Especially the use of Silwett L-77, clay nanosheets or other carriers is a very promising new way of addressing the dsRNA onto the host plant and delaying degradation as much as possible. Furthermore, the dsRNA in this study was designed to target only one fungal gene. It is however possible to create a longer dsRNA molecule that targets multiple related or unrelated genes. Since it is still uncertain whether the silencing of *Chs3b* is compensated by other genes of the chitin synthase family, it might be beneficial to design a dsRNA molecule that also targets different chitin synthase genes. Besides, it is also optional to apply siRNAs onto the pathogen or detached leaves instead of the long dsRNA. At last, it is very advisable to include real-time PCR (qPCR) investigations in future work regarding this topic. The dsRNA application in this investigation only resulted in very subtle and non-significant differences between control and treatment. Thus, qPCR expression data could clarify whether no gene silencing was present at all or whether gene silencing was present but only on a level that was too low to visually see a difference in growth or virulence.

6. Conclusion

DsRNA was created for the chitin synthase 3b gene (*chs3b*) of *F. verticillioides* and *F. graminearum*, along with dsRNA designed to target the *eGFP* gene incorporated into the *F. graminearum* strain. Reproducible protocols were established de-novo to investigate the effect of the externally applied dsRNA on *F. verticillioides.* The methods were based on microscopic investigation, *in-vitro* application and detached leaf assay. On microscopic scale, treatment with *Chs3b* or *eGFP* dsRNA did not result in noticeable differences in length of germinated conidia or integrity of the cell wall. However, it was interesting to observe that the *F. graminearum* conidia treated with *Chs3b* grew more and shorter side branches, a symptom that had previously been witnessed to be a fungal stress reaction. The *in-vitro* trials showed very moderate to non-significant changes in fungal area coverage and emitted fluorescence. Yet, when the mycotoxins levels of dsRNA treated *F. verticillioides* samples were compared to the control, a small decline in fumonisin concentration was observed. At last, the following detached leaf trials did again only show very subtle to no effects of the applied dsRNA treatments.

It was unexpected to see so little effect of the *Chs3b* treatment on the fungal growth, especially since this gene had been previously found to be of vital importance to *F. graminearum.* Nevertheless, a slight reduction in fumonisin expression of dsRNA treated *F. verticillioides* samples was witnessed. This may indicate that the dsRNA possibly affects the pathogen but in a way that is too subtle for visual constatation. In this case, qPCR results would be very helpful to distinguish between either no gene silencing at all or gene silencing at a level that is too low for successful fungal inhibition.

Many reasons can lie on the basis of the ineffective nature of the applied *Chs3b* dsRNA. The *eGFP* dsRNA control used on *F. graminearum* in parallel with the *Chs3b* experiments did also not result in an observable effect. Thus, it is reasonable to assume that the problem lies in the dsRNA application method rather than the selection of target gene. In future trials, it would be recommended to experiment with some other application methods and aim for an implementation of recent innovations that have been made in this field. Of course, this does not mean that it is a bad idea to also try different *Chs3b* related or unrelated targets. It would be very interesting to focus on the fumonisin synthesis genes as well in order to reduce the pathogenicity of the fungus. Besides, it is worth trying to apply siRNAs instead of long dsRNA onto the pathogens and leaves, as this might have an effect on successful gene silencing as well.

In short, solid and reproducible protocols were established to investigate the effectiveness of dsRNA treatment on *F. verticillioides* and *F. graminearum.* Many questions regarding this mechanism and application method remain unanswered and there are countless options left to be explored. Hence, this study opens the way for future research to experiment further on the established protocols and to find solutions for the encountered restrictions and difficulties.

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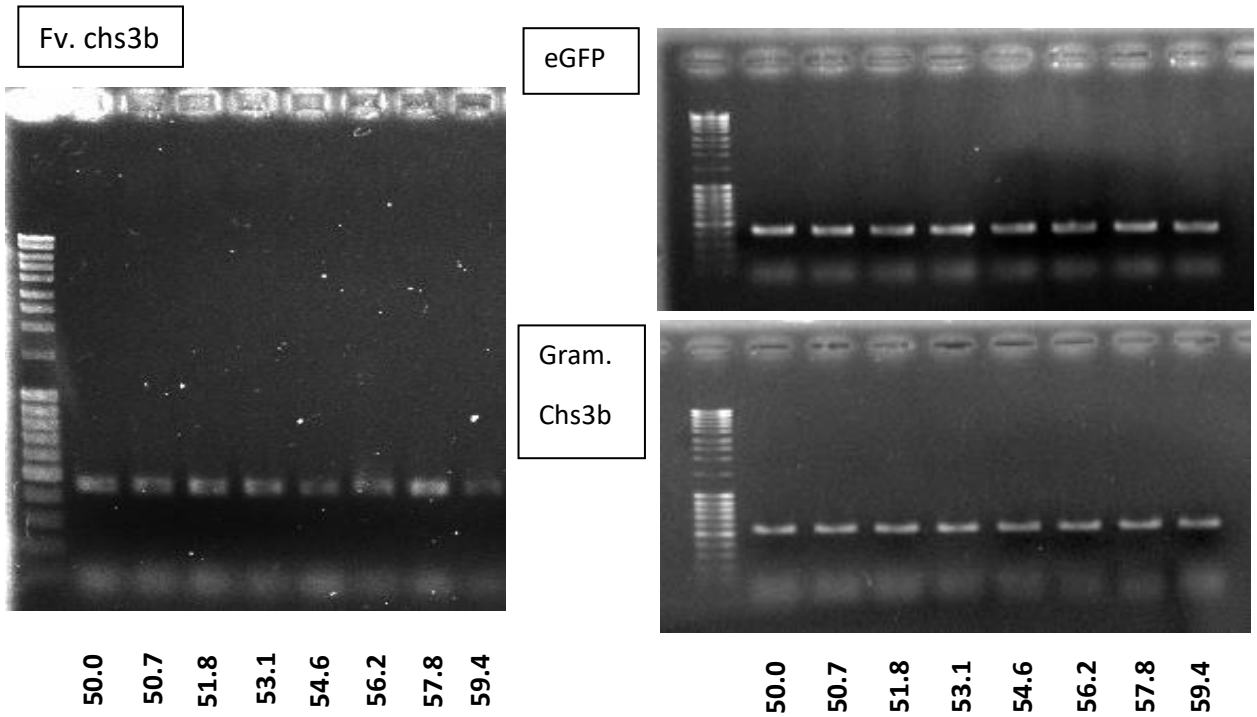
Supplement I

