FACULTY OF BIOSCIENCE ENGINEERING

RNAI AGAINST FUSARIUM TO PROTECT MAIZE AND REDUCE MYCOTOXIN PRODUCTION

Word count: 23.944

Janelle De Decker

Student number: 01906167

Supervisors: Prof. dr. ir. Kris Audenaert, dr. ir. Nji Tzi Clauvis Taning

A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in de biowetenschappen: land- en tuinbouwkunde: plantaardige en dierlijke productie

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PREFACE

The road to the finish line of this dissertation was full of challenges, and the successful completion of this paper would not have been possible without the help of many people. First and foremost, I must acknowledge my two co-promotors, prof. dr. ir Kris Audenaert and dr. ir. Clauvis Taning. Without their advice and feedback on my thesis, I would not have been able to deliver a manuscript that I can be proud of. I am also very grateful to them for the opportunity to work in their research labs for the practical aspects of this thesis. I realize that I am very lucky to have had my first practical lab experience on my own in such well-equipped and open environments. Any question or problem presented was almost always immediately solved, and they were always ready to help me with practical challenges when asked. Of course, I am grateful as well for the new insights and knowledge I have gained, both throughout conducting my own research, as well as through the expertise that was offered to me by both my supervisors.

In addition, I would like to thank the lab members of LAMP, in particular Noémie De Zutter, Boris Bekaert, Larissa De Troyer and Silke Deketelaere. They offered me a lot of help during the practical research in the lab and were always ready to answer any question I had at any moment (and I had a lot). Boris helped me out greatly during the protoplast production and always offered to jump in when things got hard. Noémie was always ready to help me with the phenotyping robot and the processing software, and I will not forget her expert knowledge, but especially her kindness to me when there were unexpected difficulties. Overall, the lab environment was very open and welcoming, and I am grateful to have been part of this team for the past year.

Lastly, there are many people in my personal life I have to thank for being able to now hand in this dissertation. Of course, I must mention my family, who were always at the ready with words of encouragement. Secondly, I would like to mention my dear friends, and especially Elise, Nele, Jolien and Rosalie, who will be graduating alongside me. I would not have made it through this year without the ever-present reassurance and kind words of our ‘mental support group’. And lastly, the person I have to thank the most for supporting, encouraging, consoling, and helping me, not only this past year but since I have known them, is my dear partner Niobe. My pievie, thank you, I could not have done this without you.

ABSTRACT (DUTCH)

Verschillende schimmels van het *Fusarium*-genus zijn verantwoordelijk voor verwoestende gewasziekten en verliezen, zoals aarfusarium veroorzaakt door *F. graminearum*, of *Fusarium*-kolfrot veroorzaakt door *F. verticillioides*. Deze ziekten leiden niet alleen tot grote economische verliezen, maar de geproduceerde mycotoxinen zijn ook schadelijk voor de gezondheid van mens en dier. De bestrijding van deze schimmels steunt doorgaans op het gebruik van synthetische fungiciden. Echter, ongunstige milieugevolgen en toenemende fungicide-resistentie bij schimmels hebben geleid tot steeds strengere reguleringen en daarmee tot de behoefte aan duurzame, milieuvriendelijke alternatieven.

Een opkomende strategie die veelbelovend lijkt, is RNA-interferentie (RNAi), en meer specifiek 'spray-induced gene silencing' (SIGS). Het doel van deze studie was om een proof-of-concept van RNAi te leveren en een SIGS-protocol te ontwikkelen, met als uiteindelijk doel eenstrategie gebaseerd op RNAi te ontwikkelen voor de bestrijding *van F. verticillioides*-infecties in maïs. Hiervoor werden dsRNA en siRNAs ontworpen om een eGFP-gen te targeten in een getransformeerde *F. graminearum*. Reproduceerbare experimentele set-ups werden ontwikkeld, waarbij geëxperimenteerd werd met verschillende media en met de lengtes van het dsRNA-construct. Het praktische onderzoek bestond uit microscopische analyse, *in vitro* behandelingen op petriplaten, en een *in vivo* detached leaf-assay op tarwe. De resultaten toonden aan dat protoplasten van de schimmel in staat waren het dsRNA op te nemen met behulp van nanopartikels, en dat het dsRNA de GFP-productie in de protoplasten kon verminderen, zij het op een voorbijgaande manier en niet consistent voor elke behandeling. Dezelfde resultaten werden gevonden voor siRNA-fragmenten. De verschillende incubatie- en inoculatiemedia werden geëvalueerd en aanbevelingen voor verder onderzoek op dit gebied werden opgesteld. Het SIGS-protocol werd geïnitieerd, maar vertoonde geen significante resultaten.

Kernwoorden: *Fusarium graminearum*, eGFP, RNAi, protoplasten, dubbelstrengig RNA, siRNA, SIGS

ABSTRACT (ENGLISH)

Several fungi of the *Fusarium* species are responsible for devastating crop diseases and losses, such as *Fusarium* head blight caused by *F. graminearum*, or *Fusarium* ear rot caused by *F. verticillioides*. Not only do these cause major economic losses, the mycotoxins produced by these pathogens are also detrimental to human and animal health. Control of these pathogens typically relies on synthetic fungicides, however, adverse environmental consequences and rising fungal resistance has led to increasingly strict government regulations and subsequently the need for sustainable, environmentally friendly alternatives.

An emerging strategy that shows great promise is RNA interference (RNAi), more particularly spray-induced gene silencing (SIGS). The objective of this study was to deliver a proof of concept of RNAi and develop a SIGS-protocol, with the eventual goal of developing an RNAi-based strategy for the control of *F. verticillioides* infections in maize. For this, dsRNA and siRNA were designed to target the eGFP-gene in a transformed *F. graminearum*. Reproduceable experimental set-ups were developed, experimenting with different media and lengths of the dsRNA-construct. The practical research consisted of microscopic analysis, *in vitro* application on petri plates, and an *in vivo* detached leaf analysis on wheat. The results showed that protoplasts of the cells were able to absorb the dsRNA with the aid of a nanocarrier, and that the dsRNA was able to reduce the level of GFP-production in the protoplasts, yet in a transient way and not consistently for each treatment. The same results were found for siRNA fragments. The different incubation and inoculation media were assessed and recommendations for further research into this topic were drawn up. The SIGS-protocol was initiated, but did not show significant results.

Keywords: *Fusarium graminearum*, eGFP, RNAi, protoplasts, double-stranded RNA, small interfering RNA, SIGS

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LIST OF ABBREVIATIONS

AGO	Argonaute protein
BCA	Biological control agent
Bp	Base pair
CAP	Common agricultural policy
cDNA	Complementary DNA
DCL	Dicer-like protein
DMI	Demethylation inhibitor
DON	Deoxynivalenol
Dpi	Days post inoculation
dsRNA	Double-stranded RNA
eGFP	Enhanced green fluorescent protein
ELEM	Equine leuko-encephalomalacia
FB	Fumonisin B
FER	Fusarium ear rot
FGSC	*Fusarium graminearum* species complex
FHB	*Fusarium* head blight
GMO	Genetically modified organism
GUS	β-glucuronidase
HIGS	Host-induced gene silencing
hpRNA	Hairpin-RNA
IPM	Integrated pest management
MDMV	Maize dwarf mosaic virus
miRNA	Micro RNA
mRNA	Messenger RNA
nHST	Non-host-specific toxin
Np	Nanoparticle
Nt	Nucleotide
PBS	Phosphate buffered saline
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PPA	Plant protecting agent
PPO	Porcine pulmonary oedema
PTGS	Post-transcriptional gene silencing
RdRP	RNA-dependent RNA polymerase

RISC	RNA-induced silencing complex
RNAi	RNA interference
SAM	Sphinganine-analogue mycotoxin
SIGS	Spray-induced gene silencing
siRNA	Small interfering RNA
sRNA	Small RNA
ssRNA	Single-stranded RNA
ZEN	Zearalenone

LIST OF FIGURES

1. INTRODUCTION

Ensuring global food security in the 21st century presents one of the most pressing challenges for agriculture, particularly due to the exponentially rising world population. One of the greatest threats to food security in today's agriculture is the risk of crop losses. Even though current agricultural practices are heavily reliant on chemical plant protection agents, 20 to 40% of potential global yield is still lost to plant diseases and pests (FAO, 2022). It is clear that synthetic pesticides alone are not able to provide enough crop protection today, let alone in the future when food demand will only get higher. However, the main problem concerning excessive use of chemical agents is the detrimental effect this common practice has on the environment and our entire ecosystem: contamination of surface- and groundwater causes off-target effects in the entire system, from essential insects like pollinators and useful predators, to even humans and other mammals. Combine this with the fact that many pests and pathogens can and have developed resistance against chemical control agents, and it becomes undeniable that new, sustainable ways of crop protection need to be brought forward.

Fungal pathogens and their subsequent diseases represent an important subset within crop stressors. Among these, the *Fusarium* genus holds a central position as a major contributor, especially to several cereal crop diseases. *Fusarium graminearum* is a predominant species, causing several devastating diseases in a broad range of crops. The most notorious disease of this pathogen is *Fusarium* head blight (FHB), commonly seen in wheat as well as other small grains. A lesser known, yet rapidly emerging species is *F. verticillioides*, the main causal agent of *Fusarium* ear rot (FER). Both of these diseases can cause a near-total harvest destruction when left untreated, yet the main concern is the accumulation of mycotoxins in affected plants. The secondary metabolites produced by these fungi are classified as carcinogenic and pose a great danger to safety in the food- and feed-chain.

In order to ensure an adequate crop protection while still reducing environmental and ecosystem impact, the European Commission introduced the European Green Deal, limiting the use of dangerous chemical agents while promoting the development and implementation of more sustainable and integrated strategies. One of these emerging strategies that shows great promise is RNA interference (RNAi). The technology behind this is based on naturally occurring pathways in most eukaryotic cells. These pathways ensure that the fungus can regulate gene expression post-transcriptionally by inhibiting

messenger RNA (mRNA). This mechanism is activated by the presence of double stranded RNA and can thus also be externally induced. This principle is the basis for the emerging RNAi-based biocontrol: exogenous dsRNA, either originating in the plant or externally applied to the fungus, is taken up into the pathogen where it is able to inhibit certain gene translations. When the dsRNA is generated in the host plant, the mechanism is called host-induced gene silencing (HIGS). For this technique, the plant has to be genetically modified to produce dsRNAs for the target gene. However, as there are not many transformation protocols available, and also due to the uninviting attitude of the public and the EU towards transgene crops, attention was quickly redirected to alternative RNAi-techniques. One of these, which is also the focus of this study, is spray-induced gene silencing (SIGS). Here, the dsRNA is applied to the external surface of the plant or pathogen, after which the dsRNA can enter the pathogenic cells. This technique is highly specific and causes no off-target gene silencing, making it a valuable, promising alternative to traditional pesticides.

The objective of this dissertation was to deliver a proof of concept of RNAi in *F. graminearum* and optimize a SIGS-protocol, with the eventual goal of developing an RNAi-based strategy for the control of *F. verticillioides* infections in maize. Therefore, the literature review of this dissertation focusses on the cultivation of this crop, its known pathologies and control strategies, before delving into the *Fusarium* species, mycotoxins, and finally delivering an in-depth review of RNAi-mechanisms and their past uses as control strategies. The subsequent section focusses on the experiments executed for this research: a microscopic trial, along with *in vitro* and *in vivo* assays were established to investigate the effect of dsRNA or siRNA on fungal protoplasts, under diverse growing conditions. An eGFP-tagged strain of the fungus was used, to visualize the (lack of) gene silencing of the different eGFP-dsRNA treatments. The resulting GFP values were compared and checked for significance in R, after which a discussing section attempts to explain the results and offer guidance and suggestions for further research into this topic.

2. LITERATURE REVIEW

2.1 Agriculture in Europe

Agriculture has always shaped a significant part of European landscapes, both in the past and at present. Today, in more rural areas, one can still look around and only see fields, crops, and the occasional farm. This is not illogical, as the agricultural sector is one of the main land users in Europe: according to Eurostat, in 2018 farmland accounted for 39.1% of total land use in the European Union, amounting to the largest portion when comparing agriculture, forestry, unused and abandoned areas, services and residential purposed areas, heavy environmental activity areas, and fishing, as shown in Figure 1.

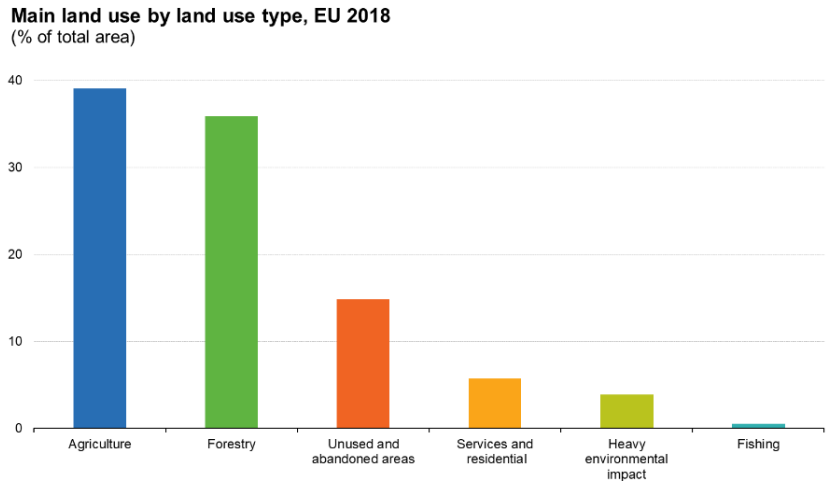


Figure 1: Main land use in EU 2018 (source: Eurostat)

Agriculture is the first and also one of the main links of the food- and feed chain; without primary production, there wouldn't be any food or feed to process, sell, and consume. Yet, society's perception towards farming seems to worsen over time. This is unsurprising, as many agricultural practices contribute to environmental pollution, which in turn leads to human health problems, economic losses, biodiversity loss, ecosystem disturbance and, on a much larger scale, global warming and climate change (Adegbeye et al., 2020).

The European Environment Agency collected data in 2020 to identify the major impacts made by agricultural practices. The list includes but is not limited to:

- **Ammonia emissions:** Agriculture is responsible for 94% of the ammonia emissions, mainly through the use of both manure/slurry and inorganic fertilizers.
- **Water use:** More than 50% of water used in Europe goes towards agricultural purposes, putting pressure on renewable water sources.

- **Nitrates** in surface- and ground waters: Nitrate mainly comes from intensive agriculture. In many regions, the concentrations of nitrate in surface- and ground waters are still too high (European Environment Agency, 2020).
- **Pesticides:** Thirteen to thirty percent of monitored surface waters in Europe showed a concentration of pesticides higher than the designated allowed threshold (European Environment Agency, 2021).

But of course, European agriculture also has many positive aspects. European agriculture produces a particularly wide variety of products, all of which are ensured to be safe for consumption (European Parliament (EP), 2021). It is also predominantly self-sufficient when it comes to primary resources. Additionally, the EU holds the superior position when it comes to global agri-food trade (European Commission, n.d.-b; European Environment Agency, 2020). Agriculture is also one of the sectors that has contributed most to efforts to reduce greenhouse gas-emissions, with a reduction of 24% over the last thirty years (Copa and Cogeca, 2022).
However, as a logical response to the abundant negative impacts, many of the EU's strategies to reduce the carbon footprint concern agriculture. It is clear that an evolution towards a more sustainable agricultural system is needed, to ensure a more sustainable management of natural resources.

2.1.1 Pest control

Ever since people started cultivating land for food, the need to protect crops from diseases has been omnipresent. Today, plant pests and diseases would be able to eliminate more than half of the world's cultivated crops if they weren't being eradicated systematically through the use of pesticides, or plant protecting agents (PPA). Despite the use of PPA's, between 20 and 40% of potential global yield is lost to plant diseases and pests. Without the use of PPA's, these numbers could easily double (FAO, 2022). The pests and diseases that pose a threat to food crops are profuse, and include weeds, insect pests, rodents and other animals, fungi, but also bacteria and viruses (US EPA, n.d.).
However, the active ingredients in pesticides often don't only impact the targeted pest or disease. Off-target effects on useful predator insects, pollinators, animals – including humans – and even further, the entire ecosystem – are of serious concern when applying agrochemicals. This is due to the fact that the active ingredient in a pesticide does not easily deteriorate, combined with the fact that the substance can drift from the place it was applied: it seeps into soil and groundwater or becomes airborne and gets blown away. This way, the active substances do not only locally show an impact (meaning on the field), but

can impact systems to over 1000 kilometres further than their site of application (Heinrich Böll Stiftung et al., 2022).

The residues of the pesticides can be particularly harmful for human health. Around 385 million cases of pesticide poisoning are reported worldwide every year, primarily affecting farmers in the Global South. One of the most commonly used herbicides in the world is glyphosate, commercially named Round-Up, and it has been classified as ‘probably carcinogenic’ by the United Nations’ World Health Organization. Other examples are paraquat, a herbicide that is highly toxic to humans, and chlorantraniliprole, which is labelled as “highly hazardous to aquatic organisms”. While many of the most toxic pesticides – to human at least – have been banned in the EU, the global use of them is still frequently occurring (Heinrich Böll Stiftung et al., 2022).

2.1.1.1 The European Green Deal

In December of 2019, a set of proposals entitled the European Green Deal was launched by the European Commission. The reasoning behind this is that the European Union wants to achieve a climate-neutral status by 2050, hereby transforming the European economy into a more modern one that is resource-efficient yet still competitive. This Green Deal strives towards three achievements:

1. no net emissions of greenhouse gases by 2050,
2. economic growth decoupled from resource use,
3. no person and no place left behind (European Commission, n.d.-a).

The ways to achieve these goals are plentiful; the focus of the plan lies on decarbonising the energy sector, reducing energy usage and supporting innovation, as well as on environmental objectives: providing fresh air, clean water, healthy soil and biodiversity, in addition to healthy and affordable food (European Commission, 2019). Naturally, to achieve this, the way land is currently cultivated must drastically change.
One of the major pillars of the Green Deal is a systematic shift in the European agriculture and food system towards a more sustainable system, seeing as agriculture is the first link in the food-production chain. The EU’s specific goals for the agricultural sector will require a change in mentality of farmers in Europe: their attention will have to be delegated between continuing and optimizing their traditional production activities, while also taking measures to contribute to the goals of the deal. These agronomic goals include better and more environmental protection, increasing biodiversity, supporting/preserving existing

ecosystems and restoring destroyed ones, implementing a Farm to Fork-strategy, etcetera (Wrzaszcz & Prandecki, 2020).

A key tool to achieve the objectives of the Green Deal is the new common agricultural policy (CAP) for 2023-2027. The CAP is an association between Europe and its farmers, and thus between society and agriculture. It's a common policy, applying to all countries in the European Union. The policy, when introduced in 1962, presented 5 aims:

- Support farmers and improve agricultural productivity, ensuring a stable supply of affordable food;
- Safeguard European Union farmers to make a reasonable living;
- Help tackle climate change and the sustainable management of natural resources.
- Maintain rural areas and landscapes across the EU;
- Keep the rural economy alive by promoting jobs in farming, agri-food industries and associated sectors. (European Commission, 2022)

While this version of the CAP has proven adequate as legislation for the past 60 years, the introduction of the European Green Deal has put a drive for change in motion. The regulations in the CAP have evolved over the years, however, the growing threat and increasingly noticeable impact of climate change calls for a more hands-on, effective strategy. Thus, a new CAP was proposed in 2018 and approved in 2021, to be implemented at the beginning of 2023 (European Commission, 2022; Pub Affairs Bruxelles, 2022).
This new policy, informally titled "a greener and fairer CAP", poses 10 new and improved objectives, three of which focus directly on the environment and climate – mainly incorporating climate change, management of natural resources, and biodiversity – while the other 7 objectives cover the other dimensions of sustainability, namely the economic and social aspects, hence the "fairer" part in the title of the policy (*Factsheet - a Greener and Fairer CAP*, n.d.). The new common policy does not define a specific set of rules to be followed by every country in the EU. Rather, it poses the ten objectives and puts responsibility at a national level to create a strategic plan appropriate for the respective country, based on their strengths, weaknesses, opportunities and threats (SWOT-analysis). These stratagems were submitted before the beginning of 2022, and should, as aforementioned, be put into action at the start of 2023 (European Commission - Agriculture and rural development, 2022).

Even currently, without the new CAP in place, implementations to achieve the objectives in the Green Deal have already made – and will continue to do so – a considerable impact on agriculture as we know it. These implementations concern 4 major aspects of traditional agriculture: pesticide- and fertilizer-use, use of antimicrobials in animal production, and organic farming. Specific targets have been set regarding these 4 pillars, which should be reached by 2030. The target goals are:

- Reducing the use of chemical pesticides and related risks by 50%;
- Reducing the use of more hazardous pesticides by 50%;
- Reducing nutrient losses by at least 50%, while preventing deterioration in soil fertility;
- Reducing the use of fertilizers by at least 20%
- Reducing the sale of antimicrobials for farmed animals and in aquaculture by 50%;
- 25% of utilized agricultural area should be used in accordance with organic farming rules. (Wrzaszcz & Prandecki, 2020)

These targets are generally regarded as very ambitious, and there are several concerns as to whether these strategies to work towards a sustainable resource management can go hand-in-hand with the global rising food-demand due to growing populations. A consideration posed by Blake (2020) is that the expansion of organically cultivated land does not go together with the “produce more using less”-concept, which is necessary to keep food production levels up to global demand. This is because organic farming leads to lower yields, warranting that more land is needed to maintain current production levels. With this, and other points, Blake aims to prove that decisions made for the European Green Deal are politically driven, as opposed to what they should be in the first place: science based. Politicians (generally) possess fewer knowledge about these subjects than scientists, and this leads to choices mainly based on public views rather than science. Similarly, concerning pesticide use, the arbitrary target of 50% reduction is not consistent with the aimed-at production levels: as aforementioned, without the pesticides currently used, at least 40% of crops would be lost to pests, diseases, weed-competition, … Emphasis in the Green Deal should be put on more controlled and integrated use of pesticides, but seeing as they are negatively perceived, they make an easy target to phase out (Blake, 2020). However, if the EU were to hold on to policies that were in place before the Green Deal, the net emission of greenhouse gasses would only be reduced by 60% by 2050. This would have been diametrically opposed to the aim of the EU to make Europe

the first climate-neutral continent in the world. Additionally, in the years leading up to the proposal, it became apparent that more focus had to be put on preserving and restoring ecosystems, as well as protecting and increasing biodiversity. Lastly, it became increasingly clear that more attention had to go to a sustainable food system with a positive environmental impact, whilst ensuring public health and nutrition. This was implemented in the Green Deal via the Farm to Fork-strategy. These objectives had been overlooked for a long time – seeing as it has been hard enough to put global warming on the international agenda – and thus the need for new proposals concerning a greener Europe were much needed (Fetting, 2020).