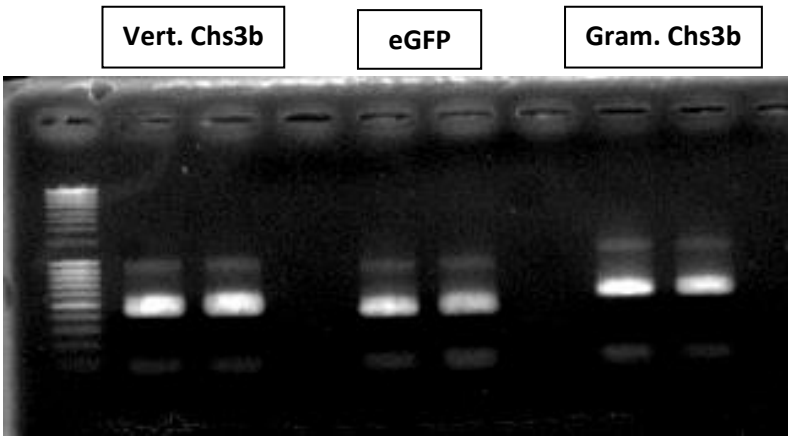
Gene	Primer	sequence	Product size
Chitin synthase 3b Vert.	F	**TAATACGACTCACTATAGGGAGA**TGGGCAA GAAGGGTATTGAC	409 bp
	R	**TAATACGACTCACTATAGGGAGA**TGGTATC CCGAGAGAACCTG	

Gene	Primer	sequence	Product size
Chitin synthase 3b Gram.	F	**TAATACGACTCACTATAGGGAGA**GTCGGAA CACCACAGGTTCT	508 bp
	R	**TAATACGACTCACTATAGGGAGA**TTGTTCC CCAAGACACATCA	

Gene	Primer	sequence	Product size
Gram. eGFP	F	**TAATACGACTCACTATAGGGAGA**CCTGAAG TTCATCTGCACCA	382 bp
	R	**TAATACGACTCACTATAGGGAGA**GTTGTGG CGGATCTTGAAGT	

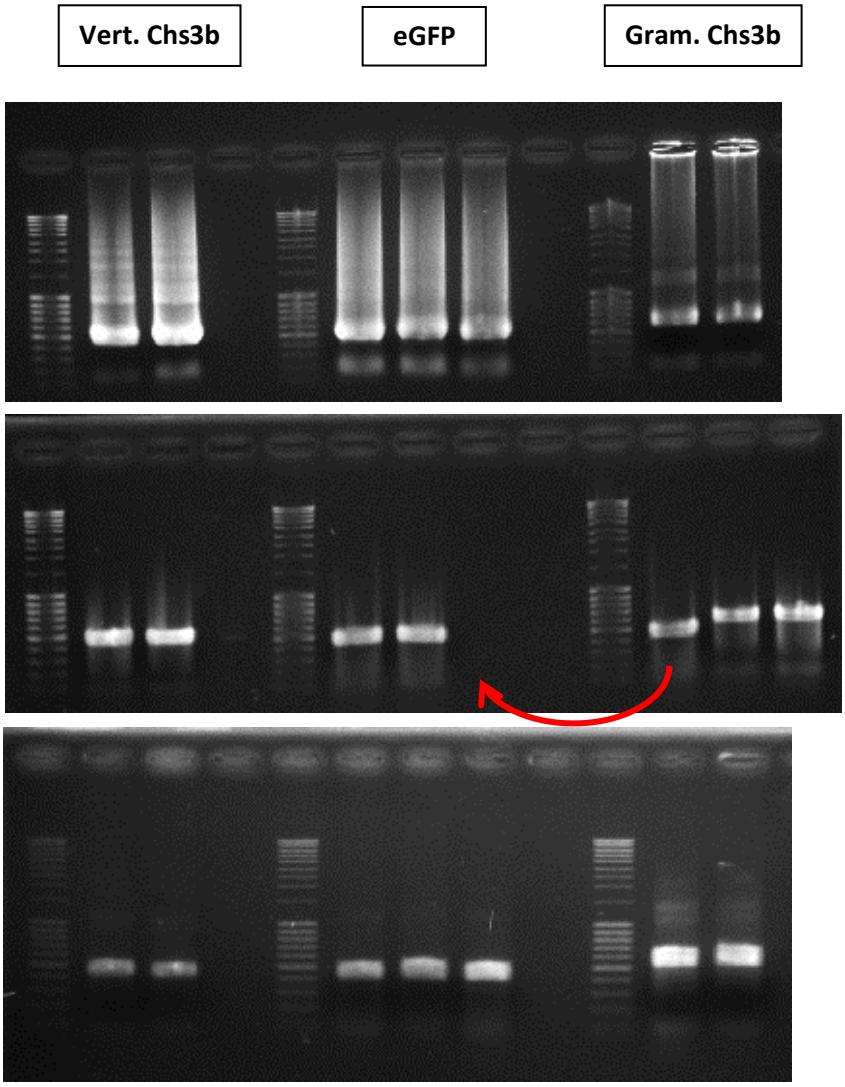


Final PCR product concentration (used as template for dsRNA synthesis)



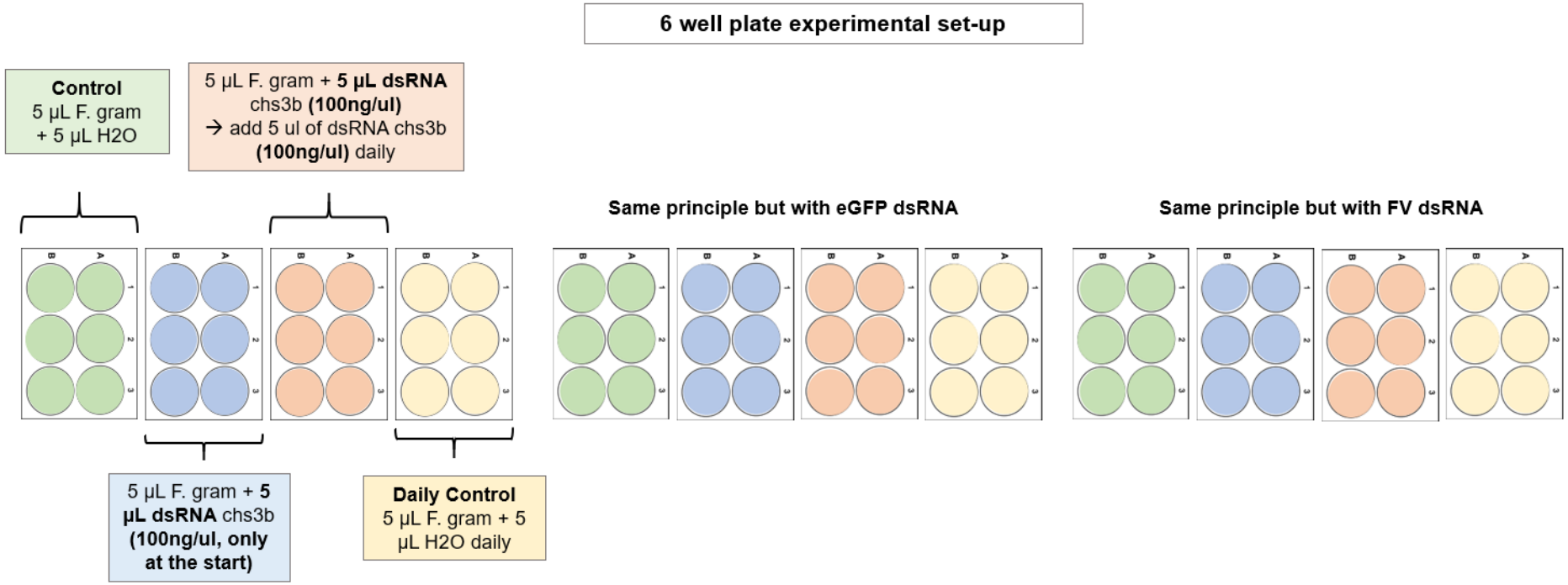
Gene	ng/uL
Vert. chs3b	377.70
eGFP	347.72
Gram. chs3b	346.75

dsRNA synthesis process



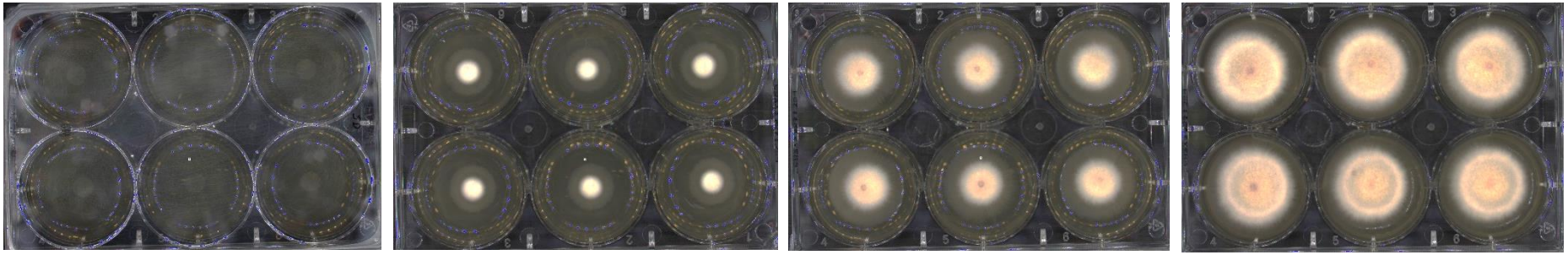
	ng/uL
fV chs3b 1	2814.24
2	2282.05
Fg eGFP 1	2255.07
2	2132.48
3	2728.52
Fg chs3b 1	3491.96
2	2367.93