2.1.1.2 Alternative control strategies

In 1959, Stern et al. published a paper entitled 'The Integrated Control Concept', which became a basis for more modern, innovative pest control. The paper introduced the concept of IPM, or integrated pest management, which entails a combination system of biological and chemical control, integrated to fit the crop and its environment. Many definitions of IPM exist, but a widely accepted one is that of Kogan (1998): "IPM is a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analyses that take into account the interests of and impacts on producers, society, and the environment." The tactic doesn't aim at eradication of plant pests, but at keeping the pest at low numbers, below levels of (economic) injury (Karlsson Green et al., 2020).

Focus shifted from intervention-based measures to preventive strategies. IPM introduced new concepts, such as inserting additional natural enemies of the targeted pests, and modifying the environment through irrigation, introduction of cover crops, development of a more heterogenous plant population, etc. This way, over-reliance on a single method is prevented, avoiding resistance to chemical controls, elimination of natural enemies, and other negative consequences associated to synthetic pesticides (Barzman et al., 2015). However, the usage of pesticides remains an integral part of pest control in IPM, so much so that IPM is presently more often dubbed as 'integrated pesticide management' than its true meaning. True, pesticides are used in a more calculated, precise, and carefully curated way, in an attempt to minimalize risks – both for farmers and the ecosystem – and reduce resistance and adverse effects. Ideally, pesticides would represent a last-resort solution to pest-related problems. However, in reality this is not always the case. And even with a more precise and thought-over usage, the 50% pesticide reduction demanded by the

European Green Deal is not a feasible goal with current implementations of IPM (Zhang & Peshin, 2014).

Luckily, the use of pesticides is not the only way plant pests and diseases can be controlled. While the use of synthetic chemicals is a direct, reactive way to practice pest management, many emerging strategies – often based on principles introduced by IPM – focus on a more indirect and preventive approach to reduce damage induced to crops by pests and diseases. These new strategies often require a restructuring of the entire crop production system (Parvatha, 2017). For example, plant pests and diseases thrive in the commonly used monoculture cropping system. Thus, it is necessary to move away from frequent use of this, and move towards alternative, more sustainable methods of agriculture, based on plant diversification. This is an adequate strategy for preventive and sustainable pest management, and can be implemented in several ways: think crop rotation, intercropping, relay cropping, trap cropping, or even agroforestry for certain crops. These methods show the most noticeable results in weed management, but significant results can also be found in reduction of invertebrate pests and increase in natural enemies, as well as obstruction of airborne diseases (He et al., 2019; Zhang & Peshin, 2014).

Another interesting strategy is biological control, often shortened to biocontrol. Although there are many definitions out there for this term, a brief but adequate description of biocontrol could be ‘the use of (the activity of) biological control agents (BCAs) to reduce a plant disease or pest’. However, this is a broad interpretation, and many different subdisciplines of plant biology have come up with their own terminologies to signify biocontrol. For example, entomologists often use terminology related to the release of a certain organism (for example ‘mass release of predators’), while plant pathologists tend to speak of ‘application of antagonists or competitors’, focussing more on the process through which the biocontrol happens. Still, it all boils down to the same thing, simply put: using active BCAs against other antagonistic organisms that are in any way harmful. Biocontrol can be used to counter all biotic sources of crop losses: weeds, animal pests, and pathogens (including viruses and fungi) (Eilenberg et al., 2001; Wagemans et al., 2022).
It seems that biocontrol is becoming increasingly important in terms of fungal pathogen control, seeing as there are few alternatives to the traditional use of fungicides. However, usage of biocontrol practices on the field is not always feasible, and methods of application often prove to be much more labour-intensive and time-consuming than traditional treatments (Hajek & Eilenberg, 2018; Tariq et al., 2020).

2.2 Maize and its pathologies

Maize (*Zea mays* L.) is a very important crop in agriculture worldwide. It's the third most cultivated crop in the world: the total harvested area amounts to over 200 million hectares (Chemura et al., 2022; Ramirez-Cabral et al., 2017). It is the second most cultivated crop in the European Union, second only to wheat (*Triticum aestivum* L.), and the most cultivated crop in Africa as well as in the United States (Erenstein et al., 2022; U.S. Department of Agriculture, 2021). However, in terms of dry grain production, maize shows notably higher yields than both wheat and rice (*Oryza sativa* L.), the other two most significant cereals in the world. Maize yields total at 1137 million tonnes of dry grain annually, in comparison to the other two, each totalling at 757 million tonnes (Erenstein et al., 2022).

Maize is primarily used as animal feed: 56% of the global production is designated for animal consumption. However, in large parts of Africa – mainly Sub-Saharan Africa – it is the primary staple crop for human consumption: in some countries (such as Zambia and Lesotho), up to 50% of the population's caloric intake stems from consumption of maize (Cairns et al., 2012). Figure 2 shows the global caloric intake from maize in million kcal per region in 2022, mapped by Erenstein et al.

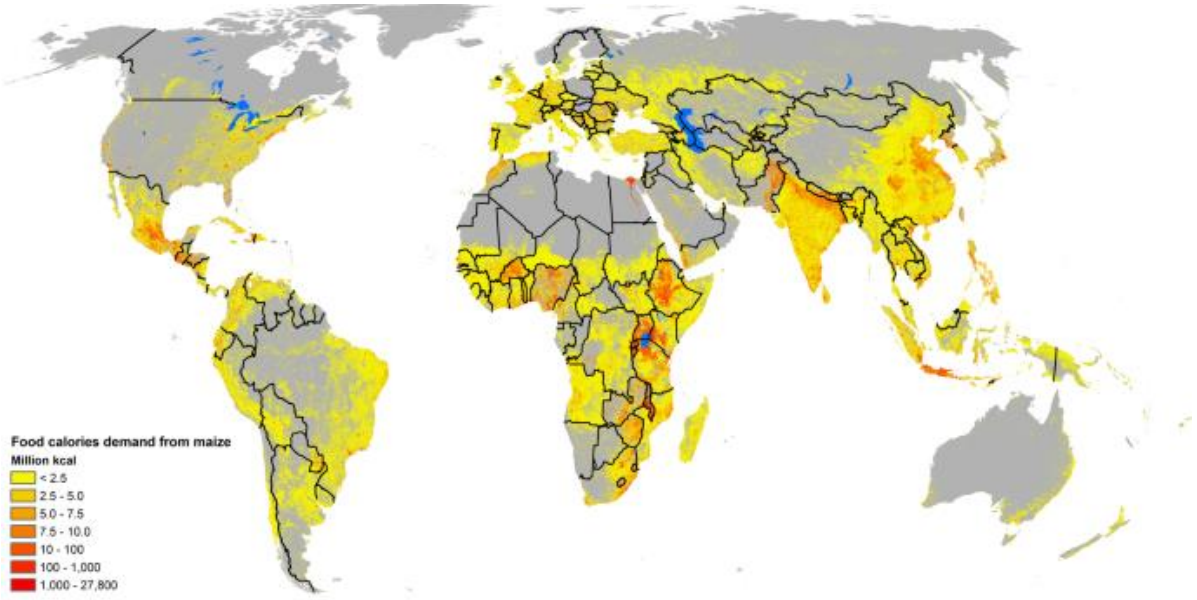


Figure 2: Global caloric demand from maize (source: Erenstein et al., 2022)

Between 1980 and 2008, global potential maize production has been reduced by 3.8% due to the effects of climate change (Lobell et al., 2011), and these numbers are only expected to rise. Breeding advantages and recent technological innovation cannot even up these losses. Meanwhile, global population only keeps on rising, especially in the African

continent. Major crop losses are thus detrimental for global food security. It's clear that there is a need for increased productivity (by means of breeding programs for example) to maintain this security, while still striving for high quality and ecosystem safeguarding (Li et al., 2017).

2.2.1 Cultivation

The cultivation of maize does not come without its own specific challenges, like with any other crop. Maize is very sensitive to freezing temperatures, ensuring that in many Western-European countries, sowing is not possible until mid-spring: if temperatures were to drop below -2°C, seedlings would not be able to survive. Likewise, germination is only possible at temperatures starting from 10°C, although 16-18°C is more favourable and leads to faster emergence (Haesaert, 2022). Every stage of a growing maize plant is susceptible to frost damage, to a point where even grain filling can be adversely affected; in every stage of growth, higher temperatures are beneficial. This makes maize an ideal crop for regions with overall high temperatures or temperate summers, which is visualized in the map in Figure 3, showing regions globally with high amounts of maize cultivation. It is clear that in northern regions like Canada, Scandinavia and Russia, the acreage of maize is close to non-existent (CGIAR, 2014; *FAOSTAT*, 2021).

The map also shows the universality of maize. This is due to the fact that maize, being a C4-plant, has the potential to thrive under a wide array of circumstances, as long as the required temperatures and insolation are fulfilled. Consequently, maize can perform well in both (sub)tropic as well as temperate regions. Even more arid regions are still considered suitable for maize production: the crop shows relatively high drought-tolerance compared to other cereals, as a result of its deep and well-developed root system (Cairns et al., 2012).

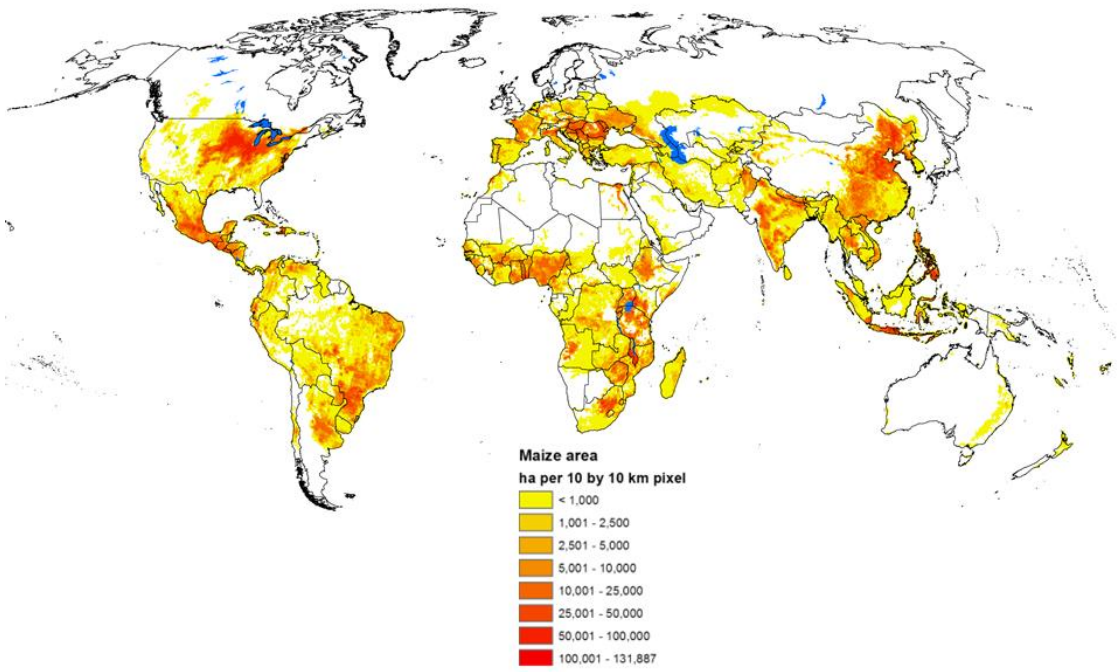


Figure 3: Global areas of maize cultivation (source: CGIAR, 2014)

More often than not, intensive maize cultivation presents itself under the form of monoculture. For the farmer, this is an effective and efficient way to sow, grow and harvest the crop. However, monocultured crops show many downsides, both short- and long-term. Short-term, the crop is more susceptible to weeds, due to lower competitiveness of a sole crop compared to multiple species together, and to pests and diseases. This is because one crop does not attract enough natural predators to compete with its plant-specific pests, who in their turn can spread diseases. Monoculture also sustains specific soil-bound diseases, that are able to return year after year if there aren't any other crops in rotation. After some time, the environment will be completely adapted to the one specific crop, so much so that biodiversity is almost completely lost. Besides that, monocultures deplete the soil of nutrients and lead to soil-degradation. In the long run, this inevitably leads to soil exhaustion, which is detrimental for sustainable, yet intensive agriculture (Balogh, 2021; Fuchs et al., 2021; Haesaert, 2021).

2.2.2 Pathologies

Plant pests and diseases can cause up to 40 % of global yield losses every year. Many of these pests and diseases have one specific host crop. Most occurring pests and diseases are also particular to the region in which the crop is cultivated. However, some of the major

ones – that also have the largest impact on yields – show a global presence. An example of such pests in maize are stalk borers, of which the European corn-borer (*Ostrinia nubilalis*), a moth with stalk-boring larvae, is most widespread. Stalk borers cause a lot of economic damage, as the damaged plant often can no longer produce cobs (Capinera, 2020; *Maize Pests and Diseases* | *The Encyclopedia of World Problems*, 2020). Aphids are also commonly seen in maize cultivation. The aphids themselves only cause insignificant damage, however these insects are often vectors of plant viruses, such as maize dwarf mosaic virus (MDMV). This virus leads to leaf chlorosis and necrotic lesions, which in turn causes delay in flowering and poor grain filling. Yield losses can rise up to 70% (Kannan et al., 2018). Other species causing significant damages and losses around the world are armyworms, rootworms, seed corn maggots and beetles, and maize cyst nematodes.

Aside from pests and viruses, many diseases caused by fungi also occur. Symptoms of infection can appear on the vegetative parts of the plant (leaves, roots, stalk), as well as on reproductive organs (flowers and eventually fruits). Except for severe infections, fungi on vegetative plant parts usually won't cause major economic losses. Northern corn leaf blight (caused by *Exserohilum turcicum*), eyespot (*Kabatiella zeae*) and common rust (*Puccinia sorghi*) are all leaf-level diseases that normally – under good management – will not cause significant losses (Czarnecka et al., 2022; *Maize Pests and Diseases* | *The Encyclopedia of World Problems*, 2020; Malvick, 2018; Wise, n.d.). Naturally, exceptions to this rule occur, often in the form of stalk diseases, and more particularly stalk rot. This disease can be caused by different pathogens, but shows the same main symptom: a hollow, rotted and weakened stalk, usually leading to wilting and/or lodging of the crop. Infection often starts in the roots but can also spread from the leaves or base of the plant. Wounds and insect cavities are most vulnerable to infection. The four most common stalk rot diseases are Anthracnose Stalk Rot, Gibberella Stalk Rot, Diplodia Stalk Rot, and Fusarium Stalk Rot, respectively caused by *Colletotrichum graminicola*, *Fusarium graminearum* (which used to be known as *Gibberella zeae*, hence the name), *Diplodia maydis*, and *Fusarium verticillioides* (Belisário et al., 2022; Identifying and Managing Stalk Rots, 2023; Jackson-Ziems et al., 2009).

Contrary to diseases on vegetative plant parts, those on reproductive organs are almost always detrimental to the harvest, and more often than not cause significant losses. Disease can strike during the flowering period, damaging tassels and silks, and thus later also the ears. There is also a possibility that the plant is infected in an earlier stage, but

only shows symptoms at the time of flowering, grain-setting or -filling. An example of such a disease is head smut, caused by the basidiomycete *Sporisorium reilianum*. This fungus often infects the maize plant at germination stage, through spores present in the soil, and grows unnoticeably within the plant until flowering. At that point, the inflorescence tissue gets deformed, forming smut galls instead of ears and kernels. After maturing, the galls burst and release teliospores into the environment, almost always ensuring the start of a new cycle, since the spores remain viable in soil for up to four years (Chemeltorit & Suresh, 2020; Jackson, n.d.). *Ustilago maydis* is a different smut fungus, causing common smut in maize. Similar to head smut, the fungus also grows tumour-like galls, filled with teliospores. The main difference between the two is that common smut only causes a local infection, meaning that an infection of the stem or leaves does not immediately lead to an affected ear. However, due to the high prevalence of the disease in Western-Europe, economical damages can quickly add up (Haesaert, 2022; Pataky & Snetselaar, 2006). Luckily, smut-affected maize can still be used as silage, salvaging a big part of the original profits. This is because smut fungi do not produce mycotoxins. However, the damage to the plants does provide an entrance for other toxigenic fungi, such as *Aspergillus* spp. and *Fusarium* spp., increasing the risk of mycotoxin contamination (Abbas et al., 2017).
One of the most common diseases affecting maize production globally, is Fusarium ear rot (FER), caused by *Fusarium verticillioides*. Elaboration on this disease, the fungus, and its pathologies can be found in part 2.3.3.

2.3 *Fusarium* spp.

Fusarium fungi are part of the Phylum of the Ascomycota, Order of the Hypocreales and Family of the Nectriaceae. Depending on the species, the fungi produce different types of spores: asexual micro- or macroconidia, sexually produced ascospores, and (asexual) chlamydospores as survival structures. The different species of the genus have an extensive range of host crops, both monocots and eudicots, many of which are of economic importance. The pathogen can infect different organs of the crop, such as roots, stems, leaves and heads, as well as various growth stages, like seedlings or flowering plants. The fungi spores can be distributed in various ways but are most often dispersed in the atmosphere by wind after drying. Dispersal through water – think rain or irrigation – is also common, while insect-mediated dispersal is less frequent (Askun, 2018; Bahadur, 2022; Karlsson et al., 2021; Rampersad, 2020).

The *Fusarium* genus is arguably one of the most researched fungal genera, due to its major global impact on both agriculture and human health. This is largely because of its toxigenic nature: nearly all of the *Fusarium*-species produce toxic secondary metabolites, known as mycotoxins, as a natural defence mechanism during stress or as an aid to the infection process. These toxins can be detrimental to animal and human health and are therefore strictly regulated. Consequently, much of a harvest can be lost due to the presence of small amounts of mycotoxins in the crop. It's no surprise that these species can therefore have a huge effect on food production and security, and are accordingly of great concern to both farmers and researchers worldwide (Askun, 2018; Summerell, 2019). Furthermore, multiple *Fusarium*-species can even directly – so not by mycotoxin intake – cause a fungal infection in humans, known as fusariosis. The rise of mortality in immunocompromised individuals as a result of this infection is another immediate reason for concern about the *Fusarium* fungi (Bansal et al., 2019).

2.3.1 Classification

Due to the global importance of the different fungi making up the *Fusarium*-genus, it is essential to be able to identify and classify a specific species correctly within the genus. However, due to the complex nature of many of these species, this has proven a difficult task. Ever since the discovery of different *Fusarium* species, confusion about the classification has been present: different publications used different classification methods, causing inconsistencies in research results between different researchers. It is no surprise that there was a strong need for clarification with the rising economic, scientific and medical impact of the fungi.

Before 1997, species were identified through their phenotypic characteristics, again causing many inaccuracies: estimates of the number of species in the genus ranged from 9 to 75 (based on morphological species recognition). However, much has changed with the rise of molecular identification techniques. In the past two decades, it has been determined that there are at least 300 phylogenetically different species in the genus, of which over 50% have not yet been formally described or named (Aoki et al., 2014; O'Donnell et al., 2018). Nowadays, a new taxonomy has been defined, classifying the known species in 23 phylogenetical species complexes, consisting of closely related, morphologically nearly indistinguishable sibling-species. Examples are *Fusarium fujikuroi* species complex (including *F. verticillioides*), the *Fusarium graminearum* species complex (including *F. graminearum*) and the *Fusarium oxysporum* species complex (including *F. oxysporum*) (Aoki et al., 2014; Summerell, 2019).

In addition to identification, the nomenclature of Fusarium species has presented a challenge as well. In order to address issues, the nomenclature system for fungi was revised in 2013, leading to the abandonment of the common double nomenclature for the asexual anamorph and sexual teleomorph stages. Likewise, preexisting double names had to be unified. For instance, *Fusarium graminearum*, which was frequently referred to as its teleomorph *Giberella zeae*, is now solely recognized by the former name to avoid confusion and ensure consistency (Aoki et al., 2014).

2.3.2 *Fusarium graminearum*

Fusarium graminearum is a hemibiotrophic fungal pathogen that infects a broad range of crops, but mainly grain cereals like wheat, barley (*Hordeum vulgare*), and maize. The pathogen thrives in a warm humid environment; however, mycelial growth can occur within a temperature range between 10-30°C (Shah et al., 2018). The fungus is a part of the *Fusarium graminearum* species complex (FGSC) and is considered the main causal agent in central Europe, North America, and Asia for *Fusarium* head blight (FHB) in wheat. *F. graminearum* surpasses in this way its sibling-species of the FGSC and other causal *Fusarium* species like *F. culmorum* or *F. poae* (Tan et al., 2020; van der Lee et al., 2015; Vaughan et al., 2016). Infection usually takes place at the anthesis stage of the wheat plants; however, the ears remain vulnerable until the soft dough age (Al Masri, 2018). The pathogen can infect the crop by both sexual ascospores and asexual macroconidia, the latter of which have a distinctive long, slender, and slightly curved shape, are multicellular (5-7 cells divided by septa) (Figure 4A), and can be formed in either mycelium or chlamydospores. The way the conidia reach the ears for infection is often through rain splash, while the sexually produced ascospores are forcefully expelled from the perithecia. These structures grow on crop residues from the year prior and are a way for the fungus – aside from chlamydospores – to overwinter on the field. The initial infection often stems from ascospores, while further spread of the disease is then facilitated by the conidia (Haesaert, 2021; Shah et al., 2018).

A  B 

Figure 4: A) macroconidia of Fusarium graminearum; B) Fusarium head blight symptoms in wheat (Al Masri, 2018; Cambaza, 2019)

The disease primarily manifests as bleached ears and spikelets, as seen in Figure 4B, which inevitably leads to shrunken or undeveloped kernels and diminishes the yield potential (Al Masri, 2018). In addition, an unmistakable symptom of the disease is the presence of small orange to brown spots on the spikelets. These are called sporodochia and contain many macroconidia spores. From these structures, hyphae can grow and gain access to the intercellular space, eventually growing to the rachis and rachilla, and thus colonizing the entire spikelet (Shah et al., 2018). However, the main danger of FHB is not the loss of yield, but rather the accumulation of mycotoxins in the kernels. *F. graminearum* and other causal species produce a broad spectrum of mycotoxins, including but not limited to zearalenone (ZEN) and trichothecenes such as deoxynivalenol (DON). ZEN is a nonsteroidal estrogenic mycotoxin, and mainly leads to reproductive disorders, and kidney and liver disease in mammals (Drakopoulos et al., 2020; Gupta et al., 2018), while DON, a sesquiterpenoid compound, mainly impairs gastro-intestinal and neural activity, and weakens the immune system by binding to ribosomes (Cope, 2018; Drakopoulos et al., 2020). According to a study by Chen et al. (2019), DON is the most detected mycotoxin in cereals globally.