Supplement II



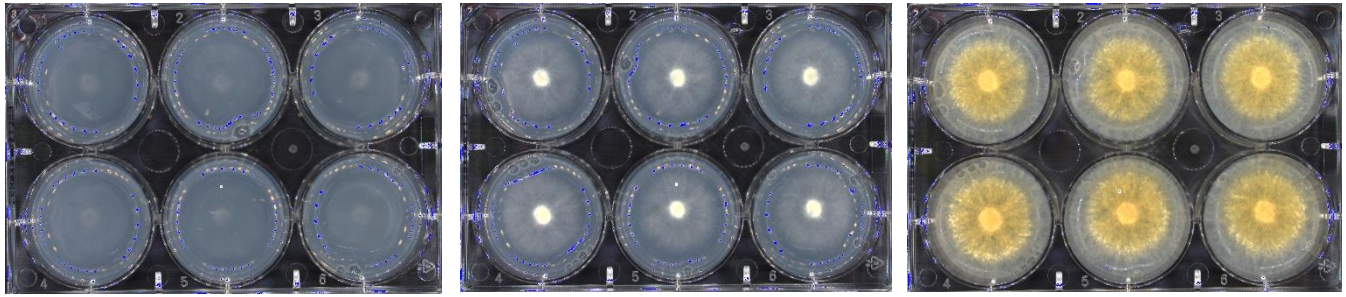
Control pictures

F. verticillioides



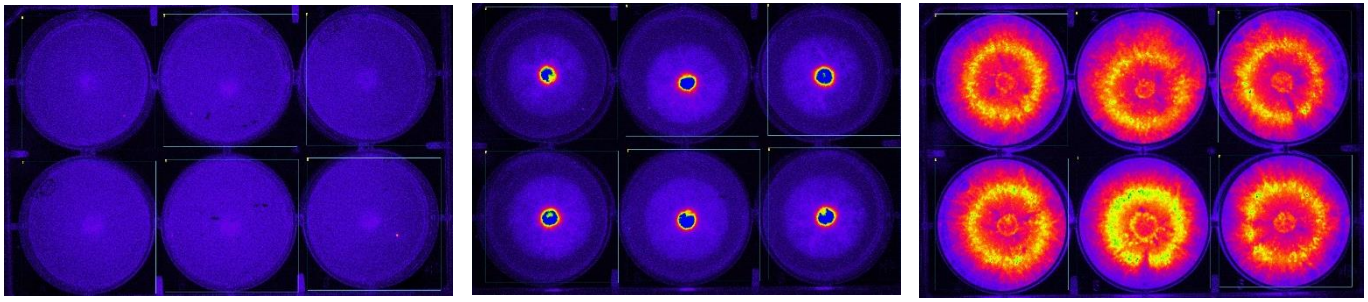
1 dai ***2 dai*** ***3 dai*** ***4 dai***

F. graminearum



1 dai ***2 dai*** ***3 dai***

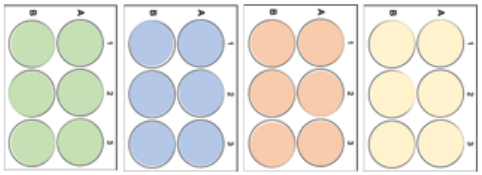
cGFP



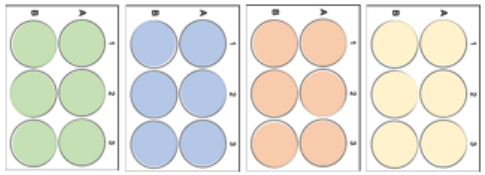
6-well plate experiment with extra added media types