Management and control of the disease currently entails crop rotation, the use of resistant varieties, adequate soil preparation (deep tillage), limiting irrigation around the anthesis period, and numerous other management practices to ensure that impact of the disease does not surpass economic thresholds (Alisaac & Mahlein, 2023). However, main prevention and treatment of FHB still relies on fungicides: triazoles of the fungicide group of demethylation inhibitors (DMIs), such as tebuconazole, metconazole, prothioconazole and benzimidazole, have been proven to be most effective against FHB in wheat, as well

as prochloraz, an imidazole derivate that is widely used in Europe. With these spray treatments, it is imperative that the fungicide be administered after the ears of the entire crop have emerged. Furthermore, empirical evidence shows that weather conditions that are beneficial to the pathogen are often suboptimal for fungicide spraying. These two factors ensure that the timing of a fungicidal treatment is challenging, often leading to varying and/or insufficient treatment results (Shah et al., 2018). It will likely be necessary to implement new techniques, considering the major possible impact of climate change on the incidence of FHB: rising temperatures will ensure that more environments become susceptible to severe outbreaks (Dweba et al., 2017). Besides, to meet the EU's required reduction of chemical treatments, it is crucial to look for alternatives. Without fungicidal treatment, crop losses due to FHB can reach up to 80%, a near-total destruction of the harvest. Lastly, resistance of *Fusarium* species to certain fungicides has already been reported, making it even more pressing to start implementing alternatives (Alisaac & Mahlein, 2023).

2.3.3 *Fusarium verticillioides*

Another important, emerging pathogen of the *Fusarium* genus is *Fusarium verticillioides* (formerly known as *F. moniliforme*). This fungus can infect different cereal crops, including wheat and rice, however, it is known to mainly affect maize. Its optimal growth temperature is 25°C, causing the fungus to mainly occur in warmer regions. The disease has been majorly reported in Southern Europe, the USA, South America, Australia and parts of Africa and Asia (Omotayo & Babalola, 2023; Pitt, 2014). Still, the pathogen is also present in cooler regions, seeing as growth can take place between 3-37°C (Pitt, 2014). As aforementioned, this species is the main causal agent for FER in maize, or more specifically for *F. verticillioides*: pink ear rot, after the pinkish colour of the mycelium. As the name implies, the disease causes the ears of maize to rot, drastically damaging the crop yield. The disease initially presents itself as white or light pink mould or a tan to brown discolouration on random, scattered kernels, as seen in Figure 5. The kernels occasionally also show a starburst pattern of discolouration, starting where the silk was attached (Lanubile et al., 2017; Tiru et al., 2022). Yet again, as with FHB, the main danger of FER is not yield-loss, but loss of quality and health endangerment due to mycotoxin contamination. *F. verticillioides* is one of the most prevalent species globally that produces fumonisins, a group of carcinogenic mycotoxins (Samsudin, 2015). More on this group follows in 2.3.3.1.

Figure 5: Initial symptoms of pink ear rot, caused by F. verticillioides, in maize ears (source: Oldenburg et al., 2017)

F. verticillioides is a soil-borne pathogen, present in the spermosphere or rhizosphere of the affected crop. In early stages of the plant development, infection can take place via two routes: either through the roots of the already developed plant, or through the germinating seed. The pathogen then often grows endophytically within the plant, waiting for optimal conditions to emerge and cause systemic and symptomatic diseases. In later stages of the growing season, the plant can get infected through wound sites in the stalk, leaves or kernels – more specifically the pericarp or the pedicel of a kernel. This happens through horizontal contamination: a primarily infected plant spreads the disease throughout the rest of the field, mainly via airborne dispersion, to plants that have suffered from insect herbivory or bird damage. The above-mentioned European corn-borer is a major promotor of *F. verticillioides* infections like these, as well as thrips and earthworms (Omotayo & Babalola, 2023; Samsudin, 2015). Additionally, infection can also occur during flowering through the emerging silks, however, this is less common for pink ear rot (Oldenburg et al., 2017). Contrary to *F. graminearum*, *F. verticillioides* produces both macro- and microconidia as asexual spores, instead of only macroconidia. Besides, perithecia play a much less significant role in the survival and disease development of the fungus (Sikhakolli et al., 2012). It is believed that mainly microconidia can facilitate an infection, due to their morphological adaptations to wind, rain, and vectored dispersal: the conidia are small, hyaline and mostly unicellular, and grow abundantly in long chains on infected plant tissue – either in debris in the soil or other infected plants on the field (Glenn, 2006).

Again, as with FHB, treatment of FER is not straightforward. The ideal approach entails a combination of good agricultural practices and other, more direct treatments. The agricultural practices again consist of crop rotation, crop residue management (to reduce sources of inoculum on the field), timely sowing and harvesting, use of resistant/tolerant varieties, adequate fertilization- and irrigation management, managing insect pests, … This last one is not to be missed, seeing as insect dispersal is one of the main routes of infection of maize kernels. In maize, the use of Bt maize (*Bacillus thuringiensis)* that is genetically engineered to produce proteins that are poisonous to insects, is a promising way to reduce *F. verticillioides* infections and its additional damage. However, this only works for insect-mediated dispersal, and resistance of certain pests – such as the corn stalk borer and its larvae – to the *B. thuringiensis* proteins has already been reported. Thus, Bt maize is obviously not a quick fix, however, combined with other practices, it has proven to show results (Madege et al., 2018; Omotayo & Babalola, 2023). However, good agricultural practices are not a foolproof way of treating the crop: it is very subjective to weather conditions of the season and cropping cycle. This is why it's usually combined with other treatments, most often of chemical nature. Fungicides like captan and fludionixil, in respective combinations with thiabendazole and metalaxyl-M – each time a sead treatment with a spraying agent – have been used, as well as triadimenol + tebuconazole. Still, these treatments also come with limitations: again, fungicides are subject to severe restrictions in the EU, *Fusarium* spp. are starting to show resistance to triazole fungicides, and additionally, multiple studies have shown that suboptimal concentrations of fungicidal treatments may actually increase mycotoxin production of *F. verticillioides*, even while growth is inhibited (Madege et al., 2018; Miguel et al., 2015). Alternatively, fungicides could be paired with insecticides like endosulfan for better results, to again prevent insect damage as a means of infection (Madege et al., 2018). However, this requires yet another chemical treatment, which is counterproductive to the objective of reducing chemicals in agriculture.

Other alternatives may be found in biological control. *Trichoderma* species are often seen as universal BCAs because of its mycoparasitism abilities and production of antibiotics and other antifungal metabolites (Tiru et al., 2022). A study by Ferrigo et al. (2014) showed that seed treatment with *T. harzanium* has great promise to control *F. verticillioides* kernel colonization and fumonisin accumulation in an eco-friendlier way. Furthermore, several strains of *Bacillus* spp. are able to produce chitinase and glucanase, which are extracellular cell-wall degrading enzymes. And plus, *B. amyloliquefaciens* can interfere with *F.*

verticillioides in the soil through the competitive exclusion principle: the bacteria outcompete the fungus for nutrients, while also producing toxic, pathogen-inhibiting compounds. Biocontrol potential with this species is also promising, either by coating the seeds or by inoculating the soil before sowing (Mita et al., 2022; Tiru et al., 2022). Other microorganisms, such as *Lactobacillus* spp., *Saccharomyces cerevisiae*, *Pseudomonas solanacearum*, and many others show great potential as BCAs against *Fusarium* spp. as well. Needless to say, biocontrol already presents a great alternative to traditional treatment methods, however, many BCAs have not yet proven their efficacity in the field, and some species – while protecting the plant from *F. verticillioides* – could still pose a threat to the health of the crop at some point in their life cycle (Nagaraj et al., 2021).

A novel strategy for the control of fungal pathogens, is based on the use of RNAi, or RNA interference. This protection strategy relies on the uptake of double-stranded RNA (dsRNA) by the fungus, which in turn can then silence essential or virulence genes. This leads to either the elimination of the fungus, or renders it essentially harmless to the plant (Šečić & Kogel, 2021b; Wytinck, Manchur, et al., 2020). The research conducted in this study will focus on the possible application of such techniques to control (the damage induced by) *F. verticillioides*. More on RNAi mechanisms will be explained in 2.4.

2.3.3.1 Fumonisins

The main mycotoxins produced by *F. verticillioides* are fumonisins, named after the fungus' obsolete name: *F. moniliforme* (Samsudin, 2015). The group of metabolites is divided into different sub-series according to their molecular structures, and are each assigned a letter (Fumonisin A, B, C, and P). Fumonisins of the B series (FBs) are the most naturally occurring, with fumonisin B1 (FB1) being the most toxically potent and most prevalent. FB2 and FB3 are also found in significant quantities in maize products but are less dangerous than FB1 (Dickman & Grollman, 2010; Pitt, 2014). Fumonisins have a linear C19 or C20 polyketide-derived backbone, an amine group, several hydroxyl side groups – the amount and positions depending on the type – and two tricarboxylic ester groups (Kamle et al., 2019). The B-series have a 20-carbon backbone and are structurally the same, but can be distinguished by their different hydroxylation patterns, as seen in
Figure 6. Fumonisin B1 (FB1) has a molecular formula of $C_{34}H_{59}NO_{15}$ and is a relatively heat stable and water-soluble molecule (Dickman & Grollman, 2010). In *Fusarium* spp., one gene cluster, known as the *FUM* gene cluster, is responsible for the biosynthesis of the mycotoxins. Within the clusters of the different toxin-producing species, a total of 21

different genes have been identified, known to encode the different enzymes involved in their biosynthetic pathway. The cluster of *F. verticillioides* contains 17 genes (J. Chen et al., 2020; Stępień et al., 2011).

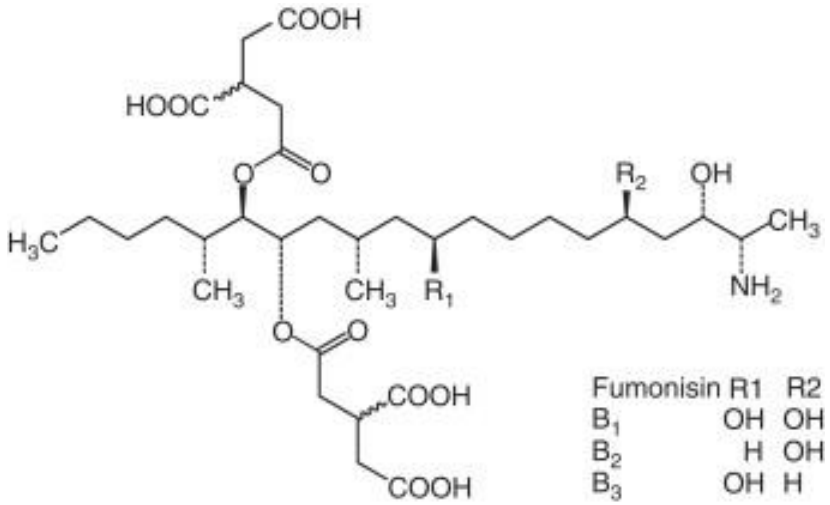


Figure 6: Chemical structures of fumonisins B1, B2 and B3 (Trucksess & Diaz-Amigo, 2011)

In 1970, it was discovered that isolates of *F. verticillioides* had been responsible for a field outbreak of equine leuko-encephalomalacia (ELEM) in South Africa. Later, cases of oesophageal cancer in humans could also be linked to this fungus. Yet it was only in 1988, 18 years later, discovered that these cases could be linked to naturally occurring mycotoxins of the fungus: fumonisins. It was later determined that not only *F. verticillioides* produced this type of toxin, but many of the *Fusarium* spp. were causal agents. It is now clear that at least 18 species are capable of producing this toxin, however *F. verticillioides* and *F. proliferatum* are the most prominent (J. Chen et al., 2020; Marasas, 2001). The mycotoxin causes damage to its host crop: contaminated cereal seeds display signs of endosperm degradation, in addition to the lack of a protein matrix surrounding the starch granules. This leads to a drastic reduction (75%) in radicle elongation during the germination process (Perincherry et al., 2019). Combination of these findings with the reported outbreaks of ELEM and oesophageal cancer led to the fact that fumonisins could be classified as non-host-specific toxins (nHSTs), meaning that they are not only toxic to the host plants of the toxin-producing pathogen, but to a broad range of species (J. Chen et al., 2020). Indeed, not only does the toxin adversely impact human and equine health, it is also proven to cause porcine pulmonary oedema (PPO) syndrome in pigs, acute poisoning in farm animals, and in addition shows carcinogenic, hepatotoxic and nephrotoxic properties in various rodent species (Mbundi et al., 2014). Furthermore,

oesophageal cancer is not the only risk following fumonisin uptake in humans. Effects of the toxin on neural tubes have been described: it is believed to cause neural tube defects in embryos when contaminated products are ingested during early pregnancy (Gelineau-van Waes, 2010). Naturally, regulations about the maximum tolerable daily intake were prescribed, both in food and feed. The limits for the EU are respectively 1 µg FBs/kg body weight per day and a maximum of 5 mg FBs/kg for complete feed for pigs (Terciolo et al., 2019).

The high toxicity of fumonisins stems from their structural analogy to sphinganine, the backbone precursor of sphingolipids. Some studies classify fumonisins – along with other groups of mycotoxins – as sphinganine-analogue mycotoxins, or SAMs. Due to the similarity, SAMs are able to competitively inhibit N-acetyltransferase – or ceramide synthase – and thus dysregulate the synthesis of ceramides and other complex sphingolipids. These lipids play essential structural and cellular roles in eukaryotic cells; disruption can lead to apoptosis in animals and programmed cell death in plants, and disruption of the cellular plasma membrane. Further, the competition with sphinganine and sphingosine also naturally leads to accumulation of both, which in turn disrupts cell signalling and functions, causing cellular dysfunction. These cellular dysfunctions/deaths are what cause the defects and diseases in both animals and humans earlier described (J. Chen et al., 2020; Du et al., 2008; Perincherry et al., 2019; Qu et al., 2022).

2.4 RNA interference

RNA interference (RNAi), also known as RNA silencing, is a post-transcriptional gene silencing (PTGS) mechanism. This mechanism is naturally present in a wide array of eukaryotic species, including plants, animals, as well as fungi. The machinery is triggered by the presence of dsRNA or siRNA (small interfering RNA). These RNAs can dysregulate translation, and thus inhibit protein synthesis, by mediating the destruction of mRNA (messenger RNA) (Gebremichael et al., 2021; Wytinck, Manchur, et al., 2020). RNAi is associated with several regulatory processes, like regulation of genome stability, gene expression, heterochromatin formation, control of transposon movement and protection against viral infections (Gebremichael et al., 2021; Šečić & Kogel, 2021).

The molecular mechanism behind RNAi was brought to light in 1998 by Fire et al. Along with this, the research uncovered that it was possible the utilize the naturally occurring RNAi machinery to an own means: in the study, they were able to manipulate gene

expression in the nematode *Caenorhabditis elegans* through the use of exogenous RNA. This study kicked off a new era in research about the types of RNA and their functions, and how these could be put to use. Since the paper was published, new types of functional, noncoding small RNAs (sRNAs) have been uncovered, the most significant being siRNA and miRNA (microRNA). These two show many similarities: both are small RNA molecules (respectively 21-23 and 19-25 nucleotides), abundant in plants, animals and viruses. They are however of different origins: miRNAs are synthesized from single-stranded RNA (ssRNA) precursor transcripts that become hairpin-structured, while siRNAs arise from dsRNA precursors. They also have a slightly different method of gene silencing: siRNA is highly specific and has only one mRNA target, which it fully matches, while miRNA has multiple targets and can impact mRNAs that are not fully complimentary (Dang et al., 2011; Lam et al., 2015). The following sections primarily concentrate on the siRNA pathway in fungi, as this aspect is most relevant to the research conducted in this thesis.

2.4.1 RNAi pathway in fungi

RNAi in fungi was first observed in the filamentous fungus *Neurospora crassa* in 1992, in a study conducted by Romano & Macino. They termed the gene silencing phenomenon as 'quelling', a natural process to eliminate and control transposons. The study was later uncovered to be the discovery of the RNAi-pathway in fungi. The core components of the pathway are the same for all eukaryotic organisms (see further), however some differences occur for example in RNAi-efficiency. These differences can be attributed to the efficiency of dsRNA uptake, intracellular distribution, and/or systemic dispersal of the RNAi molecules (Wytinck, Manchur, et al., 2020). Also, some species lack any RNAi machinery, such the corn smut fungus *Ustilago maydis*, or show difference in core elements, such as budding yeast *Saccharomyces cerevisiae*, which appears to lack RNA-dependent RNA polymerase (Dang et al., 2011; Machado et al., 2018).

In every species possessing an RNAi-machinery, there are 3 returning elements: Dicer, Argonaute (AGO), and RNA-dependent RNA polymerase (RdRP). As mentioned above, initiation typically requires dsRNA-presence in the cell. This dsRNA can have different origins, both endogenous (as a result of hybridization of complementary RNA transcripts, from ssRNAs that contain (near-)complementary inverted repeats and can fold back to form a hairpin structure, …) as well as exogenous (from viral dsRNA-intermediates, the application of formulated dsRNA, …) (Dang et al., 2011; Machado et al., 2018; Taning, Mezzetti, et al., 2021). The first step in the RNAi process involves the Dicer enzyme, an RNase III-enzyme. Dicer cleaves the long dsRNA into smaller strands of 20-25 base pairs (bp's), resulting in siRNAs. The double-stranded siRNAs have a 2-nucleotide overhang on

the 3'-end for both strands of the duplex (Machado et al., 2018; Paturi & Deshmukh, 2021). After this initiator step, the effector step takes place: the siRNAs are loaded into a multi-component protein complex, the RNA-induced silencing complex (RISC). This complex contains the Argonaute protein (AGO), which has an siRNA-binding domain as well as endo-nucleolytic activity for cleavage of target mRNAs. The RISC, also including GW182 protein and heat shock proteins 70 and 90 (Hsp70 and Hsp90), then guides the siRNA in a sequence-specific manner to the nearly perfectly complementary target mRNA. An siRNA-mRNA-complex is formed, causing the degradation of the mRNA or the inhibition of translation, both ultimately preventing protein synthesis (Machado et al., 2018; Taning, Mezzetti, et al., 2021; Wu et al., 2020). The actual gene silencing has now been carried out. In most fungi, RdRPs are another core element. These polymerases catalyse the replication of dsRNA from ssRNA transcripts, and thus generate new substrate for the Dicer protein to cleave and restart the gene silencing process (Pinzón et al., 2019).
In some fungi, the RNA-pathway has been extensively studied and thus described in detail. This is also the case for *F. graminearum*. In the FHB-pathogen, the RNAi-pathway plays no part in fungal growth, asexual conidia formation, abiotic stress response, or disease formation, however it regulates sexual perithecia development. The pathway consists of two Dicer enzymes (FgDicer1 and FgDicer2), two Argonaute enzymes (FgAgo1 and FgAgo2) and five RdRPs (FgRdRP1-5) (Machado et al., 2018)

2.4.2 RNAi-based pathogen control

Aside from being a naturally occurring machinery for post-translational gene silencing, RNAi can actually be used as an eco-friendly biocontrol mechanism. It has been proven possible to apply exogenous dsRNA in order to silence key genes in pathogenic organisms. Because of the target specificity of the dsRNA, typical risks associated with chemical treatments (off-target effects and environmental persistence) are diminished (Willow et al., 2021; Wytinck, Manchur, et al., 2020).

2.4.2.1 Host-induced gene silencing

RNAi molecules have the ability to transfer between organisms of different species, even of different kingdoms. Such transfers are frequently observed between filamentous fungi and host plants and animals: the sRNAs are a form of pathogen-host communication. This communication can go both ways, from pathogen to host or the other way around. For example, in fungal infections, some fungi (e.g., *Botrytis cinerea*) have been confirmed to produce siRNAs that are transferred to the plant host in order to occupy its RNAi machinery, down-regulate defence genes, and supress an inherent immune response.

Vice versa, in the pathogen *Verticillium dahliae*, host-derived sRNAs have been identified, targeting the pathogen's virulence genes to inhibit fungal invasion (Rampersad, 2020). In addition, studies have shown that RNA silencing signals in plants can extend beyond the producing cells and reach neighbouring cells through short-range transport (covering about 10-15 cells) as well as long-range transport (across the entire tissue). Plasmodesmata and plant phloem are respectively thought to facilitate these transports (Machado et al., 2018). Most of the time, the plant's immune system alone is not enough to hold off an infection. Keeping in mind that RNAi molecules can be transported both within host tissue as well as across the host-pathogen boundary, transgene host plants were created, with the goal of producing dsRNAs/siRNAs able to infiltrate fungal pathogens and silence endogenous fungal virulence genes. This biotechnological exploitation of RNA interference is called host-induced gene silencing (HIGS). The process of this gene silencing mechanism is visually shown in
Figure 8A (Koch & Wassenegger, 2021; Kuo & Falk, 2020).

The mechanism behind the transfer of siRNA between pathogen and host has not yet been fully clarified. It is hypothesized that the exchange is mediated by exosomes or extracellular vesicles, originating in intraluminal plant vesicles (Rutter & Innes, 2018). Evidence backing this hypothesis is found in the observation that exosomes of plant cells multiply during an infection process (Machado et al., 2018). Endocytosis could then possibly facilitate fungal uptake: in the necrotrophic fungus *Sclerotinia sclerotiorum* the clathrin-mediated endocytosis pathway has been observed during RNAi-based treatments (Šečić & Kogel, 2021; Wytinck et al., 2020). Figure 7 shows the movement of vesicles, that may contain dsRNA/siRNA, across plant-pathogen borders.

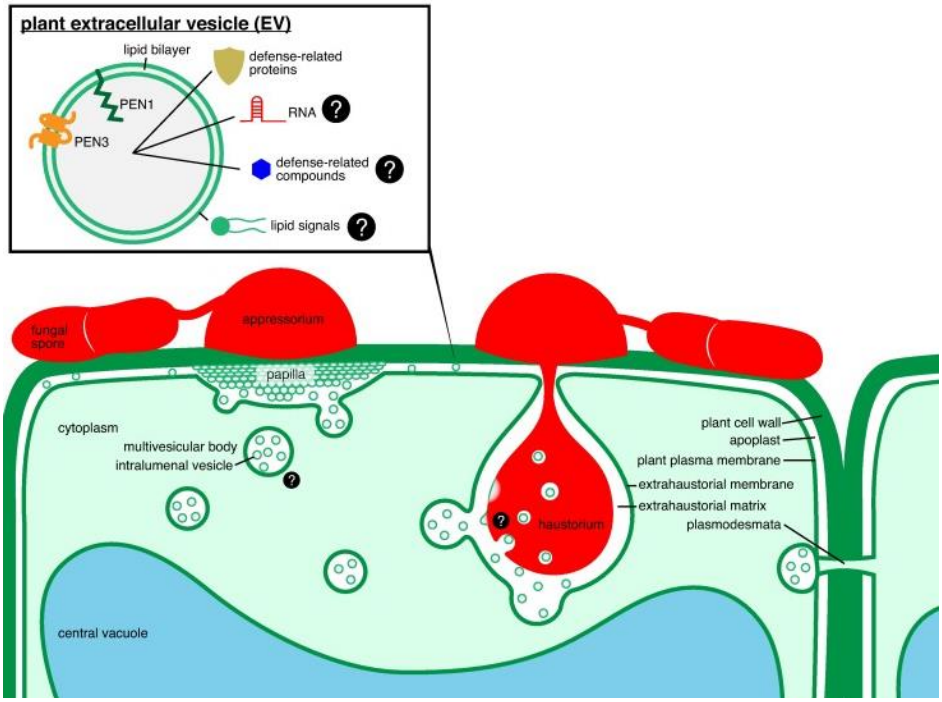


Figure 7: Extracellular vesicles, formed inside of intraluminal multivesicular bodies, accumulate at infection sites of the pathogen and enter the fungal haustoria through undiscovered mechanisms. The vesicles may contain defence-related proteins or -compounds, as well as RNA or lipid signals. Fungal cells may take up the vesicles by endocytosis or fusion with the fungal membrane. (Source: Rutter & Innes, 2018)

In 2010, HIGS was first demonstrated in filamentous fungi. Transgenic tobacco plants were used to disable β-glucuronidase (GUS) in *Fusarium verticillioides*. The tobacco plant was modified to produce hairpin RNA (hpRNA), in order to reduce the GUS-expression through the RNAi-machinery of the fungus itself. RNAi has also demonstrated specific gene expression inhibition potential in numerous other phytopathogenic filamentous fungi, including *Magnaporthe oryzae, Phytophthora sojae, Aspergillus nidulans, A. fumigatus, A. oryzae, Bipolaris oryzae, Colletotrichum lagenarium, Coprinus cinereus, Fusarium solan*i, and *Mucor circinelloides*. These fungi were transformed by introducing plasmid constructs that expressed self-complementary hairpin RNA molecules (Tinoco et al., 2010).

It can be concluded that HIGS could be a promising new method of plant-protection. However, due to the general scepticism regarding the genetically modified organisms (GMOs) necessary for the practical integration of this method, and the fact that not all plants are easily genetically transformed, research is now more focussed on exploring non-GMO based approaches using RNAi. The spotlight is now mainly on spray-induced gene silencing (SIGS), where the needed dsRNA is exogenously applied to the plant and pathogens (De Schutter et al., 2022).

2.4.2.2 Spray-induced gene silencing

After the topical application of dsRNA or siRNA to the plant's surface, there are two possible routes in which the RNA can reach and affect the fungal pathogen. Firstly, the sprayed-on dsRNA can be directly taken up by the pathogenic fungus, where the fungal RNAi-machinery is activated to cleave the dsRNA to siRNAs and degrade the targeted mRNAs. The other route is more indirect: the dsRNA is taken up by the host cells first, before being able to affect the pathogen. Either the dsRNA is cleaved by the plant's Dicer enzymes, after which siRNA is transferred to the fungal cells, or the dsRNA is passed along as is. An overview of the SIGS-method is visually represented in

Figure 8B (Wang & Jin, 2017). The transfer of dsRNA or siRNA from the host to the pathogen is subject to certain challenges, especially in the case of necrotrophic fungi. In case of severe damage to the plant cells, it could become impossible to transfer any vesicles to the pathogen – if this is at all the transfer method (see earlier) (Majumdar et al., 2017). Studies show that when the RNA is taken up by the plant, it is also effective in other (non-treated) tissue, thus showing that the dsRNA is able to translocate within the plant and silence genes of pathogenic tissue on other parts of the plant as well. In fungi, this systemic spread of RNAi molecules is facilitated by RdRP enzymes (Cagliari et al., 2019). SIGS is generally more accepted than HIGS, seeing as the pathogen's genes are silenced without the host genome being modified (Sang & Kim, 2020).

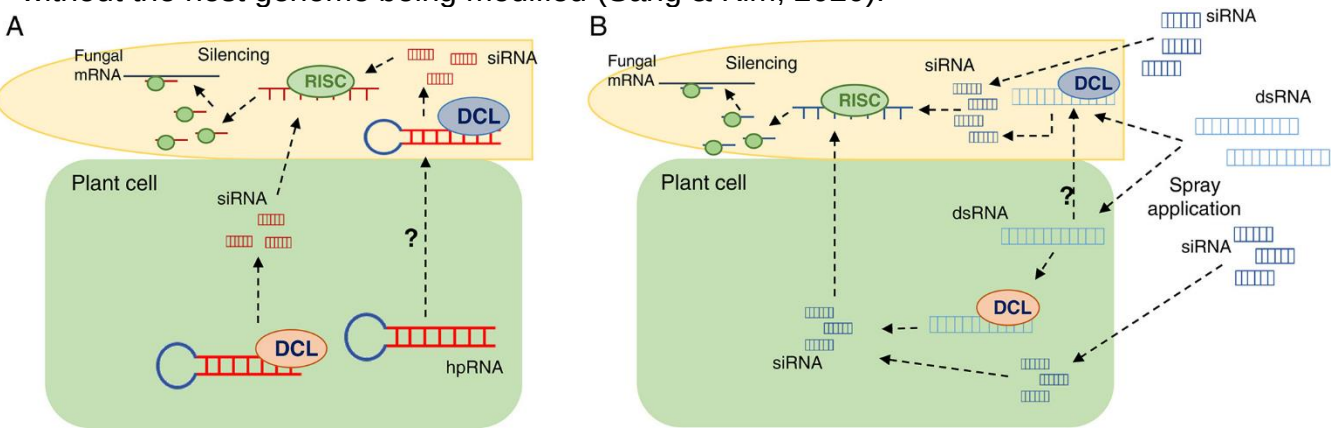


Figure 8: Illustration of RNAi-mediated gene silencing. A) HIGS: GM plants produce hpRNA/dsRNA to silence target genes in the fungal pathogen. Dicer-like proteins (DCL) in either host or pathogen cells cleave the dsRNA, after which the RNAi-machinery of the fungus binds the siRNA in the RISC, targeting pathogenic mRNA. B) SIGS: possible pathways of dsRNA/siRNA after topical application are either direct fungal uptake or transfer to the pathogen after passage through the plant cell and possibly its RNAi machinery. RNAi machinery also consists of DCL and RISC (Machado et al., 2018)

There have been several successful trials of SIGS with different fungal pathogens. In 2016, Wang et al. successfully reduced grey mould disease (*Botrytis cinerea*) in a variety of target fruits, flowers and vegetables by SIGS of *DCL1* and *DCL2*-genes. That same year, Koch et al. were able to inhibit biosynthesis of fungal ergosterol in *F. graminearum* on barley, resulting in inhibited fungal growth, through the usage of noncoding dsRNA (791 nt CYP3-dsRNA). The dsRNA targets the three fungal cytochrome P450 *lanosterol C-14α-demethylases CYP51A*, *CYP51B*, *CYP51C*, which are also the targets of azole fungicides. However, further field trials will most definitely be necessary before SIGS-products can become marketable.

2.4.2.3 Practical challenges

As with every new pest-control technique, certain challenges present themselves. The most challenging part of turning SIGS into a marketable biocontrol application has to do with either dsRNA-uptake efficiency, or with dsRNA degradation in the environment. Before entering the fungus, the dsRNA must pass the hyphal cell wall, which is composed of chitin, polysaccharides, and glycoproteins. Next, dsRNA has to migrate beyond the plasma membrane, which consists of lipids and protein/lipid heterogeneous domains. Lastly, fungal-produced nucleases could possibly also form an obstruction. There is an obvious difference in dsRNA-uptake efficiency between different species, however the mechanisms behind this have not yet been cleared up. Studies do show that both short RNA duplexes of 21 nucleotides (nt) long and longer dsRNA of up to 800 nt can enter fungal cells without obstruction (Gebremichael et al., 2021; Šečić & Kogel, 2021). Aside from uptake efficiency, dsRNA stability presents another challenge. Naked dsRNA shows a short period of stability in the environment, because of both nuclease activity and surface particle adsorption. Although this means that run-off is strongly reduced, this also ensures that efficient and rapid incorporation within the pathogen cells is necessary. Otherwise, RNAi efficiency is strongly diminished due to not having sufficient amounts of exogenous dsRNA in the cells (Gebremichael et al., 2021; Parker et al., 2019; Vurro et al., 2019). Thus, according to Taning et al. (2021), SIGS-formulations are generally designed for improving dsRNA stability, as well as ensuring effective delivery of dsRNA to pests or pathogens. The formulation of these products proves itself challenging and most of the times has to be evaluated case-by-case. Possible solutions to stabilize dsRNA in the field include the use of nanomaterials as carriers, such as “BioClay”, a layered double hydroxide clay nanosheet, or chitosan, a naturally occurring linear polysaccharide (Vurro et al., 2019)

Other potential risks, and an often-used counterargument for GMO's, is the chance of off-target effects. Even though the RNAi technology shows great selectivity, off-target gene silencing effects have not been studied enough to be completely ruled out. Especially because of the mismatch "forgivingness": it has been observed in human and plant cells that siRNAs may still silence genes, in spite of a slight sequence mismatch. Therefore, it is essential to search for possible off-target genes before and during dsRNA-design. Computational tools using sequence complementarity-based approaches, such as BLAST similarity searches against databases could assist in this process (Gebremichael et al., 2021; Taning, Gui, et al., 2021).
Despite the risks that come along with the SIGS-technology, its future as a new, environmentally friendly form of pest and disease control is promising. It is expected that RNAi-based products will reach the market in the form of sprayable products for foliar application, trunk injection, root dipping, or seed treatment. It is particularly the high sequence specificity of the method that makes it so interesting, especially as an alternative to often broad-spectrum chemical fungicides/pesticides. However, before the technique can be brought forward on the commercial market, legal frameworks for non-GMO RNAi-products as well as well-defined risk assessment procedures must be put into place (De Schutter et al., 2022)

2.4.2.4 Target genes

With the use of RNAi-based technologies comes the challenge of selecting suitable target genes. The downregulation of the genes should be able to reduce pathogenicity and disease progression of the targeted pathogen, without affecting genes in non-target organisms (Majumdar et al., 2017).

2.4.2.4.1 *CYP51*

Chosen target genes in fungi are often based on fungicidal targets. Azole fungicides are currently one of the most common control strategies against diseases caused by *F. graminearum*. These chemicals target the cytochrome P450 sterol 14α-demethylase encoded by *CYP51* genes. This enzyme catalyses sterol 14α-demethylation, an essential reaction in the ergosterol biosynthesis pathway in eukaryotes. Inhibition of the pathway causes depletion of ergosterol, resulting in growth restriction and cell death because of its interference with membrane integrity (Höfle et al., 2020; Lepesheva & Waterman, 2007). Despite being present in most eukaryotic organisms, the average nucleotide similarity between *CYP51* genes from different species is remarkably low, typically ranging from 25%

to 30%. This is why the *CYP51* genes prove valuable targets, not only for fungicides, but also for gene silencing methods (Machado et al., 2018).

As aforementioned, Koch et al. (2016) have been able to successfully inhibit *F. graminearum* growth in barley through silencing of the three *CYP51* genes (*FgCYP51A*, *FgCYP51B* and *FgCYP51C*) by spraying a 791 nt dsRNA (CYP3RNA) on the plants. These results could pose an example for further studies involving HIGS or SIGS in other fungi and *Fusarium* species.

2.4.2.4.2 *Chs3b*

A main difference in fungi cells compared to others involves the cell wall. The most important components of fungal cell walls are chitin microfibrils, which are absent in cell walls of plants and mammals. These microfibrils are of major importance to the integrity and shape of the cells (Ren et al., 2022). This makes genes coding for the chitin biosynthesis ideal targets for antifungal agents, including HIGS and SIGS. A study by Cheng et al. (2015) used the former technique in wheat to attempt silencing the enzyme chitin synthase of *F. graminearum*. This enzyme catalyses chitin formation and translocation across the membrane, and is encoded in 8 *Chs* genes (*Chs1*, *Chs2*, *Chs3a*, *Chs3b*, *Chs4*, *Chs5*, *Chs6*, and *Chs7*). Through the use of knockout mutant strains, the study determined that the *Chs3b* gene is most essential for the survival of the pathogen and was thus the best target for gene silencing. They generated five RNAi constructs to match the 2718 bp long *Chs3b*-sequence. The co-expression of 3 of these constructs in wheat resulted in significant resistance to *F. graminearum* infection. Again, these results could be used as a guideline for PTGS in *F. verticillioides*. It is unknown whether the same *Chs* gene would result in lethality in both these fungi; knockout strains of *F. verticillioides* could possibly offer more insight.

2.4.2.4.3 *FUM*

Studies concerning RNAi in *F. verticillioides* are scarce; much more research has been conducted on *F. graminearum*. In 2018, Johnson et al. were one of the first research groups to attempt PTGS in the FER-pathogen, paving the way for further research into HIGS or SIGS. They used plasmid constructs to transform the pathogen and silence the first two genes in the fumonisin biosynthesis pathway (*FUM1* and *FUM8*, respectively encoding polyketide synthase and α-oxoamine synthase). In several fungal isolates, RNAi was able to successfully reduce the amount of fumonisin B1 produced by the fungus. These results

suggest that in the future, HIGS or SIGS could possibly be implemented in maize production. However, reducing fumonisin production does not necessarily mean a reduction of disease symptoms and crop damage, so if implemented, this method would still have to be combined with other fungal agents, which is not ideal.

2.4.2.4.4 *eGFP*

The target gene for PTGS used in this thesis is *eGFP*, short for enhanced green fluorescent protein. Green fluorescent protein (GFP), initially isolated from the jellyfish *Aequorea victoria*, has become widely used as a reporter for localization and monitoring gene expression. In eukaryotic cells, GFP expression results in green fluorescence when exposed to blue or (near-)ultraviolet light. To enhance its fluorescence intensity, the improved variant eGFP was developed, which is expressed and folded differently, ensuring a brighter signal (Werbrouck, 2021). For this study, the *F. graminearum* strain (PH-1) used is genetically marked with eGFP and serves as a control for PTGS.

The focus in this dissertation is on reducing virulence and pathogenicity of *F. verticillioides* infections on maize crops through the use of SIGS. For this, dsRNA against *eGFP*-genes was applied to *F. graminearum* containing an eGFP tag, as a way to deliver a proof of concept of RNAi in *Fusarium* species. *F. graminearum* was used because the fungus has been widely studied, and RNAi has already been found to work on this pathogen.

3. METHODOLOGY

3.1 Species and sampling

3.1.1 Spore suspension

The *F. graminearum* isolate used for the conducted research is a PH-1 reference strain, marked with an eGFP-tag (Tan et al., 2020). The fungus was inoculated on solid potato dextrose agar (PDA) (39 g/L) plates and placed under a UV-blacklight regimen of 12h of UV-light and 12h of darkness for at least one week, in order to promote fungal sporulation. The conidia were then harvested using sterile PBS (phosphate buffered saline) + 0.01% Tween80, and then filtered through 2 layers of Miracloth to remove any mycelium from the spore solution. The spores were then counted under a microscope in a Burker haemocytometer and diluted to a final concentration of 1×10^7 conidia/mL.

3.1.2 Protoplast production

For the RNAi experiments, protoplasts of *F. graminearum* were needed. To produce these protoplasts, shake flasks of 100 mL potato dextrose broth (PDB) (24g/L) were inoculated with spores of 1×10^7/mL concentration. After 12-16h of incubation in the orbital shaker, young mycelia were harvested in a Whatman cellulose filter and washed with sterile H_2O. The mycelia were then incubated with a 'protoplasting mix' in a rotary shaker at 30°C and 100 rpm for 4h. This mix consists of 25 g/L driselase, 0.05 g/L chitinase, 5 g/L lysing enzyme of *Trichoderma harzianum* and 0.8 M KCL (20 mL). The resulting protoplasts were then separated from any remaining mycelia through 4 layers of Miracloth, centrifuged at 1500g for 10min, and resuspended in 0.5 mL STC-buffer (consisting of 1.2 M sorbitol, 50 mM CaCl and 10 mM Tris/HCl pH8). The protoplasts were stored on ice or immediately used for further experiments. The concentration was calculated with a Burker haemocytometer, and the solution was accordingly diluted to 2×10^7 protoplasts/mL.

3.2 dsRNA-synthesis and -labelling

To silence the eGFP-signal of the used *F. graminearum* strain, dsRNA was synthesized out of cDNA of the eGFP-sequence. This cDNA (complementary DNA) is the result of a PCR-amplification with two T7-primers. The PCR reaction mix (20 µL) contains 2 µL 10x PCR-buffer, 0.6 µL $MgCl_2$ (50 mM), 0.6 µL dNTP mix (10 mM), 0.6 µL of the forward primer (10 mM) and 0.6 µL of the reverse primer (10 mM), 0.9 µL eGFP-DNA template (± 100 ng)

and 14.5 µL nuclease-free water. Lastly, 0.2 µL Taq polymerase was added, and the PCR-reaction was initiated. The program comprised 3min at 94°C, 39 times (45sec at 94°C, 30sec at 58°C, 45sec 72°C), and 10min at 72°C. A clean-up and purification of the resulting PCR-products was performed by use of the Wizard SV gel and PCR clean-up kit® (Promega, Madison, US), following the included protocol. The two resulting DNA-samples were then visualised through gel electrophoresis: the fragments were loaded on 1.5% agarose gel at 135V and stained with ethidium-bromide. The concentration of the DNA was measured using a Denovix Nanodrop spectrophotometer. The resulting DNA-concentrations can be found in supplement I.

The cDNA was then used as a template for the synthesis of the needed dsRNA. For this, the MEGAscript™ RNAi Kit (Thermo Fisher Scientific, Waltham, U.S.A.) was used. The transcription reaction assembly, RNA annealing reaction, nuclease digestion and purification of the dsRNA were carried out according to the protocol provided by the producer. After each reaction (transcription, annealing and clean-up), a 10X diluted sample of the dsRNA was subjected to gel-electrophoresis, as a control method in case of failure, and to visually confirm the correct fragment size. The concentration of the final product was then again measured using the spectrophotometer. Visualization and the final dsRNA-concentration are listed in supplement I.

Before usage in the experimental setups, the dsRNA was fluorescently labelled for microscopic localisation using the Mirus *Label* IT® siRNA Tracker™ Intracellular Localization Kit, Cy®5. The Cy5 fluorophore is excited at 649 nm and has an emission wavelength of 670 nm. Under a fluorescence microscope, the labelled dsRNA will be visualised in an orange to red colour. The labelling was carried out according to the Mirus-protocol. No adjustments were made for working with dsRNA instead of siRNA, only the siRNA dilution buffer for suspension of the RNA was replaced by nuclease-free water.

3.3 *In vitro* dsRNA assay

The experiments executed in this study were centred around confirming and/or facilitating dsRNA-uptake of *F. graminearum* cells to induce RNAi. Earlier experiments conducted by Dangreau (2022) confirmed that neither the fungal spores nor mycelial protoplasts could take up dsRNA/siRNA without an assisting carrier. Therefore, these experiments now focus on using nanoparticles to deliver the dsRNA into the targeted cells. The chosen carrier for

this study is Lipofectamine™ 3000 Transfection Reagent, a solution of lipid nanoparticles produced by Invitrogen (Thermo Fisher Scientific, Waltham, USA).

The produced *F. graminearum* protoplasts were resuspended in PBS or 10X diluted PDB (1/10 PDB), diluting them to a concentration of 1×10^7 protoplasts/mL. The concentration of the labelled dsRNA was measured, after which an amount according to 5 µg (volume depending on the dsRNA-concentration) was combined with the reagents of the Lipofectamine™ 3000 Transfection Reagent kit at a 1:2:3 ratio (5 µg dsRNA : 10 µL P300 : 15 µL lipofectamine™ 3000 Reagent) and incubated for 5min at room temperature. This mixture was then added to both the protoplasts in PBS and in 1/10 PDB. Positive and negative controls were also installed. The positive controls consisted of protoplasts in both PBS and 1/10 PDB without dsRNA added, and the negative control consisted of only dsRNA without any fungal cells added. After the first experiment, this negative control was abolished, to avoid wasting dsRNA and labelling reagents. The treatments were then incubated in the dark at room temperature for 24h, before being inoculated on 3 or 6 small petri-dishes of both PDA and 10X diluted PDA (1/10 PDA). The experimental setup is visually represented in supplement II. The dishes were then incubated at 25°C to enhance growth of the protoplasts. The treatments were monitored daily for several days, until the fungus had fully overgrown the dish (at 72h or 96h). The monitoring consisted of taking pictures every 24h with a Wiwam automated multispectral phenotyping robot (PathoViewer), present in the lab. This robot can capture RGB values, chlorophyll fluorescence (Chl), and GFP fluorescence at a high spatial and temporal resolution. For the in-vivo experiment monitoring, the corrected GFP-value was of importance. The images were later processed using CropReporter software.

The same protocol was repeated several times with slight alterations. Instead of dsRNA, four different siRNAs were also used, each tested separately as well as combined together. These treatments were only plated on regular PDA and not diluted PDA. The concentration for the siRNAs was 25ng/µL. These siRNAs were designed by Dangreau (2022) and ordered through Integrated DNA Technologies (Integrated DNA Technologies, Coralville, USA). The properties of the siRNAs are shown in Table 1.

Table 1: Properties of siRNAs chosen out of the dsRNA eGFP strand.

Name	Sequence	Base pairs	Molar mass (g/mol)
siRNA eGFP 1	5'-GAACGGCAUCAAGGUGAACUU-3' 3'-CUUGCCGUAGUUCCACUUGAA-5'	21	13,369
siRNA eGFP 2	5'-GGCACAAGCUGGAGUACAACU-3' 3'-CCGUGUUCGACCUCAUGUUGA-5'	21	13,384
siRNA eGFP 3	5'-AAGCAGCACGACUUCUUCAAG-3' 3'-UUCGUCGUGCUGAAGAAGUUC-5'	21	13,369
siRNA eGFP 4	5'-AACGGCCACAAGUUCAGCGUG-3' 3'-UUGCCGGUGUUCAAGUCGCAC-5'	21	13,399

Lastly, to confirm the entry of dsRNA in the protoplasts, the incubated samples of protoplasts + labelled dsRNA were visualised under a fluorescence microscope, as the PathoViewer is unable to detect the Cy5-signal of the labelled dsRNA. The samples were compared to control samples of only protoplasts, to confirm both entry of the dsRNA and the functioning of the RNAi-machinery. See also supplement II.

3.4 Detached leaf siRNA assay

The chosen siRNAs were also tested for SIGS in wheat. For this, six leaf pieces of 10-days old wheat were detached and transferred to a square petri-dish containing 0.5% water-agar + Benzimidazole (40mg/L). The siRNAs were mixed and diluted to a concentration of 25ng/µL, and, using a spray flask, the leaves were evenly sprayed (3-4 puffs). To allow some time for siRNA-uptake, the sprayed leaves were incubated for 48h at room temperature before wounding them with a scalpel and inoculating with 10µL of the *F. graminearum* protoplast suspension (1×10^6 protoplasts/mL). A positive control assay was also installed, consisting of six leaf pieces sprayed with sterile H_2O, before being inoculated with the protoplast suspension. The setup is visualised in supplement IV. The treatments were monitored using the multispectral phenotyping robot (PathoViewer). A standard software protocol for detached leaf assays in wheat was used, monitoring the GFP-signal of the fungus. The images were processed using CropReporter software.

The visualisations of the experimental setups (see supplement II, III and IV) were created using BioRender.com.