F. Verticillioides chs3b: 4 days

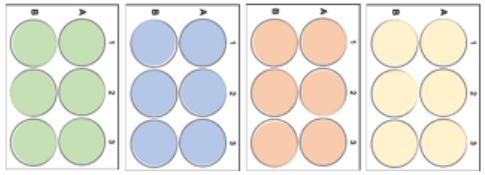
Normal PGA



PGA 10X diluted

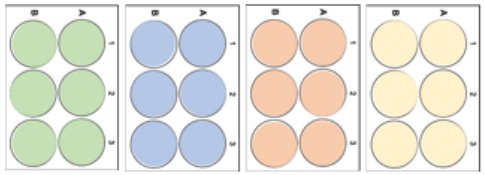


H2O medium

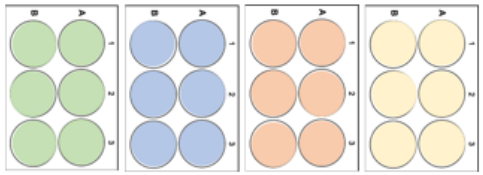


F. Graminearum chs3b: 3 days

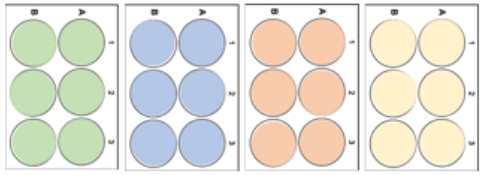
Normal mineral medium



mineral medium 10X diluted

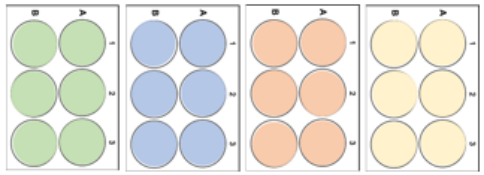


H2O medium

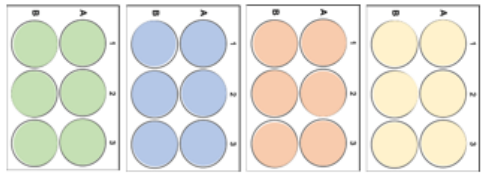


F. Graminearum eGFP: 3 days