4. RESULTS

4.1 Microscopic assay

In order to confirm or disprove the hypothesized uptake of dsRNA within fungal cells, protoplasts of *F. graminearum* were treated with labelled dsRNA and subjected to fluorescence microscopy. For this, the dsRNA was fluorescently labelled using a Cy®5 fluorophore as a means to visualize the dsRNA under the microscope. The fluorophore binds to the RNA and ensures that visualization will show an orange to red signal where dsRNA is present. This way, it could either be confirmed or disproved that dsRNA enters the fungal protoplasts. The same sample setup as the further *in vitro* trials was used: 1×10^7 protoplasts/mL complexed to nanocarriers in a 1:2:3 ratio (dsRNA, P3000 reagent and Lipofectamine particles respectively).

Figure 9 shows the results of the samples that were incubated in PBS-medium. The first thing to be noticed is that there is some fungal tissue still present in the samples (see supplement V), raising the concern that the Miracloth filters to separate the produced protoplasts from the remaining mycelia (during protoplast production) are not enough to ensure complete obstruction of mycelia. Aside from that, the protoplasts and fungal tissue in the PBS medium show an obvious uptake of dsRNA within the cells, and as there is also a clear GFP-signal being emitted from the fungal cells, it is evident that there has been little to no gene silencing. This proves that dsRNA-uptake does not necessarily lead to gene silencing, and raises the question that perhaps the lack of GFP-reduction is not because of inhibited passing through the cell membrane, but rather due to lack of incorporation of the dsRNA within the RNAi-machinery.

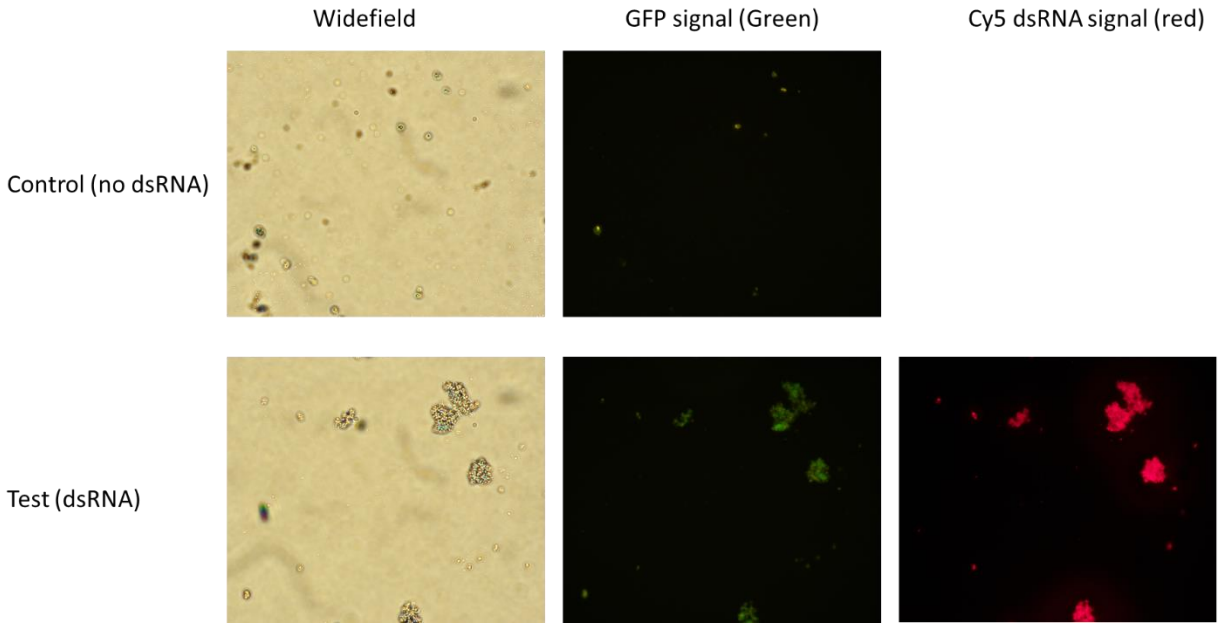


Figure 9: Microscopic images of protoplasts (1 × 10^7 protoplasts/mL) incubated in PBS (control sample) and protoplasts (1 × 10^7 protoplasts/mL) treated with dsRNA + P3000 reagent + Lipofectamine nanoparticles in 1:2:3 ratio in PBS (test sample). Samples were incubated at room temperature for 24h before microscopy.

Contrary to the PBS-incubated samples, the samples in 10X diluted PDB (Figure 10) show no red dsRNA-signal, neither in the protoplasts nor anywhere in the sample. This could possibly indicate high nuclease presence in this particular sample, causing degradation of the dsRNA. Meanwhile, the protoplasts are visible and do still emit GFP-signal, proving that there are protoplasts present, and the GFP-production was not silenced. The possible nuclease contamination does cause an inconclusive result, as it is not clear whether the protoplasts can take up the dsRNA or not. However, combined with the result found in the PBS-medium, the probable conclusion is that the protoplasts would be able to absorb the dsRNA in the absence of any nucleic acid degrading enzymes and with the help of a lipid nanocarrier. Yet, the dsRNA does not seem to be able to silence GFP-signalling.

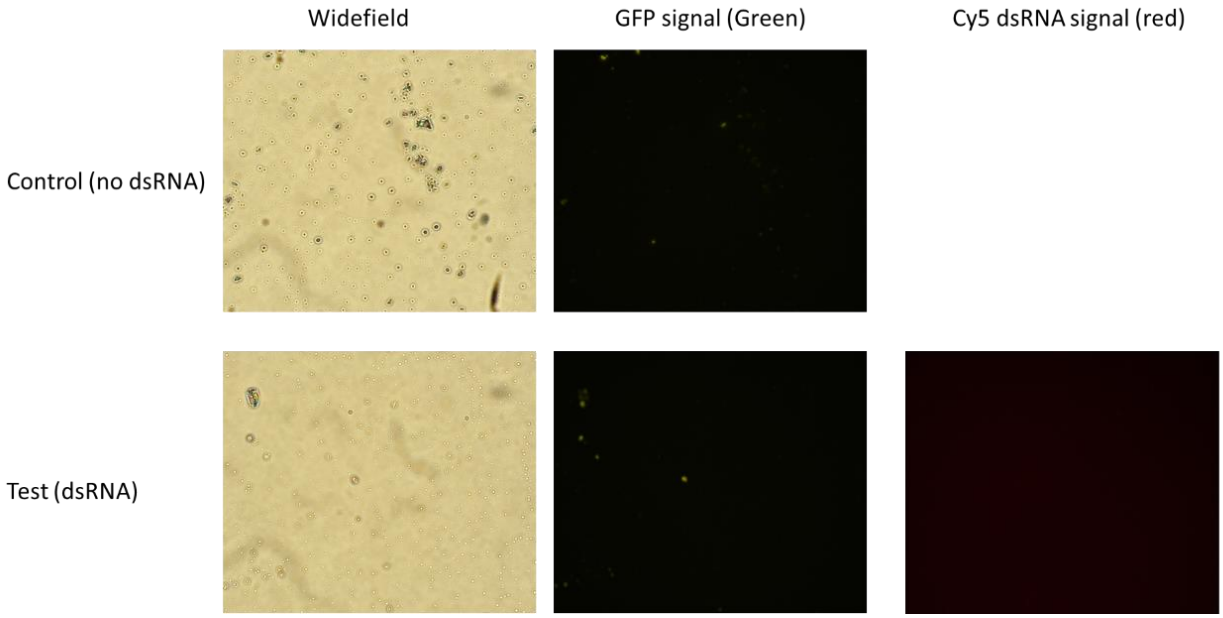


Figure 10: Microscopic images of protoplasts (1 × 10^7 protoplasts/mL) incubated in 10X diluted PDB (control sample) and protoplasts (1 × 10^7 protoplasts/mL) treated with dsRNA + P3000 reagent + Lipofectamine nanoparticles in 1:2:3 ratio in 10X diluted PDB (test sample). Samples were incubated at room temperature for 24h before microscopy.

4.2 *In vitro* dsRNA assay

The goal of the experimental setup in this study was to deliver a proof of concept of post-transcriptional gene silencing in an eGFP-transformed *F. graminearum* strain (PH-1), by diminishing or completely reducing the emitted GFP-signal through RNAi. An experimental setup, based on results of Dangreau (2022), was established: 1 × 10^7 protoplasts were resuspended in either PBS or 10X diluted PDB (1/10 PDB), and inoculated on petri dishes of both PDA and 10X diluted PDA (1/10 PDA). These setups were monitored for either 3 or 4 days post inoculation (dpi), depending on the growth rate of the protoplasts. Along with the experimental treatments, positive and negative controls were installed, respectively consisting of protoplasts suspended in both PBS and 1/10 PDB, inoculated on PDA and 1/10 PDA, and dsRNA suspended in PBS and 1/10 PDB, inoculated on PDA and 1/10 PDA. The positive controls are expected to show normal growth and GFP-signalling, while the negative controls are supposed to show no growth and thus no GFP-signalling. The GFP-values of the latter could then be used as a corrective factor for background signalling. However, not all of the experiments included a negative control group. The GFP-signals of these setups were corrected by using the GFP-value at timepoint 0: seeing as there is no growth yet, and thus no GFP-signal from the fungus, these signals should also represent

the background noise. One of these corrections was used for the data in each graph in this section.

Due to the small scale of the experiments (3 or 6 replicates), the conditions for a parametric analysis were not met (normality or homogeneity of variances). Thus, statistical data-analysis for this study consisted of a non-parametric test followed by a post-hoc pairwise comparison when the results showed significance. A Kruskal-Wallis test was used followed by a post-hoc Dunn's test, this for each timepoint separately. For each test, a significance level of p=0.05 was applied.

4.2.1 Use of long dsRNA

For the 2 first trials, the synthesized dsRNA was used in combination with Lipofectamine nanoparticles (abbreviated to np in the datasets). Due to a monitoring error during the first experiment, data from 2-3 dpi could not be used for statistical analysis and are thus not included in the graphical representations. The experiment was therefore inconclusive about the effect of the exogenously applied dsRNA to the GFP-signal of the fungus on these days. This was however not a wasted experiment, as the data showed that there was no growth on the negative control plates (dsRNA in PBS and dsRNA in 1/10 PDB), both for PDA and 1/10 PDA, and that there was no visible effect of the dsRNA on the GFP-signal after 96h of incubation.

Figure 11A shows a graphical representation of the cGFP-values for timepoints 0h, 24h and 96h of the different treatments (suspended in both PBS and 1/10 PDB) inoculated on solid PDA. The values were corrected for background noise using their respective negative control values (dsRNA in PBS or dsRNA in 1/10 PDB) at 0h. At 0h and 24h, there was very little signal because the protoplasts had not yet started growing or had grown very little. At 96h, no significant differences ($p<0.05$) were found for the cGFP-signal of the treatments containing protoplasts, while both negative controls showed little to no cGFP-signal, significantly differing from the dsRNA + np-treatments and positive controls. Figure 11B represents the same experiment but inoculated on 10X diluted solid PDA. 0h and 24h again show little to no signal due to no growth, and again at 96h, the treatments containing protoplasts + dsRNA + nanoparticles show no significant differences to the positive controls, while the negative controls clearly show no growth or signal. The positive control in PBS shows very little signalling, this is due to the fact that only one of the three duplicate petri dishes showed fungal growth. This fact, in combination with the presence of large

error bars (representing standard deviation), led to the installation of 3 more duplicates for each treatment in later experiments.

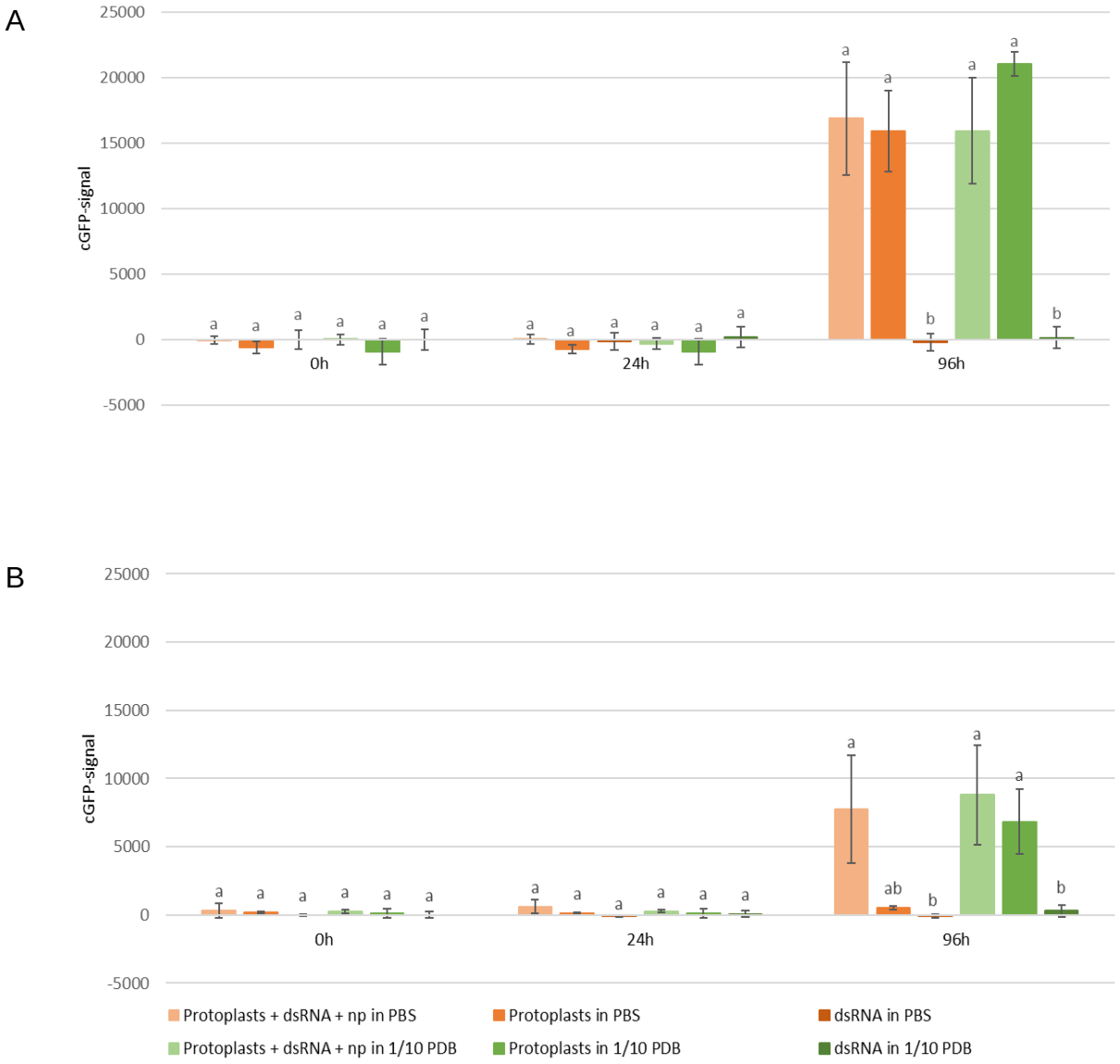


Figure 11: Graphical representation of the cGFP signal of Fusarium graminearum protoplasts treated with dsRNA and nanoparticles. Treatments contain 1 × 10^7 protoplasts/mL and a 1:2:3 ratio of dsRNA, P300-reagent and Lipofectamine 3000. A) Treatments and controls inoculated on small petri dishes of solid PDA, and B) treatments and controls inoculated on small petri dishes of 10X diluted PDA. cGFP-values were measured every 24h for 96 hours using multispectral imaging. The error bars represent the standard deviation. Each timepoint was analysed separately using the Kruskal-Wallis test as a non-parametric test, followed by Dunn's test for pairwise comparisons. The significance level is p = 0.05. The statistical analyses were performed in R version 4.1.3.

Even though the first trial showed no significant GFP-reduction at 96h of incubation, it is possible that there had been a transient effect of the dsRNA at timepoints 48h or 72h. Therefore, the experimental trial was repeated, yet with slight alterations (6 replicates of each treatment and no negative controls as they had been proven to be redundant in the last trial).

Figure 12 shows the cGFP-values of the second experiment, having used the same methods as experiment 1: 1×10^7 protoplasts/mL and 5µg dsRNA with 10µL P3000 reaction agent and 15µL Lipofectamine transfection nanoparticles, suspended in PBS or 1/10 PDB, inoculated on PDA (Figure 12A) or 1/10 PDA (Figure 12B). The experimental setups were monitored each 24h for 4 days (96h). Figure 12A, representing the treatments inoculated on PDA, shows again that there is very little GFP-signal present at 24h, however, at 48h, the protoplasts + dsRNA + np treatment shows a significantly lower cGFP-value than the other treatment and controls, indicating that the dsRNA inhibited the GFP production of the fungal cells. However, as all of the signals at this point are quite low, this effect could also possibly occur because of a slower growth initiation. The effect is transient, as the treatment shows higher cGFP-value than its control again at 72h (however not significantly). This could possibly be attributed to a compensatory effect. The treatment of protoplasts + dsRNA + np in 1/10 PDB also shows a significantly higher value than both controls at 72h, a result that goes against the expectations. By 96h of monitoring, none of the values show any significant differences, indicating that the effect at 48h was indeed transient.

Figure 12B shows cGFP-values of the treatments and controls inoculated on 1/10 PDA. Visually, it was clear that the protoplasts did not show much growth on this medium, hence the low values of cGFP at each timepoint (never higher than 5000). Some significant differences can be observed: at 24h, the protoplasts + dsRNA + np in 1/10 PDB showed a significantly higher cGFP-value than its control (protoplasts in 1/10 PDB) and the protoplasts + dsRNA + np treatment in PBS, a result that goes against the expectations. At 48h, 72h and 96h, the results are the same: the protoplasts + dsRNA + np treatment in PBS and the untreated protoplasts in 1/10 PDB show significantly lower cGFP-values than the protoplasts in PBS. Also, none of the values show much change during this interval of time, which is confirmation of the stunted growth of the protoplasts on this medium. Significant differences in cGFP-values can thus possibly be attributed to differences in growth initiation, followed by an almost complete stunt of the growth. Because of the

incoherent results on 1/10 PDA, both in experiment 1 and 2, it was decided not to use 1/10 PDA plates again for the subsequent experiment.

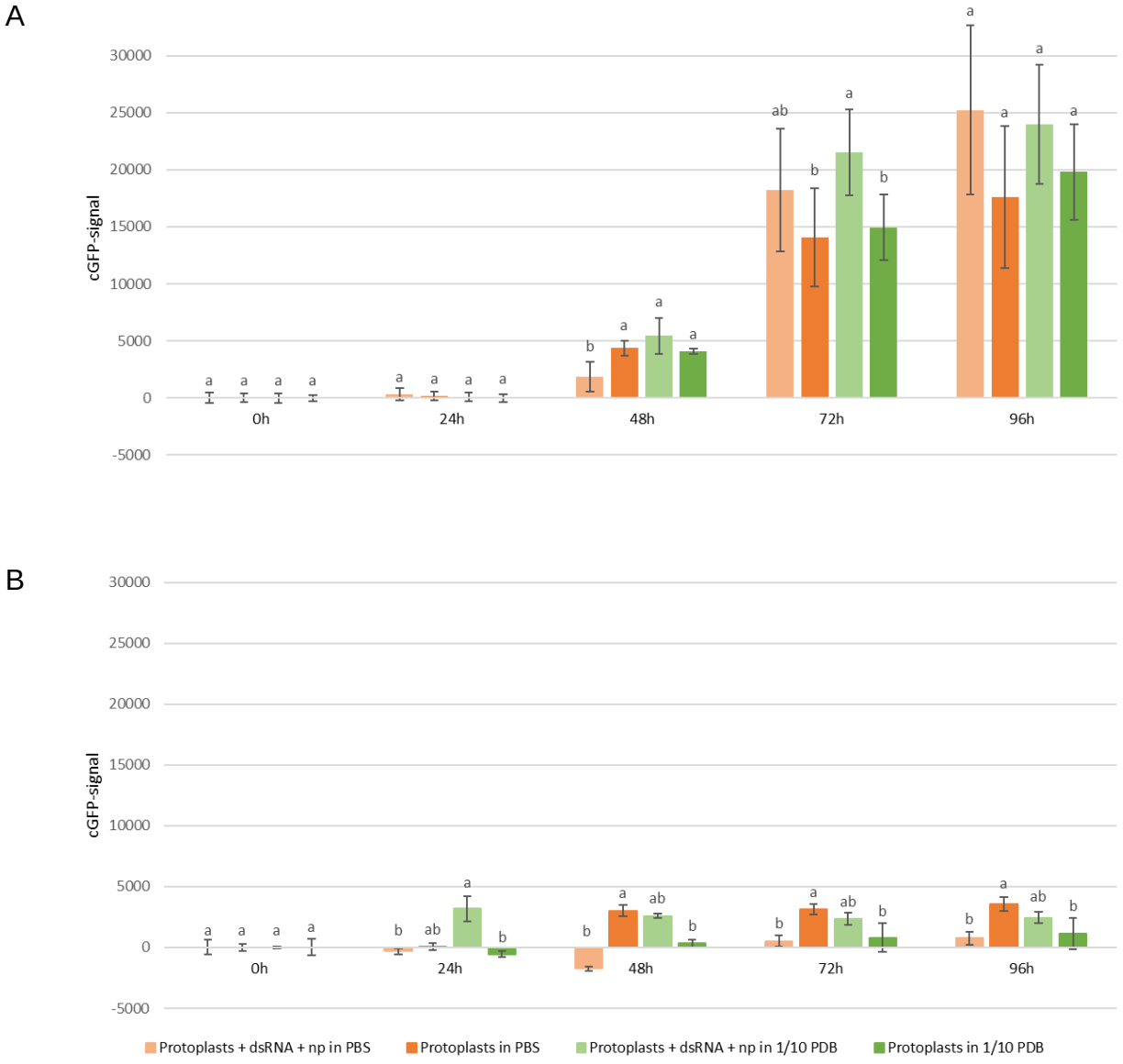


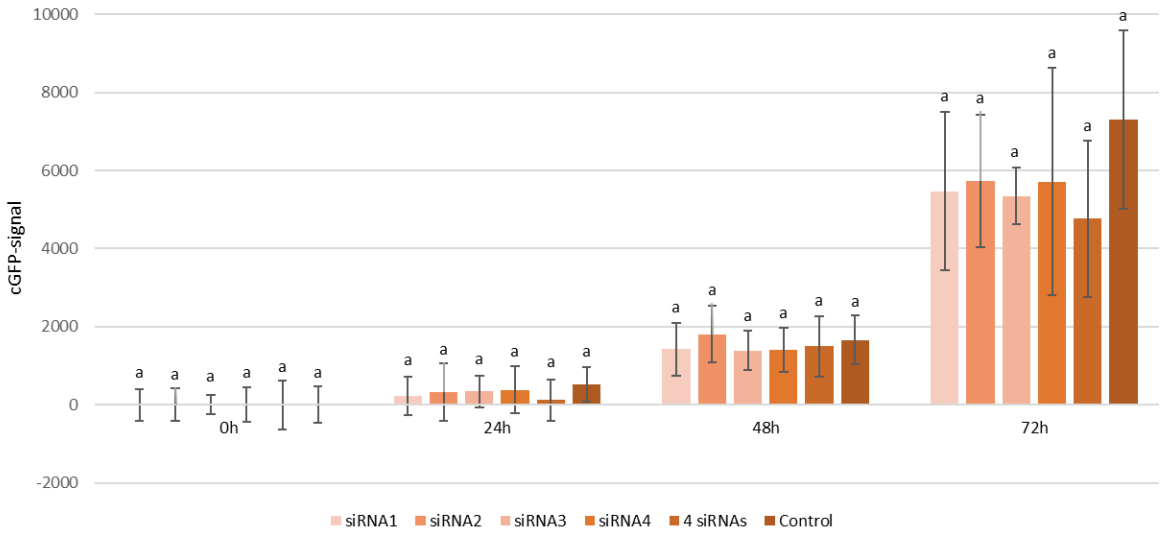
Figure 12: Graphical representation of the cGFP signal of Fusarium graminearum protoplasts treated with dsRNA and nanoparticles. Treatments contain 1 × 10^7 protoplasts/mL and a 1:2:3 ratio of dsRNA, P300-reagent and Lipofectamine 3000. A) Treatments and controls inoculated on small petri dishes of solid PDA, and B) treatments and controls inoculated on small petri dishes of 10X diluted PDA. cGFP-values were measured every 24h for 96 hours using multispectral imaging. The error bars represent the standard deviation. Each timepoint was analysed separately using the Kruskal-Wallis test as a non-parametric test, followed by Dunn's test for pairwise comparisons. The significance level is p = 0.05. The statistical analyses were performed in R version 4.1.3.