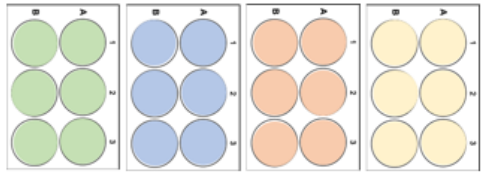
Normal mineral medium



mineral medium 10X diluted



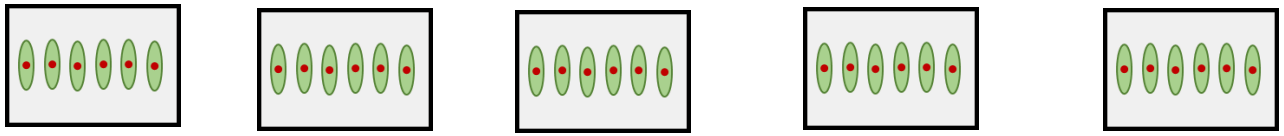
H2O medium



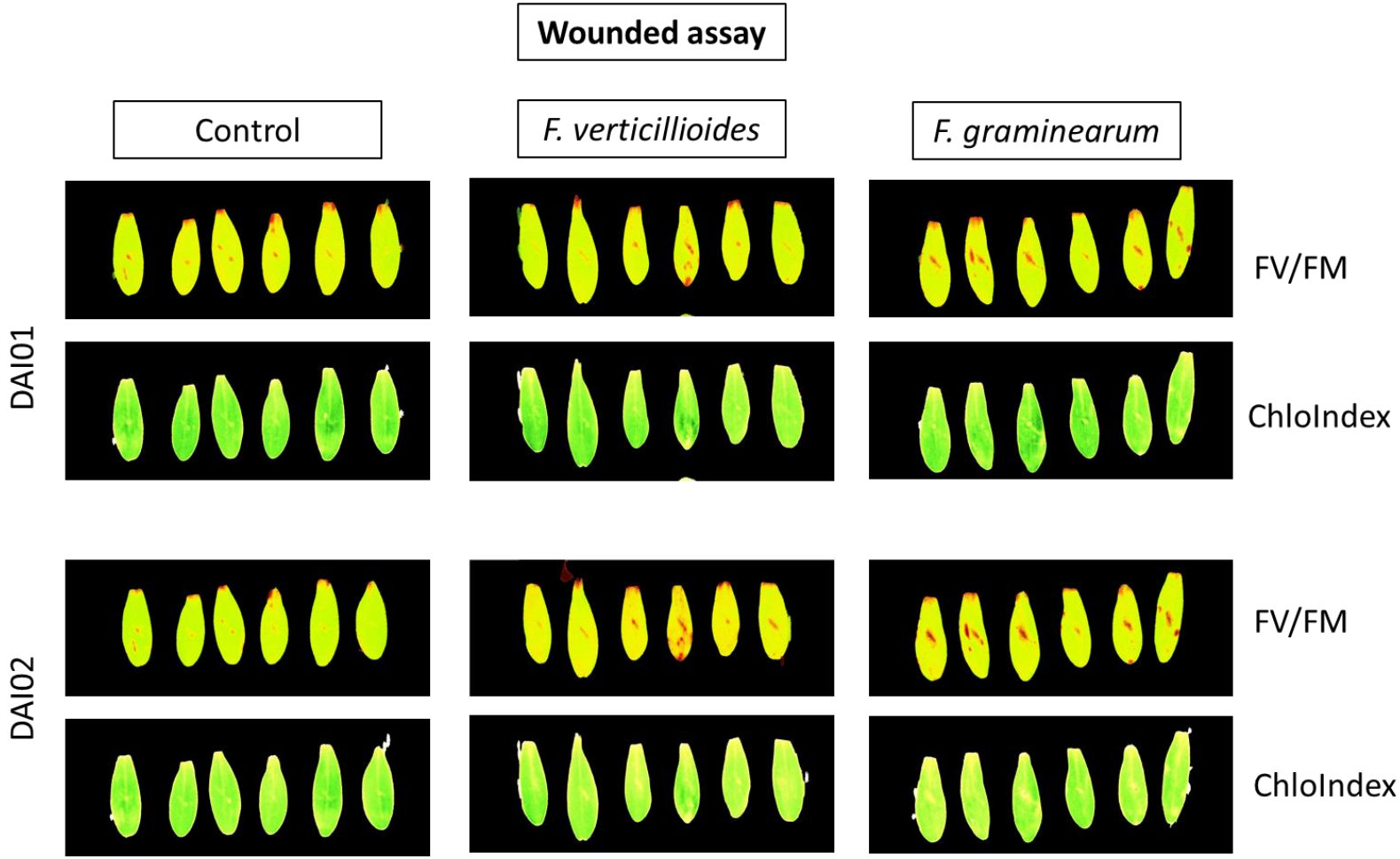
Supplement III

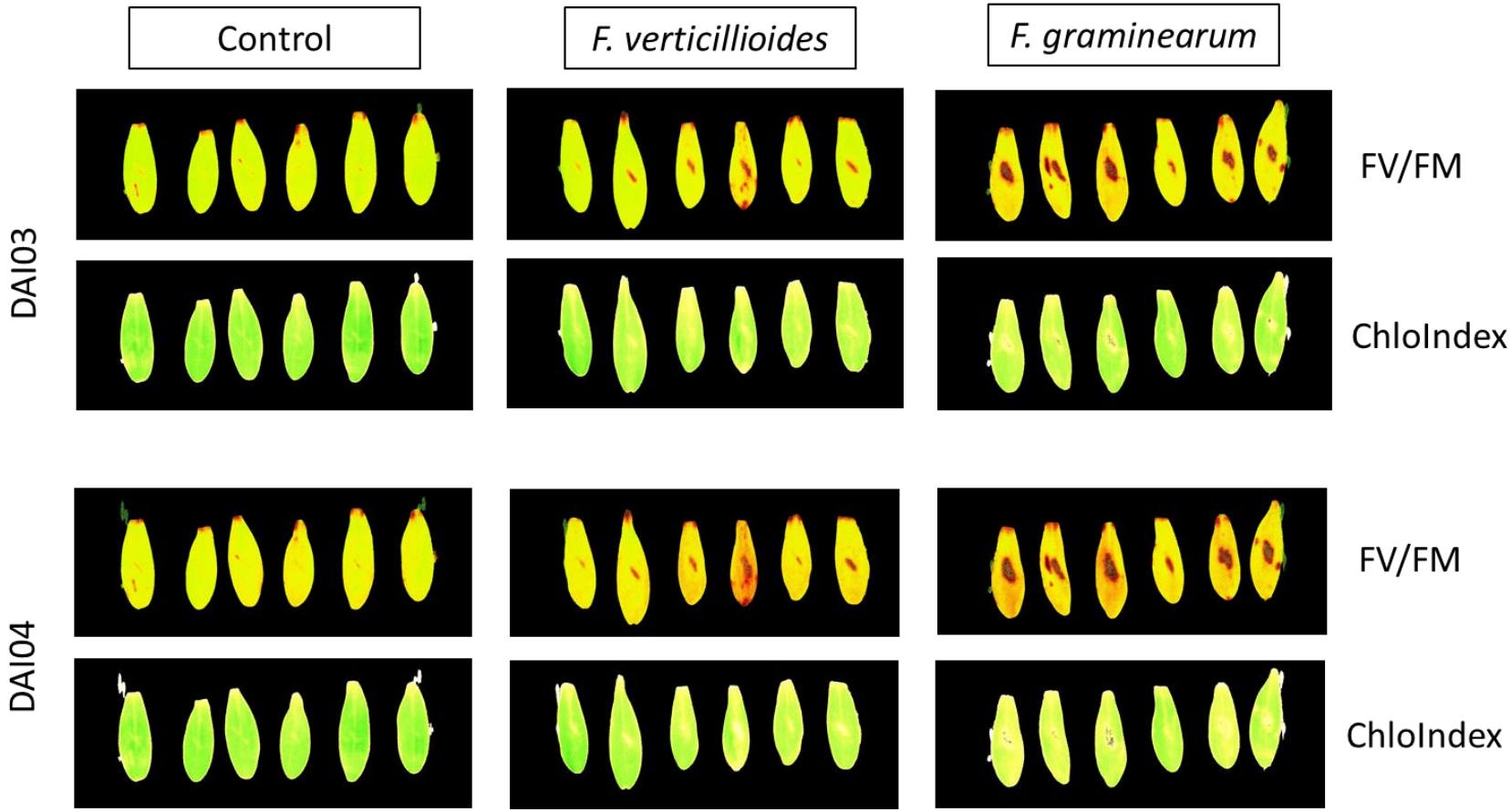
Infection trial

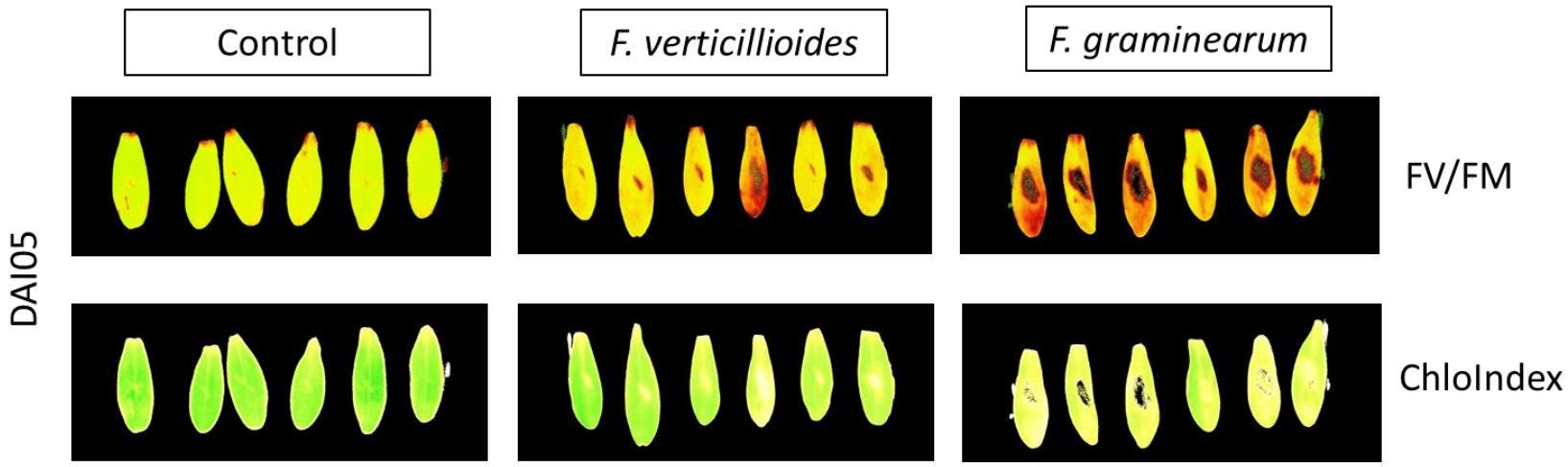
Control	**10 µL F. vert.**	**10 µL F. vert.**	**10 µL F. gram.**	**10 µL F. gram.**
10 µL Sterile water	Unwounded leaves	Wounded leaves	Unwounded leaves	Wounded leaves