4.2.2 Use of siRNA

Due to the lack of evidence for gene silencing through the use of dsRNA, the next set of experiments consisted of trials involving siRNA complexed to the nanocarriers. It is hypothesized that because of the shorter length of the siRNA-fragments, uptake in the target cells should be more efficient. Four carefully selected siRNAs were tested (see Table 1), each one separately as well as combined all together. Concentrations of the siRNA were 25ng/µL each time, before being added together with the P3000-reagent and Lipofectamine particles in a 1:2:3 ratio. These treatments were then incubated for 24h with 1×10^7 protoplasts/mL in PBS and 1/10 PDB, before being inoculated on small PDA petri dishes.

Figure 13 shows the results of these experiments. Figure 13A represents the treatments that were resuspended and incubated in PBS, before being inoculated on PDA. Over the 72h of monitoring, none of the siRNA-treatments showed significant reduction of the cGFP-signal compared to the control (protoplasts in PBS). There is a noticeable difference between cGFP-values of the protoplasts treated with 4 siRNAs + nanoparticles and the positive control, however this result does not prove significant according to a non-parametric Kruskal-Wallis test. Figure 13B represents the same treatments, only now resuspended and incubated in 1/10 PDB. For this setup, there are significant results at timepoint 24h: the first and the fourth used siRNA (complexed to nanoparticles) show significantly lower cGFP-values than the positive control (protoplasts in 1/10 PDB). The other treatments (siRNA 2, siRNA3 and the mixture of 4siRNAs) also show lower values than the control, these are however insignificant ($p>0.05$). At 48h and 72h, there are also differences in cGFP-values, however non-parametric testing again showed no significant disparities.

A



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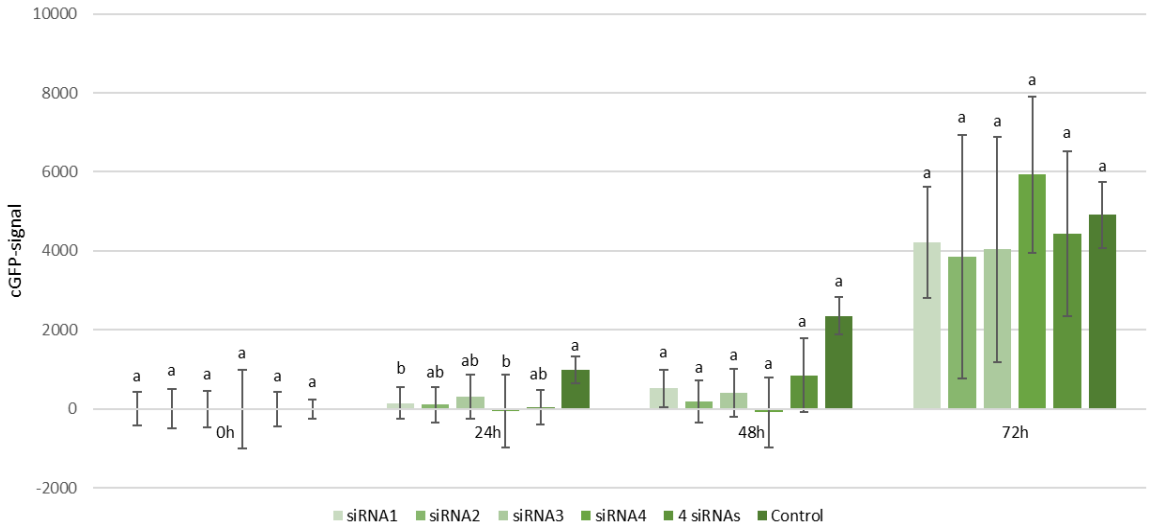


Figure 13: Graphical representation of the cGFP signal of Fusarium graminearum protoplasts treated with siRNAs and nanoparticles. Treatments contain 1 × 10^6 protoplasts/mL and a 1:2:3 ratio of siRNA, P300-reagent and Lipofectamine 3000. A) Treatments and control resuspended in PBS-medium. B) Treatments and control resuspended in 10X diluted PDB-medium. Treatments and control inoculated on small petri dishes of PDA. cGFP-values were measured every 24h for 96 hours using multispectral imaging. The error bars represent the standard deviation. Each timepoint was analysed separately using the Kruskal-Wallis test as a non-parametric test, followed by Dunn's test for pairwise comparisons. The significance level is p = 0.05. The statistical analyses were performed in R version 4.1.3.

4.3 Detached leaf siRNA assay

Not only were the siRNAs tested in an *in vitro* assay on PDA-dishes, they were also used in a trial using wheat leaf pieces inoculated with *Fusarium graminearum* protoplasts, as the host plant can also transfer siRNAs to the pathogen and activate the RNAi-system, and the eventual goal of this research is to produce results that can be implemented at field level. For this, 6 wheat leaf pieces of 10 days old were transferred to 0.5% water-agar + Benzimidazole (40mg/L) and sprayed with the mixture of 4 siRNAs (25ng/µL). For the control treatment, 6 pieces on a different petri dish were sprayed with nuclease-free water. Both were incubated for 48h, before being inoculated with 10µL of 1×10^6 protoplasts/mL. Every 24h, images were made using the multispectral PathoViewer, results of which can be found in Figure 14. The detected GFP-signals were corrected for background signalling using the values of the treatments at 0h. However, as can be observed on the graph, the GFP-signal of both remains exceptionally low over the different timepoints, which is a result of the protoplasts not growing on the leaf pieces (which was confirmed visually). Consequently, the experiment was terminated, as both the agar-plates were overtaken by contaminations while the protoplasts showed no growth.

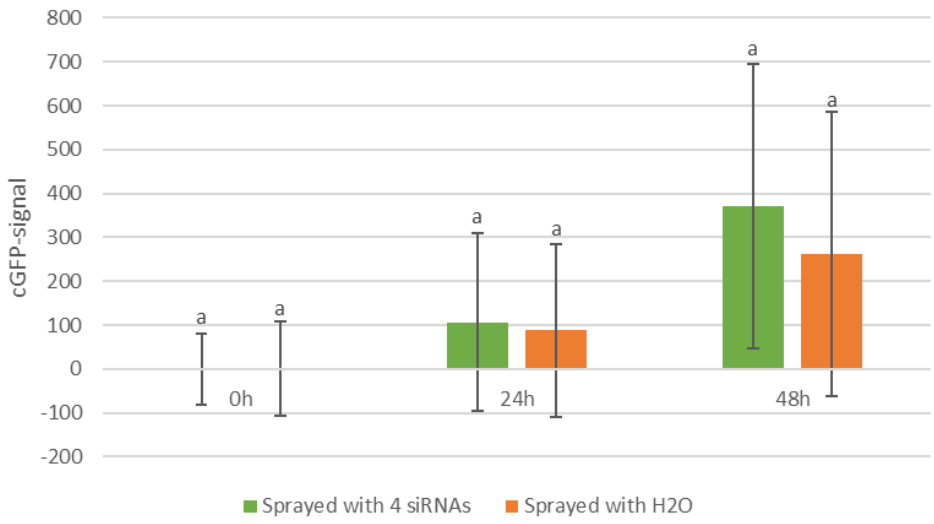


Figure 14: Graphical representation of the cGFP signal of Fusarium graminearum protoplasts treated with siRNAs and nanoparticles, inoculated on 10 days old wheat leaf pieces on 0.5% water-agar. Spray treatments contain 25ng/µL siRNA, inoculation contains 1×10^6 protoplasts/mL. cGFP-values of the 6 leaf-piece regions were measured every 24h for 48 hours using multispectral imaging. The error bars represent the standard deviation. Each timepoint was analysed separately using the Kruskal-Wallis test as a non-parametric test, followed by Dunn's test for pairwise comparisons. The significance level is p = 0.05. The statistical analyses were performed in R version 4.1.3.

5. DISCUSSION

The goal of this study was to optimize a protocol for post-transcriptional gene silencing through RNA-interference in *F. graminearum* and deliver a proof of concept, in order to eventually develop an RNAi strategy for the control of *F. verticillioides* infections in maize. For this, eGFP-tagged strains of the former species were submitted to a microscopic assay, as well as *in vitro* and *in vivo* trials. The goal was to inhibit the GFP-production of the fungus by transfecting the dsRNA or siRNA into the cells and into the RNAi-machinery of the fungus.

F. graminearum protoplast cells incubated with labelled dsRNA were subjected to a fluorescent microscopy assay, to qualitatively check whether the dsRNA was present in the incubated cells, before quantitively checking the GFP-production of the growing pathogen through *in vitro* trials. The protoplasts + dsRNA + nanoparticles-sample incubated in PBS and 1/10 PDB was compared to its control sample containing only protoplasts. The results clearly showed that the PBS-incubated cells had absorbed the dsRNA, by showing a red signal when excited at the right wavelength. Nevertheless, the fungal cells still displayed a clear green signal because of GFP-production, proving that while the dsRNA may enter the cells (aided by a lipid-based nanocarrier), this does not necessarily mean that the RNAi-machinery incorporates these molecules within its processes. A conduction from these results is that the difficulties with RNAi-techniques not working as expected might not lie in the passage of the RNA past cellular barriers, yet in the integration of the applied dsRNA within the actual RNAi-reactions of the cells. A possible solution could be found in altering the concentration of dsRNA throughout different trials, as the concentration was the same for each trial in this study (759.975 ng/µL). Contrary to the sample in PBS, the one incubated in 1/10 PDB showed no dsRNA signal anywhere in the sample, indicating that a contamination containing a ribonuclease had been able to infiltrate the sample, or that the dsRNA had degraded in a different way within the 24h of incubation. To achieve an accurate, trustworthy result of the microscopic assay, the samples should have been produced in multiple separate replicates, as the PDB-sample now does not present an accurate result of the trial, due to the probable contamination. Further experiments should thus include multiple repetitions of the treatments to achieve a reproduceable result. Confocal microscopy and Z-stacking for more in-focus, precise images could also be implemented, to confirm dsRNA entry within the cells and to determine the extent in which the molecules were able to penetrate the cells.

Knowing now that dsRNA entering the cell does not necessarily lead to GFP-silencing, further microscopic assays could be performed based on previously discarded experimental setups, as the GFP-signal is not always an indicator of lack of entry. Spores treated with dsRNA, protoplasts treated with dsRNA without a nanocarrier, …, could be reevaluated by microscopic assays to confirm or disprove entry of dsRNA within the cells. What's more is that the protoplast samples for the microscopic assay in this study accidentally contained some none-protoplasted fungal tissue, which also showed a red fluorescent signal. This indicates that either the dsRNA was also able to enter this tissue and pass the cell wall, that the dsRNA was buried in the cell walls of this tissue, or that the dsRNA simply adhered to the cells' exterior sites. In further experiments, this could be cleared up by treating the samples with a nuclease after the 24h incubation, in order to break down any dsRNA that has not entered the cell tissue (Wang et al., 2016).

The *in vitro* and *in vivo* trials for this dissertation were based on two different hypotheses: the length of the RNA-samples affects the transfection efficiency, and the nutrient conditions during transfection affects the uptake efficiency of the fungal cells. In attempt to confirm these propositions, protoplasts of the strains were treated with either long dsRNA, or shorter siRNA, to assess the importance of sample length for a successful transfection, and incubated in a nutrient containing medium, 10X diluted PDB, or in a sterile isotonic buffer, PBS. A lipid-based nanocarrier, Lipofectamine™ 3000 Transfection Reagent (Invitrogen), was added to both protect the RNA from degradation and to assist in cell-membrane passage. The samples were then inoculated on nutrient-dense PDA, as well 10X diluted PDA, containing less nutrients.

Former *in vitro* trials by Dangreau (2022) had shown that intact spores of *F. graminearum* PH-1 treated with dsRNA did not show any sign of cGFP-reduction, both when complexed to a nanocarrier or added as is. A logical explanation is that the applied dsRNA got stuck or buried in the cell wall matrix when attempting cell entry, as the cell wall is designed not to let external molecules simply enter the cell (Šečić & Kogel, 2021). To simplify the trials, and to be able to confirm or disprove that entry within the cell leads to PTGS, it was decided to further use protoplasts in the trials, lacking a cell wall. The hypothesis was that this would ensure a more efficient passage of the RNA within the cells, as former studies have already proven that protoplasts (for example of *B. cinerea*) can absorb exogenously applied dsRNA, even without an added carrier (Wang et al., 2016). Dangreau (2022) already

disproved this for *F. graminearum* protoplasts, which is why the trials in this study immediately implemented the Lipofectamine carrier.

The results of the first *in vitro* trials using long dsRNA, as well as both the nutrient-containing 1/10 PDB and the sterile PBS, showed that the treatments in the isotonic PBS often had difficulty growing, especially when inoculated on the diluted PDA-medium. The hypothesis here is that the conditions of these treatments are not good enough for the protoplasts to regenerate their cell walls and initiate mitosis. However, in trials using spores or mycelium, these conditions would probably best simulate the natural circumstances during plant infection: the pathogen usually grows in an environment containing little to no nutrients until it is able to penetrate plant tissue. It is only due to working with already fragile protoplasts that these treatments show little or no growth in 50% of the attempts. On the other hand, the treatments in PBS growing on nutrient-rich PDA dishes, thus more closely simulating natural conditions of the environment after plant penetration, did show a significant yet transient GFP-silencing effect after 48h of inoculation with dsRNA, in comparison to its control dishes consisting of untreated protoplasts (Figure 12A). At the following timepoints, 72h and 96h, the treated cells show a higher cGFP-signal than the control samples, possibly as a compensation for the lower GFP-production at 48h. This indicates that the treatment with dsRNA should probably be repeated several times over, at different points in time, which could prove challenging when it comes to implementing the technique in the field and moving RNAi-based products to the market. Contrary to the sterile PBS, the treatments in diluted PDB did lead to growth each time, both plated on PDA and 10X diluted PDA. This is probably a result of the fact that the protoplasts were already able to (partly) regenerate the cell wall and initiate growth during the 24h of incubation before inoculation on the petri dishes, because of the nutrients present in the PDB. There were some significant differences in cGFP-values between the treated and untreated dishes in this medium, with the treated ones showing higher values at several different timepoints on both PDA and 1/10 PDA, which is a counterintuitive result and could possibly mean that something had gone wrong with the untreated sample causing the concentration of the cells to be lower than anticipated, leading to slower and lesser growth. Both treatments (PBS or 1/10 PDB) however clearly show overall low signalling and growth on 10X diluted PDA, indicating that a nutrient-poor growing environment is not the ideal way to perform trials involving protoplasts. As aforementioned, this would probably be a better technique when using spores or other unaffected fungal tissue.

Although the *in vitro* trials using dsRNA showed a significant difference in cGFP-signal at one certain timepoint, other significant results went against the expectations, raising suspicion that the significant results could possibly be flukes, or simply due to measurement errors or different growth rates. This is why the following trials were executed using siRNAs, in hopes of getting unambiguous proof of RNA-interference in the cells. These trials again consisted of treatments in two different media, PBS and 1/10 PDB, and tested 4 different siRNA samples individually, as well as mixed together. The concentration of the siRNAs (25 ng/µL) was based on a study by Abdel-Hadi et al. (2011), which proved that for protoplasts of *Aspergillus flavus* and *Aspergillus parasiticus*, siRNAs of this concentration could successfully silence a key gene of the fungi. Because of former confirmation that diluted, nutrient-poor PDA was a suboptimal medium for fungal growth starting from protoplasts, these treatments were only inoculated on undiluted PDA.

The PBS-incubated samples showed no significant cGFP-reduction according to non-parametric testing; however, the data clearly show that all of the treatments have a lower cGFP signal after 72h of inoculation than the control sample, especially the treatment of all 4 siRNAs together. The same goes for the treatment in 1/10 PDB: all (but one) of the treatments show a lower signal than the control sample at 48h and 72h, however the results are not significant according to Kruskal-Wallis testing. At 24h of inoculation (and thus actually 48h of the dsRNA being incubated in 1/10 PDB with the cells), siRNA1 and siRNA4 show significantly lower cGFP-values than the other treatments and the control samples, indicating that the bp-sequences of these RNA-pieces have a high influence on the GFP-production within the fungus. The effect of the siRNAs could be further evaluated on spores in both *in vitro* and microscopic assays. The signal-reducing effect is transient, indicating that the siRNA treatment should again have to be applied at several different timepoints, creating a big practical challenge. A study by Hendrix et al. (2021) showed that siRNAs with a length of 22 nt – so 1 bp difference to the siRNAs used in this study – can deliver much better results in plants than pieces of 21 nt, so perhaps this is also worth researching for fungi in further experiments.

Alongside the *in vitro* trials using siRNA, an *in vivo* trial using wheat leaf pieces was set up, to investigate if the plant system would be a better transferor of RNA into the fungal cells. For this, the SIGS technique was employed: leaf pieces were sprayed with 25 ng/µL of the siRNA mixture, incubated for 48h so the leaves were able to incorporate the RNA within their cells, before being inoculated with a protoplast suspension. Several studies (Koch et al., 2016; Werner et al., 2020) have already shown that SIGS has great promise for gene

silencing in *F. graminearum*: both *DCL* (dicer-like) and *CYP51* genes have been successfully silenced to reduce pathogenicity by dsRNA in SIGS-trials. Since Dangreau (2022) had already disproved *in vivo* GFP-silencing when using spores, the trial in this study attempted to use protoplasts instead. However, as already observed in the *in vitro* trials using 1/10 PDA petri dishes, protoplasts often cannot initiate growth unless there are enough nutrients present. The water-agar and leaf pieces did not provide enough nutrients for the cells, causing the protoplasts to decay before growth initiation. This led to little to no signalling, and thus no reproducible result out of these trials. The experimental setup could in the future be repeated with spores – or other fungal tissue – that are able to successfully grow on the leaves, while the spray treatment could be replaced with dsRNA or siRNA in a higher concentration. To verify the transfer of the RNA from the plant tissue to the fungal cells, HIGS could be used after a vector-mediated transformation of the host plant. If this technique showed successful results, it would be confirmation that the host is capable of transferring the molecules to the pathogen, which is useful when attempting to create a protocol for SIGS in the same plant-pathogen complex.

A continuation of this study could thus entail more microscopic assays, to better understand the limits of reach of the dsRNA within different types of cells. If it becomes clear that a certain treatment is able to transfect dsRNA into the pathogen, *in vitro* assays can provide more insight into which concentration of dsRNA-treatment effectively leads to gene silencing, and what the effects of application at different timepoints and under different growing circumstances are. If a successful gene silencing method has been identified, it could then be used to target other key genes of the pathogen to suppress pathogenicity. Real-time PCR (qPCR) could then be used to prove the silencing of the targeted gene.

6. CONCLUSION

DsRNA and siRNAs were designed to target the eGFP-gene in a fungal strain (PH-1) of *F. graminearum*. Reproduceable experimental set-ups were developed and optimized to investigate the influence of externally added dsRNA or siRNA on protoplasts of the fungal pathogen. The methods were based on microscopic analysis, *in vitro* dsRNA-application on petri plates and an *in vivo* detached leaf analysis on wheat.

The microscopic assay using fluorescently labelled dsRNA proved to be a relevant aspect of the research, as it was able to confirm dsRNA-entry within the protoplasted cells when incubated in PBS. The samples showed no GFP-reduction, which provided the insight that the entry of dsRNA does not necessarily lead to gene silencing. Further research should thus look into other challenges associated with RNAi-based control as well, and not limit focus to a successful transfection into the target cells.

The *in vitro* and *in vivo* experiments were based on two objectives: determining the effect of the length of the dsRNA fragments on RNAi-efficiency, and examine whether the medium used facilitated a different RNAi response. Based on results from previous studies, nanocarriers were added to protect the dsRNA and aid the transfection process. The results made clear that inoculation of protoplasts on a nutrient-poor medium is not an ideal experimental setup, as the cells had extreme difficulty growing and thus couldn't be properly monitored for GFP-signal. The treatments growing on nutrient-rich PDA dishes showed contradictory results: the treatment in PBS indicated transient gene-silencing at 48h, while the treatment in 1/10 PB showed a stronger signal than its control. More individual replicates should be made, plus the trials should be repeated several times to verify these results. However, it appears that PBS-incubation leads to a better transfection of the dsRNA. When using shorter fragments (siRNAs), both media showed lower values for the RNA-treated samples, yet only the 1/10 PDB medium showed a transient significant result. Conclusion for both of these experiments is that any treatment applied should probably be applied repetitively at different timepoints.

The *in vivo* detached leaf assay showed no significant results, as the medium and leaves did not provide enough nutrients for the protoplasts to grow. *In vivo* assays like these are more suitable for unaffected fungal tissue.

Future research into this topic should entail more microscopic investigation of the transfection process, before moving on to *in vitro* testing, as those results can often be conflicting. Upon confirmation of dsRNA/siRNA-entry, several replicates using different treatment-concentrations could be installed, to assess the influence of this on RNAi-efficiency. Only when the right concentration and circumstances have been determined should the trials attempt at silencing different genes and should the protocol be transferred to trials in other species. The research in this dissertation opens the way for countless other trials to be performed and invites future researchers to find answers to the encountered difficulties.

7. REFERENCES

Abbas, H. K., Shier, W. T., Plasencia, J., Weaver, M. A., Bellaloui, N., Kotowicz, J. K., Butler, A. M., Accinelli, C., de la Torre-Hernandez, M. E., & Zablotowicz, R. M. (2017). Mycotoxin contamination in corn smut (Ustilago maydis) galls in the field and in the commercial food products. *Food Control*, *71*, 57–63. https://doi.org/10.1016/J.FOODCONT.2016.06.006

Abdel-Hadi, A., Caley, D., Carter, D., & Magan, N. (2011). Control of Aflatoxin Production of Aspergillus flavus and Aspergillus parasiticus Using RNA Silencing Technology by Targeting aflD (nor-1) Gene. *Toxins*, *3*(6), 647–659. https://doi.org/10.3390/toxins3060647

Adegbeye, M. J., Ravi Kanth Reddy, P., Obaisi, A. I., Elghandour, M. M. M. Y., Oyebamiji, K. J., Salem, A. Z. M., Morakinyo-Fasipe, O. T., Cipriano-Salazar, M., & Camacho-Díaz, L. M. (2020). Sustainable agriculture options for production, greenhouse gasses and pollution alleviation, and nutrient recycling in emerging and transitional nations - An overview. *Journal of Cleaner Production*, *242*, 118319. https://doi.org/10.1016/J.JCLEPRO.2019.118319

Al Masri, A. (2018). *Application of proximal sensing techniques for epidemiological investigations of Fusarium head blight in wheat under field and controlled conditions*.

Alisaac, E., & Mahlein, A.-K. (2023). Fusarium Head Blight on Wheat: Biology, Modern Detection and Diagnosis and Integrated Disease Management. *Toxins*, *15*(3), 192. https://doi.org/10.3390/toxins15030192

Aoki, T., O'Donnell, K., & Geiser, D. M. (2014). Systematics of key phytopathogenic Fusarium species: current status and future challenges. *Journal of General Plant Pathology*, *80*(3), 189–201. https://doi.org/10.1007/s10327-014-0509-3

Askun, T. (2018). Introductory Chapter: Fusarium: Pathogenicity, Infections, Diseases, Mycotoxins and Management. In *Fusarium - Plant Diseases, Pathogen Diversity, Genetic Diversity, Resistance and Molecular Markers*. InTech. https://doi.org/10.5772/intechopen.76507

Bahadur, A. (2022). Current Status of Fusarium and Their Management Strategies. In *Fusarium - An Overview of the Genus*. IntechOpen. https://doi.org/10.5772/intechopen.100608

Balogh, A. (2021, December 13). The rise and fall of monoculture farming. *Horizon: The EU Research & Innovation Magazine*. https://ec.europa.eu/research-and-innovation/en/horizon-magazine/rise-and-fall-monoculture-farming

Bansal, Y., Singla, N., Kaistha, N., Sood, S., & Chander, J. (2019). Molecular identification of Fusarium species complex isolated from clinical samples and its antifungal susceptibility patterns. *Current Medical Mycology*. https://doi.org/10.18502/cmm.5.4.2149

Barzman, M., Bàrberi, P., Birch, A. N. E., Boonekamp, P., Dachbrodt-Saaydeh, S., Graf, B., Hommel, B., Jensen, J. E., Kiss, J., Kudsk, P., Lamichhane, J. R., Messéan, A., Moonen, A. C., Ratnadass, A., Ricci, P., Sarah, J. L., & Sattin, M. (2015). Eight principles of integrated pest management. *Agronomy for Sustainable Development*, *35*(4), 1199–1215. https://doi.org/10.1007/S13593-015-0327-9/FIGURES/8

Belisário, R., Robertson, A. E., & Vaillancourt, L. J. (2022). Maize Anthracnose Stalk Rot in the Genomic Era. *Plant Disease*, *106*(9), 2281–2298. https://doi.org/10.1094/PDIS-10-21-2147-FE/ASSET/IMAGES/LARGE/PDIS-10-21-2147-FEF7.JPEG

Blake, R. (2020). Will the European Green Deal Make Agriculture More Sustainable? *Outlooks on Pest Management*, *31*(5), 198–200. https://doi.org/10.1564/V31_OCT_01

Cagliari, D., Dias, N. P., Galdeano, D. M., dos Santos, E. Á., Smagghe, G., & Zotti, M. J. (2019). Management of Pest Insects and Plant Diseases by Non-Transformative RNAi. *Frontiers in Plant Science*, *10*. https://doi.org/10.3389/fpls.2019.01319

Cairns, J. E., Sonder, K., Zaidi, P. H., Verhulst, N., Mahuku, G., Babu, R., Nair, S. K., Das, B., Govaerts, B., Vinayan, M. T., Rashid, Z., Noor, J. J., Devi, P., San Vicente, F., & Prasanna, B. M. (2012). Maize Production in a Changing Climate: Impacts, Adaptation, and Mitigation Strategies. *Advances in Agronomy*, *114*, 1–58. https://doi.org/10.1016/B978-0-12-394275-3.00006-7

Cambaza, E. (2019). *The use of color as alternative to size measurements in Fusarium graminearum growth studies and prediction of deoxynivalenol synthesis*.

Capinera, J. L. (2020, June). *European corn borer - Ostrinia nubilalis (Hubner)*. https://entnemdept.ufl.edu/creatures/field/e_corn_borer.htm

CGIAR. (2014). *Challenges and opportunities* . https://archive.maize.org/challenges-and-opportunities/

Chemeltorit, P., & Suresh, L. M. (2020). Maize Head Smut: Pathogenesis, Epidemiology, and Management Options. *African Phytosanitary Journal*, *2*(1), 63–71. https://www.researchgate.net/publication/352825299

Chemura, A., Nangombe, S. S., Gleixner, S., Chinyoka, S., & Gornott, C. (2022). Changes in Climate Extremes and Their Effect on Maize (Zea mays L.) Suitability Over Southern

Africa. *Frontiers in Climate*, *4*, 118. https://doi.org/10.3389/FCLIM.2022.890210/BIBTEX

Chen, J., Li, Z., Cheng, Y., Gao, C., Guo, L., Wang, T., & Xu, J. (2020). Sphinganine-Analog Mycotoxins (SAMs): Chemical Structures, Bioactivities, and Genetic Controls. *Journal of Fungi*, *6*(4), 312. https://doi.org/10.3390/jof6040312

Chen, Y., Kistler, H. C., & Ma, Z. (2019). Fusarium graminearum Trichothecene Mycotoxins: Biosynthesis, Regulation, and Management. *Annual Review of Phytopathology*, *57*(1), 15–39. https://doi.org/10.1146/annurev-phyto-082718-100318

Cheng, W., Song, X.-S., Li, H.-P., Cao, L.-H., Sun, K., Qiu, X.-L., Xu, Y.-B., Yang, P., Huang, T., Zhang, J.-B., Qu, B., & Liao, Y.-C. (2015). Host-induced gene silencing of an essential chitin synthase gene confers durable resistance to Fusarium head blight and seedling blight in wheat. *Plant Biotechnology Journal*, *13*(9), 1335–1345. https://doi.org/10.1111/pbi.12352

Copa and Cogeca. (2022). *European farming*. https://copa-cogeca.eu/europeanfarming

Cope, R. B. (2018). Trichothecenes. In *Veterinary Toxicology* (pp. 1043–1053). Elsevier. https://doi.org/10.1016/B978-0-12-811410-0.00075-1

Czarnecka, D., Czubacka, A., Agacka-Mołdoch, M., Trojak-Goluch, A., & Księżak, J. (2022). The Occurrence of Fungal Diseases in Maize in Organic Farming Versus an Integrated Management System. *Agronomy 2022, Vol. 12, Page 558*, *12*(3), 558. https://doi.org/10.3390/AGRONOMY12030558

Dang, Y., Yang, Q., Xue, Z., & Liu, Y. (2011). RNA Interference in Fungi: Pathways, Functions, and Applications. *Eukaryotic Cell*, *10*(9), 1148–1155. https://doi.org/10.1128/EC.05109-11

Dangreau, B. (2022). *Optimalisatie van RNAi technologie voor het bestrijden van Fusarium graminearum in Tarwe*.

De Schutter, K., Taning, C. N. T., Van Daele, L., Van Damme, E. J. M., Dubruel, P., & Smagghe, G. (2022). RNAi-Based Biocontrol Products: Market Status, Regulatory Aspects, and Risk Assessment. *Frontiers in Insect Science*, *1*. https://doi.org/10.3389/finsc.2021.818037

Dickman, K. G., & Grollman, A. P. (2010). Nephrotoxicity of Natural Products: Aristolochic Acid and Fungal Toxins. In *Comprehensive Toxicology* (pp. 433–458). Elsevier. https://doi.org/10.1016/B978-0-08-046884-6.00823-X

Drakopoulos, D., Kägi, A., Gimeno, A., Six, J., Jenny, E., Forrer, H.-R., Musa, T., Meca, G., & Vogelgsang, S. (2020). Prevention of Fusarium head blight infection and mycotoxins in wheat with cut-and-carry biofumigation and botanicals. *Field Crops Research*, *246*, 107681. https://doi.org/10.1016/j.fcr.2019.107681

Du, L., Zhu, X., Gerber, R., Huffman, J., Lou, L., Jorgenson, J., Yu, F., Zaleta-Rivera, K., & Wang, Q. (2008). Biosynthesis of sphinganine-analog mycotoxins. *Journal of Industrial Microbiology & Biotechnology*, *35*(6), 455–464. https://doi.org/10.1007/s10295-008-0316-y

Dweba, C. C., Figlan, S., Shimelis, H. A., Motaung, T. E., Sydenham, S., Mwadzingeni, L., & Tsilo, T. J. (2017). Fusarium head blight of wheat: Pathogenesis and control strategies. *Crop Protection*, *91*, 114–122. https://doi.org/10.1016/j.cropro.2016.10.002

Eilenberg, J., Hajek, A., & Lomer, C. (2001). Suggestions for unifying the terminology in biological control. *BioControl*, *46*(4), 387–400. https://doi.org/10.1023/A:1014193329979

Erenstein, O., Jaleta, M., Sonder, K., Mottaleb, K., & Prasanna, B. M. (2022). Global maize production, consumption and trade: trends and R&D implications. *Food Security 2022 14:5*, *14*(5), 1295–1319. https://doi.org/10.1007/S12571-022-01288-7

European Commission. (n.d.-a). *A European Green Deal | European Commission*. Retrieved November 4, 2022, from https://ec.europa.eu/info/strategy/priorities-2019-2024/european-green-deal_en

European Commission. (n.d.-b). *Trade and international policy analysis*. Retrieved November 27, 2022, from https://agriculture.ec.europa.eu/international/agricultural-trade/trade-and-international-policy-analysis_en

European Commission. (2019). *What is the European Green Deal?* https://doi.org/10.2775/97540

European Commission. (2022). *The common agricultural policy at a glance*. https://agriculture.ec.europa.eu/common-agricultural-policy/cap-overview/cap-glance_en

European Commission - Agriculture and rural development. (2022). *CAP strategic plans*. https://agriculture.ec.europa.eu/cap-my-country/cap-strategic-plans_en

European Environment Agency. (2020, November 23). *Agriculture* . https://www.eea.europa.eu/themes/agriculture/intro

European Environment Agency. (2021, December 9). *New indicator on pesticides in European waters*. https://www.eea.europa.eu/highlights/new-indicator-on-pesticides-in

European Parliament (EP). (2021, November 24). *EU agriculture statistics: subsidies, jobs, production (infographic)*. https://www.eumonitor.eu/9353000/1/j9vvik7m1c3gyxp/vlo6ng25okwy?ctx=vg9pir5eze8o&start_tab0=10

Eurostat. (2018). *Land use statistics - Statistics Explained*. https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Land_use_statistics

Factsheet - a greener and fairer CAP. (n.d.). Retrieved November 20, 2022, from https://agriculture.ec.europa.eu/common-agricultural-policy/cap-overview/cap-glance_en

FAO. (2022). FAO publications catalogue 2022. *FAO Publications Catalogue 2022*. https://doi.org/10.4060/CC2323EN

FAOSTAT. (2021). https://www.fao.org/faostat/en/#data/QCL

Ferrigo, D., Raiola, A., Rasera, R., & Causin, R. (2014). Trichoderma harzianum seed treatment controls Fusarium verticillioides colonization and fumonisin contamination in maize under field conditions. *Crop Protection*, *65*, 51–56. https://doi.org/10.1016/j.cropro.2014.06.018

Fetting, C. (2020). *THE EUROPEAN GREEN DEAL*.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, *391*(6669), 806–811. https://doi.org/10.1038/35888

Fuchs, A., Berger, V., Steinbauer, K., Köstl, T., Wuttej, D., & Jungmeier, M. (2021). The long-term effects of monoculture maize cultivation on plant diversity. *Phytocoenologia*, *50*(4), 397–408. https://doi.org/10.1127/PHYTO/2021/0382

Gebremichael, D. E., Haile, Z. M., Negrini, F., Sabbadini, S., Capriotti, L., Mezzetti, B., & Baraldi, E. (2021). RNA Interference Strategies for Future Management of Plant Pathogenic Fungi: Prospects and Challenges. *Plants*, *10*(4), 650. https://doi.org/10.3390/plants10040650

Gelineau-van Waes, J. (2010). Fumonisin, Folate, and Neural Tube Defects. In *Comprehensive Toxicology* (pp. 189–208). Elsevier. https://doi.org/10.1016/B978-0-08-046884-6.01519-0

Glenn, A. E. (2006). Natural variation of ascospore and conidial germination by Fusarium verticillioides and other Fusarium species. *Mycological Research*, *110*(2), 211–219. https://doi.org/10.1016/j.mycres.2005.09.004

Gupta, R. C., Mostrom, M. S., & Evans, T. J. (2018). Zearalenone. In *Veterinary Toxicology* (pp. 1055–1063). Elsevier. https://doi.org/10.1016/B978-0-12-811410-0.00076-3

Haesaert, G. (2021). *Cursus Gewasbescherming* .

Haesaert, G. (2022). *Cursus Plant- en gewaswetenschappen 1*. UGent.

Hajek, A., & Eilenberg, J. (2018). *Natural Enemies: An Introduction to Biological Control*. https://books.google.be/books?hl=nl&lr=&id=Y-RfDwAAQBAJ&oi=fnd&pg=PR9&dq=(Hajek+and+Eilenberg,+2018&ots=iwvppSJeLP&sig=vcx5-7R839MVxM3fbIHF_J4CgWs#v=onepage&q=(Hajek%20and%20Eilenberg%2C%202018&f=false

He, H. ming, Liu, L. na, Munir, S., Bashir, N. H., WANG, Y., YANG, J., & LI, C. yun. (2019). Crop diversity and pest management in sustainable agriculture. *Journal of Integrative Agriculture*, *18*(9), 1945–1952. https://doi.org/10.1016/S2095-3119(19)62689-4

Heinrich Böll Stiftung, Friends of the Earth Europe, Bund für Umwelt und Naturschutz, & PAN Europe. (2022). *Pesticide Atlas: Facts and figures about toxic chemicals in agriculture 2022*. https://eu.boell.org/PesticideAtlas

Hendrix, B., Zheng, W., Bauer, M. J., Havecker, E. R., Mai, J. T., Hoffer, P. H., Sanders, R. A., Eads, B. D., Caruano-Yzermans, A., Taylor, D. N., Hresko, C., Oakes, J., Iandolino, A. B., Bennett, M. J., & Deikman, J. (2021). Topically delivered 22 nt siRNAs enhance RNAi silencing of endogenous genes in two species. *Planta*, *254*(3), 60. https://doi.org/10.1007/s00425-021-03708-y

Höfle, L., Biedenkopf, D., Werner, B. T., Shrestha, A., Jelonek, L., & Koch, A. (2020). Study on the efficiency of dsRNAs with increasing length in RNA-based silencing of the Fusarium CYP51 genes. *RNA Biology*, *17*(4), 463–473. https://doi.org/10.1080/15476286.2019.1700033

Identifying and Managing Stalk Rots . (2023). Pioneer Seeds. https://www.pioneer.com/us/agronomy/managing_stalk_rot.html

Jackson, T. (n.d.). *Head Smut* . Institute of Agriculture and Natural Resources: Cropwatch. Retrieved April 22, 2023, from https://cropwatch.unl.edu/plantdisease/corn/head-smut

Jackson-Ziems, T. A., Rees, J. M., & Harveson, R. M. (2009). Common Stalk Rot Diseases of Corn. *University of Nebraska: Extension*.

Johnson, E. T., Proctor, R. H., Dunlap, C. A., & Busman, M. (2018). Reducing production of fumonisin mycotoxins in Fusarium verticillioides by RNA interference. *Mycotoxin Research*, *34*(1), 29–37. https://doi.org/10.1007/s12550-017-0296-8

Kamle, M., Mahato, D. K., Devi, S., Lee, K. E., Kang, S. G., & Kumar, P. (2019). Fumonisins: Impact on Agriculture, Food, and Human Health and their Management Strategies. *Toxins*, *11*(6), 328. https://doi.org/10.3390/toxins11060328

Kannan, M., Ismail, I., & Bunawan, H. (2018). Maize Dwarf Mosaic Virus: From Genome to Disease Management. *Viruses 2018, Vol. 10, Page 492*, *10*(9), 492. https://doi.org/10.3390/V10090492

Karlsson Green, K., Stenberg, J. A., & Lankinen, Å. (2020). Making sense of Integrated Pest Management (IPM) in the light of evolution. *Evolutionary Applications*, *13*(8), 1791–1805. https://doi.org/10.1111/EVA.13067

Karlsson, I., Persson, P., & Friberg, H. (2021). Fusarium Head Blight From a Microbiome Perspective. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.628373

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., Linicus, L., Johannsmeier, J., Jelonek, L., Goesmann, A., Cardoza, V., McMillan, J., Mentzel, T., & Kogel, K.-H. (2016). An RNAi-Based Control of Fusarium graminearum Infections Through Spraying of Long dsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery. *PLOS Pathogens*, *12*(10), e1005901. https://doi.org/10.1371/journal.ppat.1005901

Koch, A., & Wassenegger, M. (2021). Host-induced gene silencing – mechanisms and applications. *New Phytologist*, *231*(1), 54–59. https://doi.org/10.1111/nph.17364

Kogan, M. (1998). Integrated pest management: historical perspectives and contemporary developments. *Annual Review of Entomology*, *43*, 243–270. https://doi.org/10.1146/ANNUREV.ENTO.43.1.243

Kuo, Y.-W., & Falk, B. W. (2020). RNA interference approaches for plant disease control. *BioTechniques*, *69*(6), 469–477. https://doi.org/10.2144/btn-2020-0098

Lam, J. K. W., Chow, M. Y. T., Zhang, Y., & Leung, S. W. S. (2015). siRNA Versus miRNA as Therapeutics for Gene Silencing. *Molecular Therapy - Nucleic Acids*, *4*, e252. https://doi.org/10.1038/mtna.2015.23

Lanubile, A., Maschietto, V., Borrelli, V. M., Stagnati, L., Logrieco, A. F., & Marocco, A. (2017). Molecular Basis of Resistance to Fusarium Ear Rot in Maize. *Frontiers in Plant Science*, *8*. https://doi.org/10.3389/fpls.2017.01774

Lepesheva, G. I., & Waterman, M. R. (2007). Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1770*(3), 467–477. https://doi.org/10.1016/j.bbagen.2006.07.018

Lobell, D. B., Schlenker, W., & Costa-Roberts, J. (2011). Climate trends and global crop production since 1980. *Science*, *333*(6042), 616–620. https://doi.org/10.1126/SCIENCE.1204531/SUPPL_FILE/LOBELL.SOM.REVISED2.PDF

Machado, A. K., Brown, N. A., Urban, M., Kanyuka, K., & Hammond-Kosack, K. E. (2018). RNAi as an emerging approach to control Fusarium head blight disease and mycotoxin contamination in cereals. *Pest Management Science*, *74*(4), 790–799. https://doi.org/10.1002/ps.4748

Madege, R., Audenaert, K., Kimanya, M., Tiisekwa, B., De Meulenaer, B., Bekaert, B., Landschoot, S., & Haesaert, G. (2018). Control of Fusarium verticillioides (Sacc.) Nirenberg and Fumonisins by Using a Combination of Crop Protection Products and Fertilization. *Toxins*, *10*(2), 67. https://doi.org/10.3390/toxins10020067

Maize pests and diseases | The Encyclopedia of World Problems. (2020, October 4). http://encyclopedia.uia.org/en/problem/135352

Majumdar, R., Rajasekaran, K., & Cary, J. W. (2017). RNA Interference (RNAi) as a Potential Tool for Control of Mycotoxin Contamination in Crop Plants: Concepts and Considerations. *Frontiers in Plant Science*, *8*. https://doi.org/10.3389/fpls.2017.00200

Malvick, D. (2018). *Common rust on corn* . https://extension.umn.edu/corn-pest-management/common-rust-corn

Marasas, W. F. (2001). Discovery and occurrence of the fumonisins: a historical perspective. *Environmental Health Perspectives*, *109*(suppl 2), 239–243. https://doi.org/10.1289/ehp.01109s2239

Mbundi, L., Gallar-Ayala, H., Khan, M. R., Barber, J. L., Losada, S., & Busquets, R. (2014). *Advances in the Analysis of Challenging Food Contaminants* (pp. 35–105). https://doi.org/10.1016/B978-0-444-63406-1.00002-7

Miguel, T. de Á., Bordini, J. G., Saito, G. H., Andrade, C. G. T. de J., Ono, M. A., Hirooka, E. Y., Vizoni, É., & Ono, E. Y. S. (2015). Effect of fungicide on Fusarium verticillioides

mycelial morphology and fumonisin B1 production. *Brazilian Journal of Microbiology*, *46*(1), 293–299. https://doi.org/10.1590/S1517-838246120120383

Mita, M. M., Jannat, M., Bashar, S., Protic, I. A., Saha, P., Masud, Md. M., Islam, R., Islam, N. B., Alam, Md. Z., & Islam, Md. R. (2022). Potential Native Bacilli Reduce Fumonisin Contamination in Maize. *Agronomy*, *12*(11), 2608. https://doi.org/10.3390/agronomy12112608

Nagaraj, D., Achar, P. N., & Sreenivasa, M. Y. (2021). Current Perspectives of Biocontrol Agents for Management of Fusarium verticillioides and Its Fumonisin in Cereals—A Review. *Journal of Fungi*, *7*(9), 776. https://doi.org/10.3390/jof7090776

O'Donnell, K., McCormick, S. P., Busman, M., Proctor, R. H., Ward, T. J., Doehring, G., Geiser, D. M., Alberts, J. F., & Rheeder, J. P. (2018). Marasas et al. 1984 "Toxigenic Fusarium Species: Identity and Mycotoxicology" revisited. *Mycologia*, *110*(6), 1058–1080. https://doi.org/10.1080/00275514.2018.1519773

Oldenburg, E., Höppner, F., Ellner, F., & Weinert, J. (2017). Fusarium diseases of maize associated with mycotoxin contamination of agricultural products intended to be used for food and feed. *Mycotoxin Research*, *33*(3), 167–182. https://doi.org/10.1007/s12550-017-0277-y

Omotayo, O. P., & Babalola, O. O. (2023). Fusarium verticillioides of maize plant: Potentials of propitious phytomicrobiome as biocontrol agents. *Frontiers in Fungal Biology*, *4*. https://doi.org/10.3389/ffunb.2023.1095765