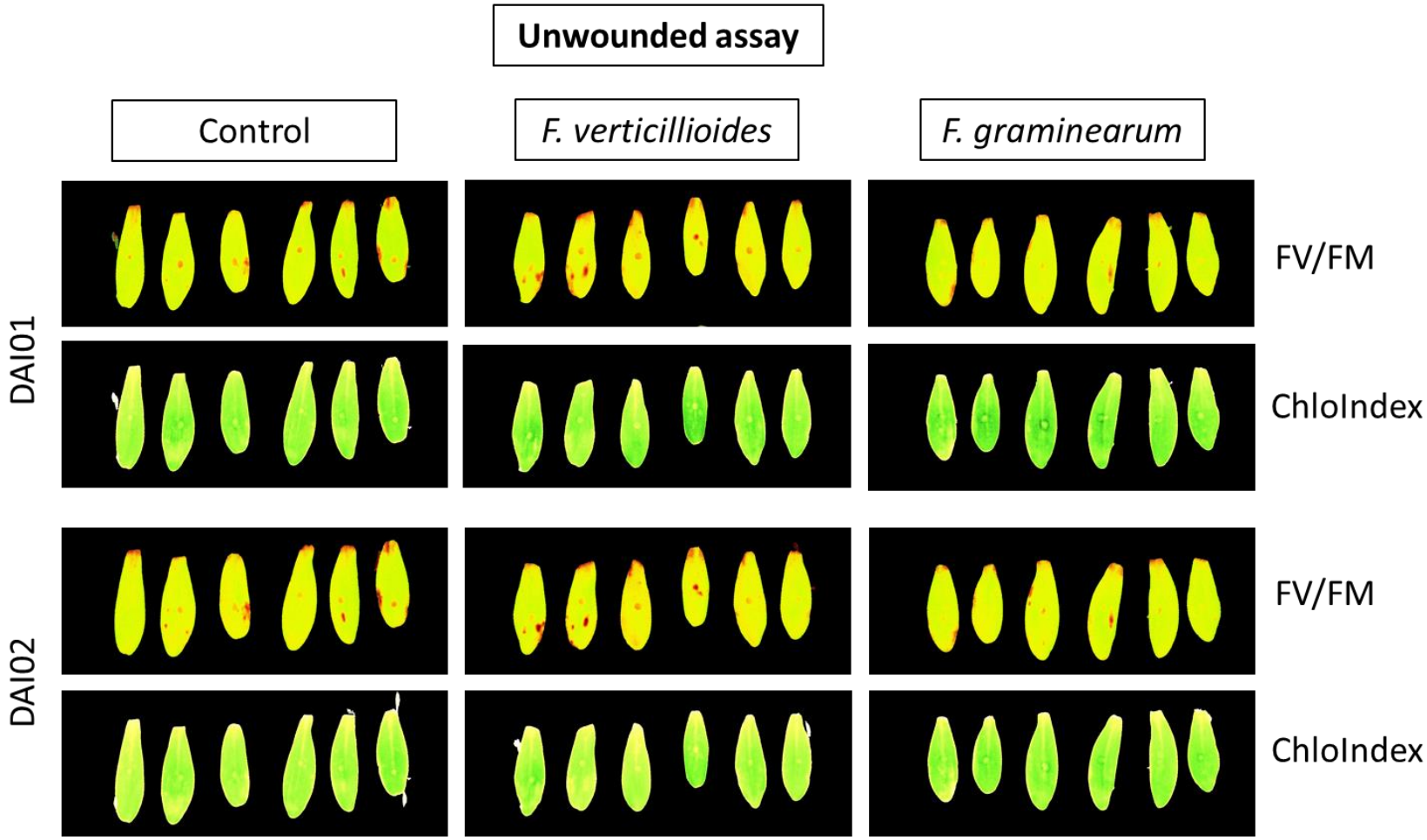


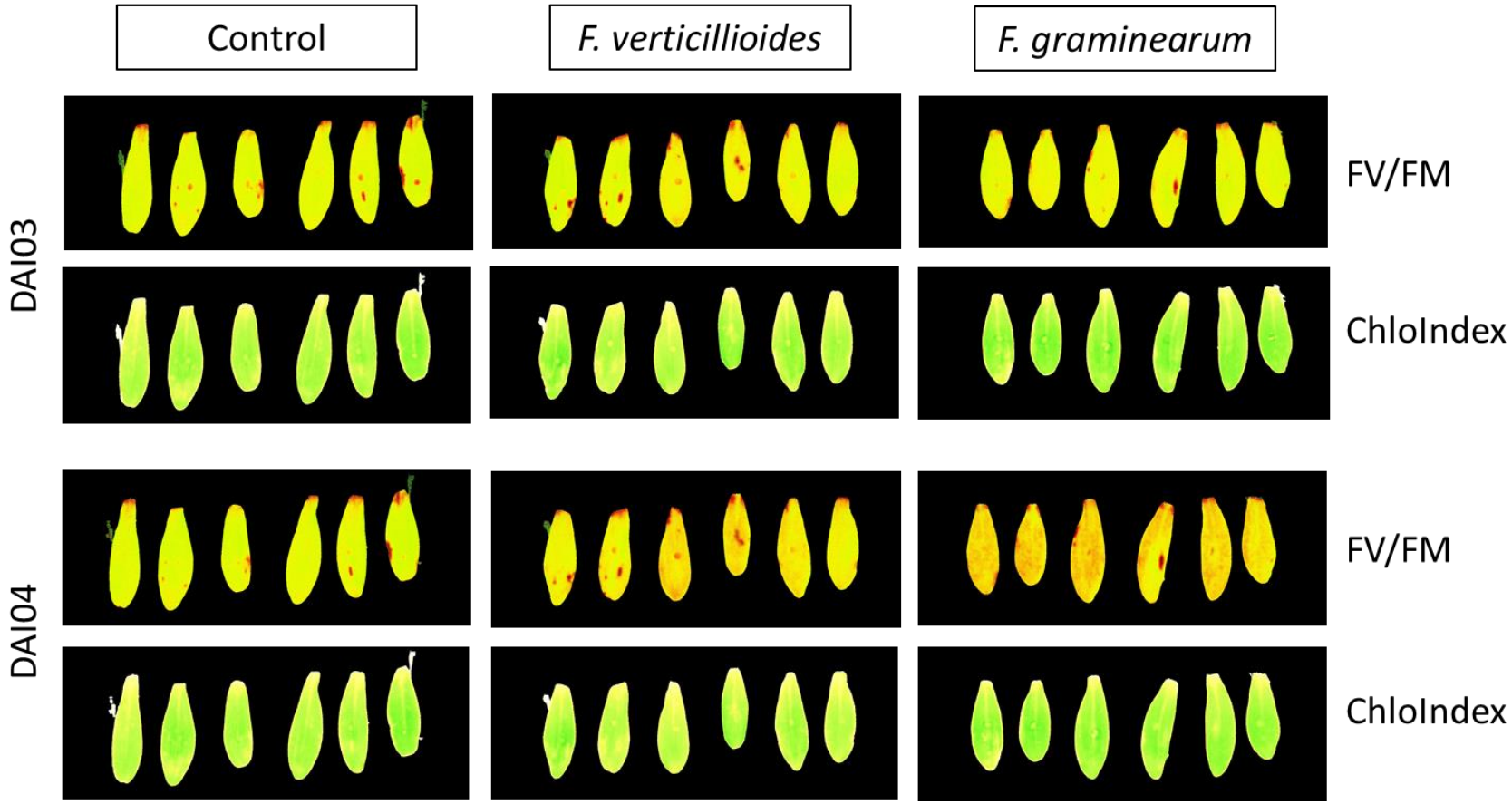
Pictures of infection trial – Fv/Fm values

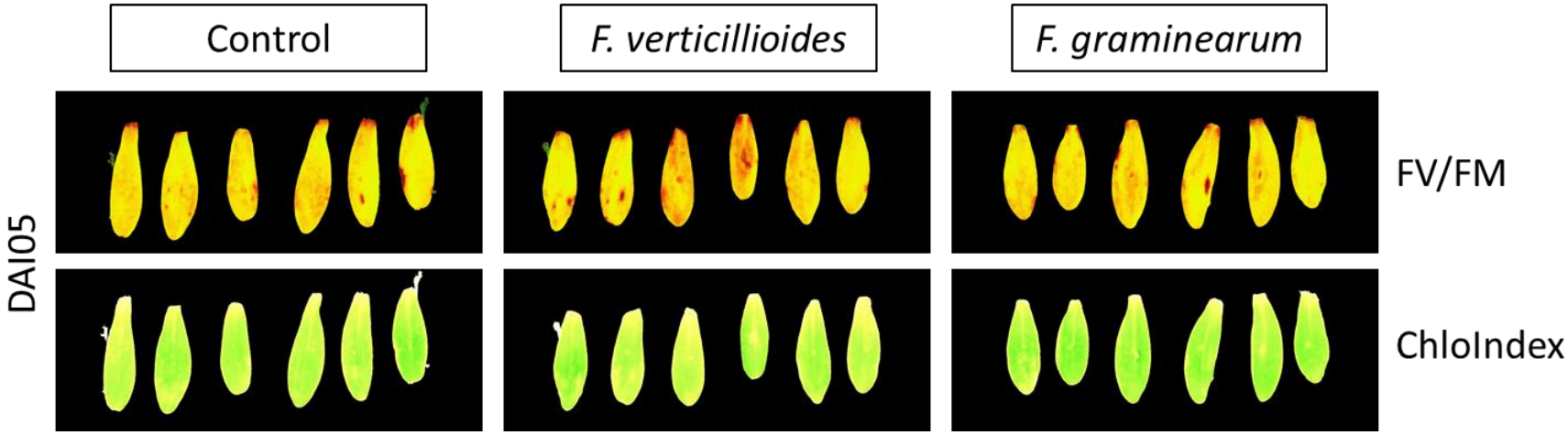



Control
F. verticillioides
F. graminearum
DAI03
DAI04
FV/FM
ChloIndex
FV/FM
ChloIndex

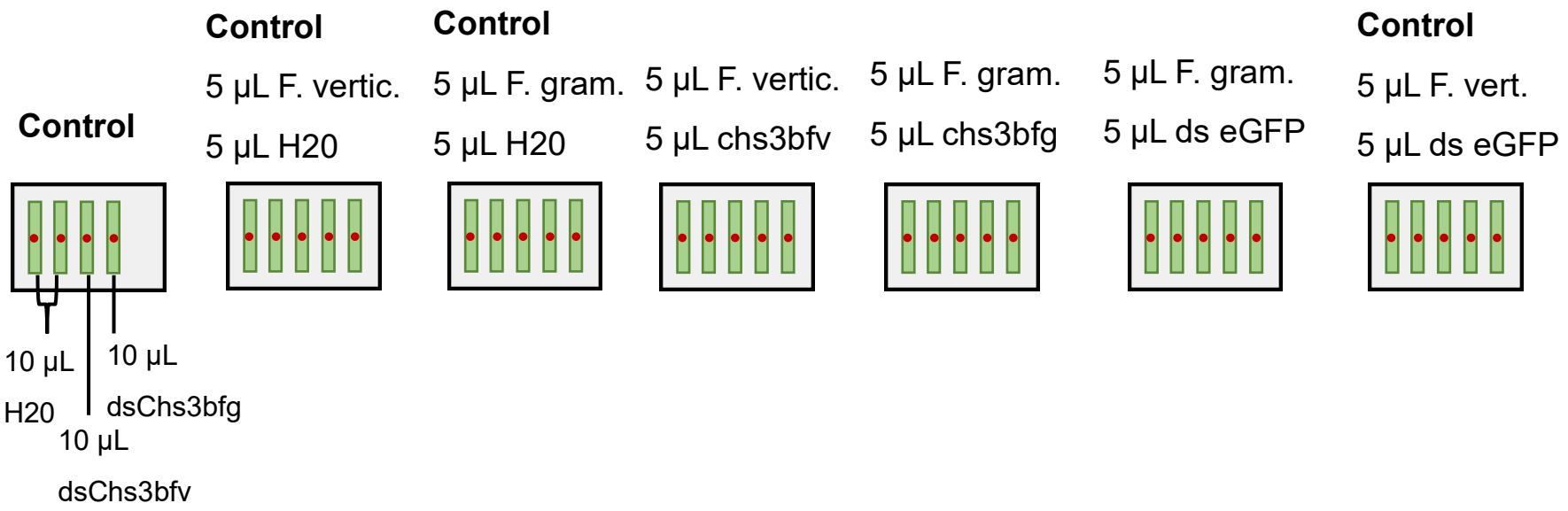

Control
F. verticillioides
F. graminearum
DAI05
FV/FM
ChloIndex


Unwounded assay
Control
F. verticillioides
F. graminearum
DAI01
DAI02
FV/FM
ChloIndex
FV/FM
ChloIndex


Control
F. verticillioides
F. graminearum
DAI03
DAI04
FV/FM
ChloIndex
FV/FM
ChloIndex


Control
F. verticillioides
F. graminearum
DAI05
FV/FM
ChloIndex

Chs3b and eGFP set-up I



Chs3b and eGFP set-up II