Parker, K. M., Barragán Borrero, V., van Leeuwen, D. M., Lever, M. A., Mateescu, B., & Sander, M. (2019). Environmental Fate of RNA Interference Pesticides: Adsorption and Degradation of Double-Stranded RNA Molecules in Agricultural Soils. *Environmental Science & Technology*, *53*(6), 3027–3036. https://doi.org/10.1021/acs.est.8b05576

Parvatha Reddy P. (2017). *Agro-ecological Approaches to Pest Management for Sustainable Agriculture*. https://link.springer.com/content/pdf/10.1007/978-981-10-4325-3.pdf

Pataky, J. K., & Snetselaar, K. M. (2006). Common smut of corn. *The Plant Health Instructor*. https://doi.org/10.1094/PHI-I-2006-0927-01

Paturi, S., & Deshmukh, M. V. (2021). A Glimpse of "Dicer Biology" Through the Structural and Functional Perspective. *Frontiers in Molecular Biosciences*, *8*. https://doi.org/10.3389/fmolb.2021.643657

Perincherry, L., Lalak-Kańczugowska, J., & Stępień, Ł. (2019). Fusarium-Produced Mycotoxins in Plant-Pathogen Interactions. *Toxins*, *11*(11), 664. https://doi.org/10.3390/toxins11110664

Pinzón, N., Bertrand, S., Subirana, L., Busseau, I., Escrivá, H., & Seitz, H. (2019). Functional lability of RNA-dependent RNA polymerases in animals. *PLOS Genetics*, *15*(2), e1007915. https://doi.org/10.1371/journal.pgen.1007915

Pitt, J. I. (2014). Mycotoxins: Fumonisins. In *Encyclopedia of Food Safety* (pp. 299–303). Elsevier. https://doi.org/10.1016/B978-0-12-378612-8.00192-X

Pub Affairs Bruxelles. (2022). *Common Agricultural Policy 2023-2027: the Commission approves the first CAP strategic plans - PubAffairs Bruxelles*. https://www.pubaffairsbruxelles.eu/eu-institution-news/common-agricultural-policy-2023-2027-the-commission-approves-the-first-cap-strategic-plans/

Qu, L., Wang, L., Ji, H., Fang, Y., Lei, P., Zhang, X., Jin, L., Sun, D., & Dong, H. (2022). Toxic Mechanism and Biological Detoxification of Fumonisins. *Toxins*, *14*(3), 182. https://doi.org/10.3390/toxins14030182

Ramirez-Cabral, N. Y. Z., Kumar, L., & Shabani, F. (2017). Global alterations in areas of suitability for maize production from climate change and using a mechanistic species distribution model (CLIMEX). *Scientific Reports 2017 7:1*, *7*(1), 1–13. https://doi.org/10.1038/s41598-017-05804-0

Rampersad, S. (2020). Pathogenomics and Management of Fusarium Diseases in Plants. *Pathogens*, *9*(5), 340. https://doi.org/10.3390/pathogens9050340

Ren, Z., Chhetri, A., Guan, Z., Suo, Y., Yokoyama, K., & Lee, S.-Y. (2022). Structural basis for inhibition and regulation of a chitin synthase from Candida albicans. *Nature Structural & Molecular Biology*, *29*(7), 653–664. https://doi.org/10.1038/s41594-022-00791-x

Romano, N., & Macino, G. (1992). Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Molecular Microbiology*, *6*(22), 3343–3353. https://doi.org/10.1111/j.1365-2958.1992.tb02202.x

Rutter, B. D., & Innes, R. W. (2018). Extracellular vesicles as key mediators of plant–microbe interactions. *Current Opinion in Plant Biology*, *44*, 16–22. https://doi.org/10.1016/j.pbi.2018.01.008

Samsudin, N. (2015). *Potential biocontrol of fumonisin B1 production by Fusarium verticillioides under different ecophysiological conditions in maize*. Cranfield university.

Sang, H., & Kim, J.-I. (2020). Advanced strategies to control plant pathogenic fungi by host-induced gene silencing (HIGS) and spray-induced gene silencing (SIGS). *Plant Biotechnology Reports*, *14*(1), 1–8. https://doi.org/10.1007/s11816-019-00588-3

Šečić, E., & Kogel, K. H. (2021a). Requirements for fungal uptake of dsRNA and gene silencing in RNAi-based crop protection strategies. In *Current Opinion in Biotechnology* (Vol. 70, pp. 136–142). Elsevier Ltd. https://doi.org/10.1016/j.copbio.2021.04.001

Šečić, E., & Kogel, K.-H. (2021b). Requirements for fungal uptake of dsRNA and gene silencing in RNAi-based crop protection strategies. *Current Opinion in Biotechnology*, *70*, 136–142. https://doi.org/10.1016/j.copbio.2021.04.001

Shah, L., Ali, A., Yahya, M., Zhu, Y., Wang, S., Si, H., Rahman, H., & Ma, C. (2018). Integrated control of fusarium head blight and deoxynivalenol mycotoxin in wheat. *Plant Pathology*, *67*(3), 532–548. https://doi.org/10.1111/ppa.12785

Sikhakolli, U. R., López-Giráldez, F., Li, N., Common, R., Townsend, J. P., & Trail, F. (2012). Transcriptome analyses during fruiting body formation in Fusarium graminearum and Fusarium verticillioides reflect species life history and ecology. *Fungal Genetics and Biology*, *49*(8), 663–673. https://doi.org/10.1016/j.fgb.2012.05.009

Stępień, Ł., Koczyk, G., & Waśkiewicz, A. (2011). FUM cluster divergence in fumonisins-producing Fusarium species. *Fungal Biology*, *115*(2), 112–123. https://doi.org/10.1016/j.funbio.2010.10.011

Summerell, B. A. (2019). Resolving Fusarium: Current Status of the Genus. *Annual Review of Phytopathology*, *57*(1), 323–339. https://doi.org/10.1146/annurev-phyto-082718-100204

Tan, J., Ameye, M., Landschoot, S., De Zutter, N., De Saeger, S., De Boevre, M., Abdallah, M. F., Van der Lee, T., Waalwijk, C., & Audenaert, K. (2020). At the scene of the crime: New insights into the role of weakly pathogenic members of the fusarium head blight disease complex. *Molecular Plant Pathology*, *21*(12), 1559–1572. https://doi.org/10.1111/mpp.12996

Taning, C. N. T., Gui, S., De Schutter, K., Jahani, M., Castellanos, N. L., Christiaens, O., & Smagghe, G. (2021). A sequence complementarity-based approach for evaluating off-target transcript knockdown in Bombus terrestris, following ingestion of pest-specific dsRNA. *Journal of Pest Science*, *94*(2), 487–503. https://doi.org/10.1007/s10340-020-01273-z

Taning, C. N. T., Mezzetti, B., Kleter, G., Smagghe, G., & Baraldi, E. (2021). Does RNAi-Based Technology Fit within EU Sustainability Goals? *Trends in Biotechnology*, *39*(7), 644–647. https://doi.org/10.1016/j.tibtech.2020.11.008

Tariq, M., Khan, A., Asif, M., Khan, F., Ansari, T., Shariq, M., & Siddiqui, M. A. (2020). Biological control: a sustainable and practical approach for plant disease management. *Https://Doi.Org/10.1080/09064710.2020.1784262*, 507–524. https://doi.org/10.1080/09064710.2020.1784262

Terciolo, Bracarense, Souto, Cossalter, Dopavogui, Loiseau, Oliveira, Pinton, & Oswald. (2019). Fumonisins at Doses below EU Regulatory Limits Induce Histological Alterations in Piglets. *Toxins*, *11*(9), 548. https://doi.org/10.3390/toxins11090548

Tinoco, M. L. P., Dias, B. B., Dall'Astta, R. C., Pamphile, J. A., & Aragão, F. J. (2010). In vivo trans-specific gene silencing in fungal cells by in planta expression of a double-stranded RNA. *BMC Biology*, *8*(1), 27. https://doi.org/10.1186/1741-7007-8-27

Tiru, Z., Mandal, P., Pratim Chakraborty, A., Pal, A., & Sadhukhan, S. (2022). Fusarium Disease of Maize and Its Management through Sustainable Approach. In *Fusarium - An Overview of the Genus*. IntechOpen. https://doi.org/10.5772/intechopen.100575

Trucksess, M. W., & Diaz-Amigo, C. (2011). Mycotoxins in Foods. In *Encyclopedia of Environmental Health* (pp. 888–897). Elsevier. https://doi.org/10.1016/B978-0-444-52272-6.00700-5

U.S. Department of Agriculture. (2021). *Corn is America's Largest Crop in 2019* . https://www.usda.gov/media/blog/2019/07/29/corn-americas-largest-crop-2019

US EPA. (n.d.). *Why We Use Pesticides* . Retrieved November 27, 2022, from https://www.epa.gov/safepestcontrol/why-we-use-pesticides

van der Lee, T., Zhang, H., van Diepeningen, A., & Waalwijk, C. (2015). Biogeography of Fusarium graminearum species complex and chemotypes: a review. *Food Additives & Contaminants: Part A*, *32*(4), 453–460. https://doi.org/10.1080/19440049.2014.984244

Vaughan, M., Backhouse, D., & Ponte, E. M. Del. (2016). Climate change impacts on the ecology of Fusarium graminearum species complex and susceptibility of wheat to Fusarium head blight: a review. *World Mycotoxin Journal*, *9*(5), 685–700. https://doi.org/10.3920/WMJ2016.2053

Vurro, M., Miguel-Rojas, C., & Pérez-de-Luque, A. (2019). Safe nanotechnologies for increasing the effectiveness of environmentally friendly natural agrochemicals. *Pest Management Science*, *75*(9), 2403–2412. https://doi.org/10.1002/ps.5348

Wagemans, J., Holtappels, D., Vainio, E., Rabiey, M., Marzachì, C., Herrero, S., Ravanbakhsh, M., Tebbe, C. C., Ogliastro, M., Ayllón, M. A., & Turina, M. (2022). Going Viral: Virus-Based Biological Control Agents for Plant Protection. *Https://Doi.Org/10.1146/Annurev-Phyto-021621-114208*, *60*, 21–42. https://doi.org/10.1146/ANNUREV-PHYTO-021621-114208

Wang, M., & Jin, H. (2017). Spray-Induced Gene Silencing: a Powerful Innovative Strategy for Crop Protection. *Trends in Microbiology*, *25*(1), 4–6. https://doi.org/10.1016/j.tim.2016.11.011

Wang, M., Weiberg, A., Lin, F.-M., Thomma, B. P. H. J., Huang, H.-D., & Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nature Plants*, *2*(10), 16151. https://doi.org/10.1038/nplants.2016.151

Werbrouck, S. (2021). *Cursus Agrobiotechnologie*.

Werner, B. T., Gaffar, F. Y., Schuemann, J., Biedenkopf, D., & Koch, A. M. (2020). RNA-Spray-Mediated Silencing of Fusarium graminearum AGO and DCL Genes Improve Barley Disease Resistance. *Frontiers in Plant Science*, *11*. https://doi.org/10.3389/fpls.2020.00476

Willow, J., Taning, C. N. T., Cook, S. M., Sulg, S., Silva, A. I., Smagghe, G., & Veromann, E. (2021). RNAi Targets in Agricultural Pest Insects: Advancements, Knowledge Gaps, and IPM. *Frontiers in Agronomy*, *3*. https://doi.org/10.3389/fagro.2021.794312

Wise, K. (n.d.). *Purdue extension Northern Corn Leaf Blight*. Retrieved April 10, 2023, from www.the-education-store.com

Wrzaszcz, W., & Prandecki, K. (2020). AGRICULTURE AND THE EUROPEAN GREEN DEAL. *Zagadnienia Ekonomiki Rolnej Problems of Agricultural Economics*, *4*(365), 156–179. https://doi.org/10.30858/zer/131841

Wu, J., Yang, J., Cho, W. C., & Zheng, Y. (2020). Argonaute proteins: Structural features, functions and emerging roles. *Journal of Advanced Research*, *24*, 317–324. https://doi.org/10.1016/j.jare.2020.04.017

Wytinck, N., Manchur, C. L., Li, V. H., Whyard, S., & Belmonte, M. F. (2020). dsRNA Uptake in Plant Pests and Pathogens: Insights into RNAi-Based Insect and Fungal Control Technology. *Plants*, *9*(12), 1780. https://doi.org/10.3390/plants9121780

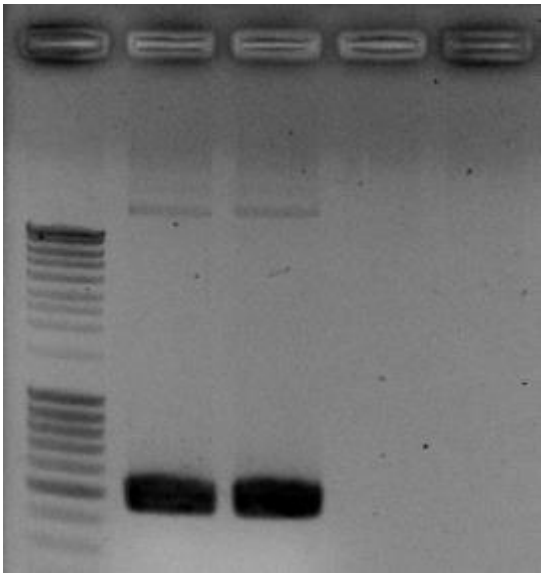
Wytinck, N., Sullivan, D. S., Biggar, K. T., Crisostomo, L., Pelka, P., Belmonte, M. F., & Whyard, S. (2020). Clathrin mediated endocytosis is involved in the uptake of exogenous double-stranded RNA in the white mold phytopathogen Sclerotinia

sclerotiorum. *Scientific Reports*, *10*(1), 12773. https://doi.org/10.1038/s41598-020-69771-9

Zhang, W. J., & Peshin, R. (2014). Integrated pest management and pesticide use. *Integrated Pest Management: Pesticide Problems, Vol.3*, 1–46. https://doi.org/10.1007/978-94-007-7796-5_1/TABLES/14

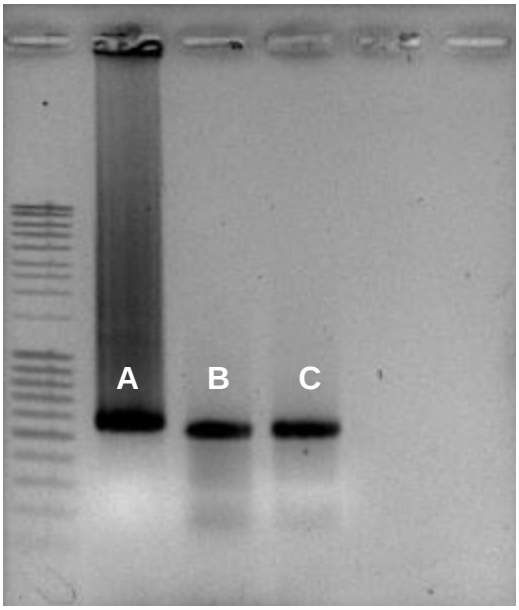
SUPPLEMENT I.

Final PCR product concentrations (sample 1 used as template for dsRNA synthesis)



Gene	Sample	Concentration (ng/µL)
eGFP	1	280.697
eGFP	2	299.118

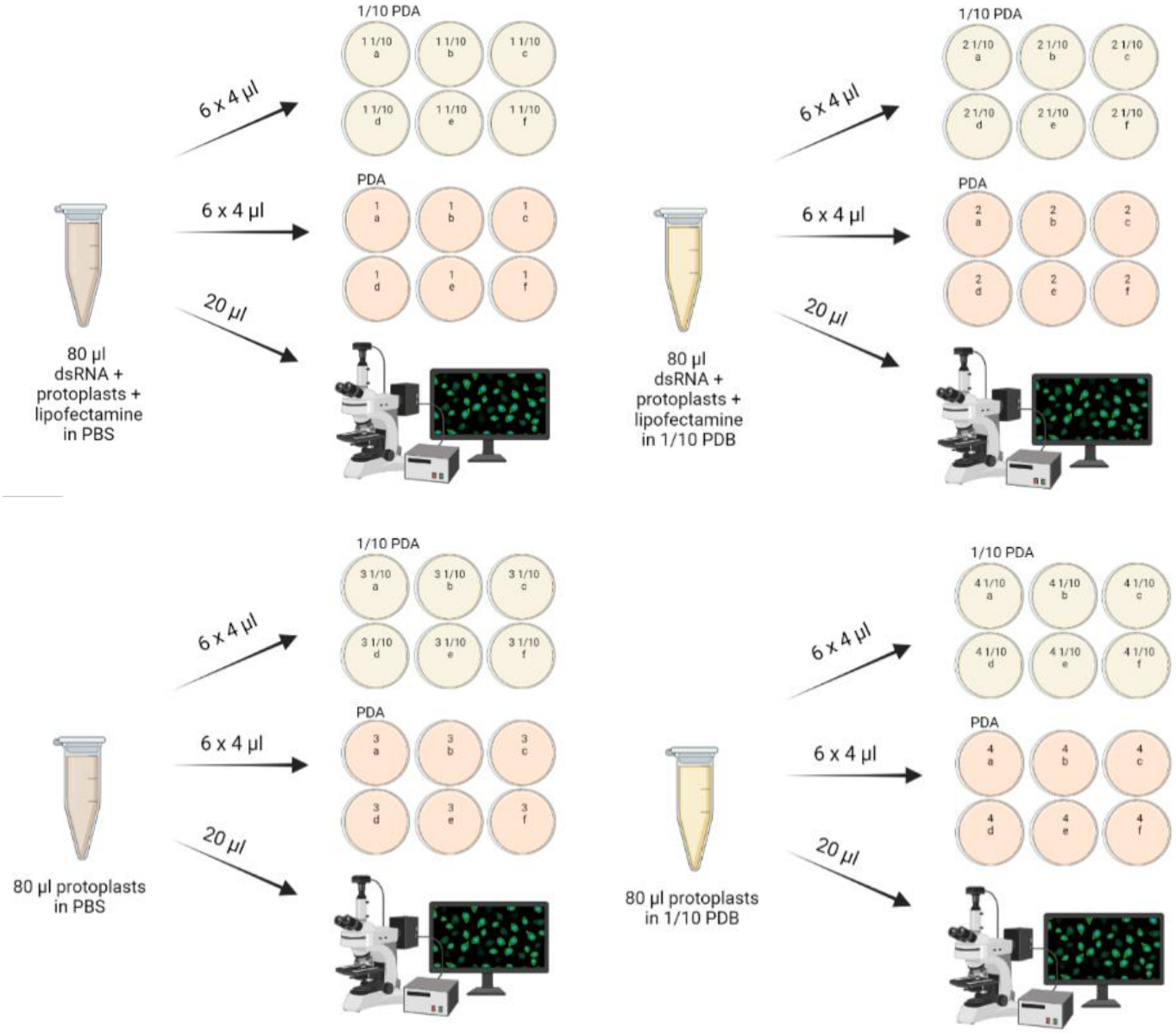
dsRNA synthesis process (A: after transcription reaction; B: after RNA annealing; C: after cleanup)



Gene	Concentration (ng/µL)
eGFP	759.975

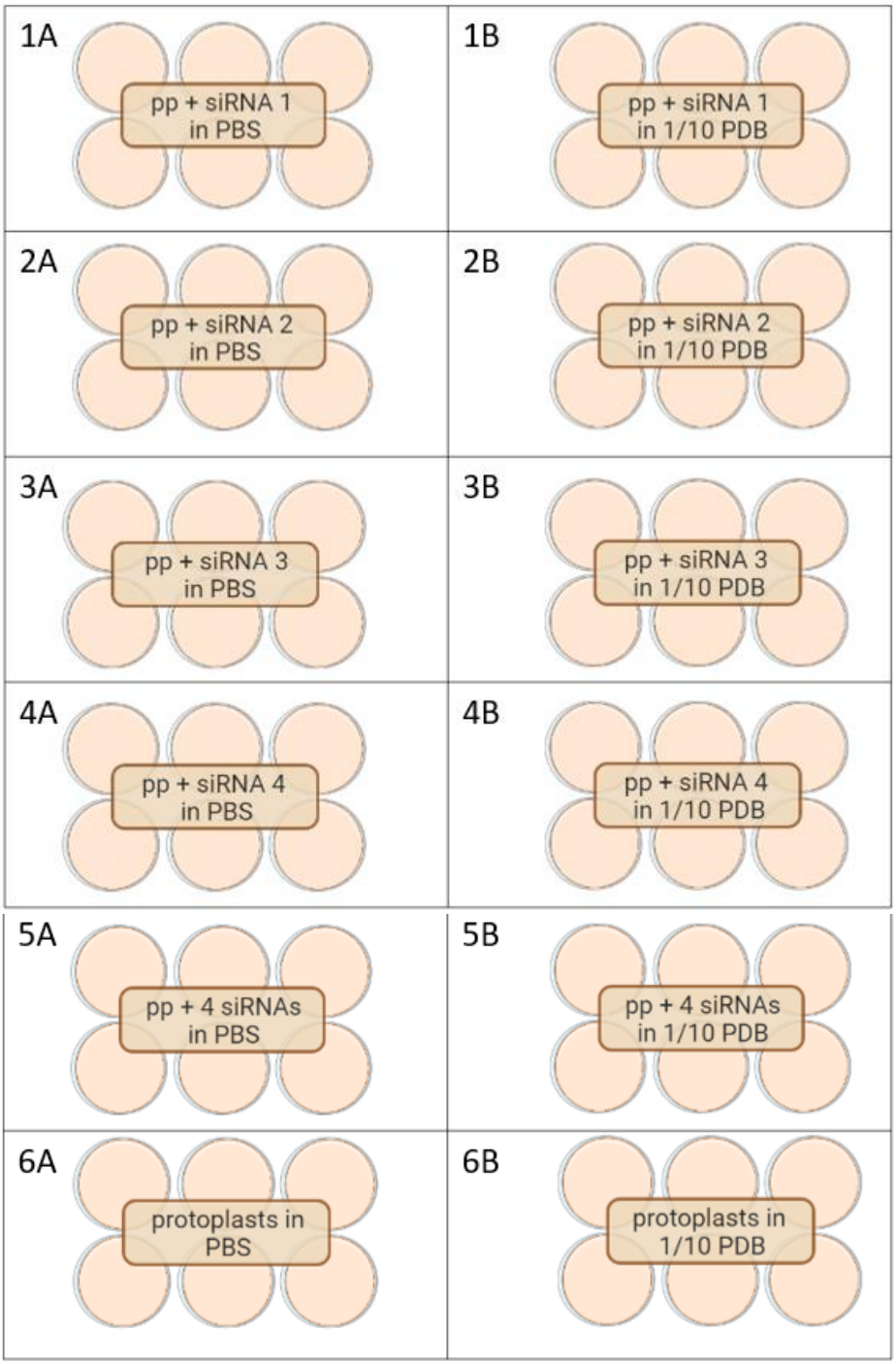
SUPLEMENT II.

Experimental setup of protoplasts + dsRNA treatments (without negative controls):



SUPPLEMENT III.

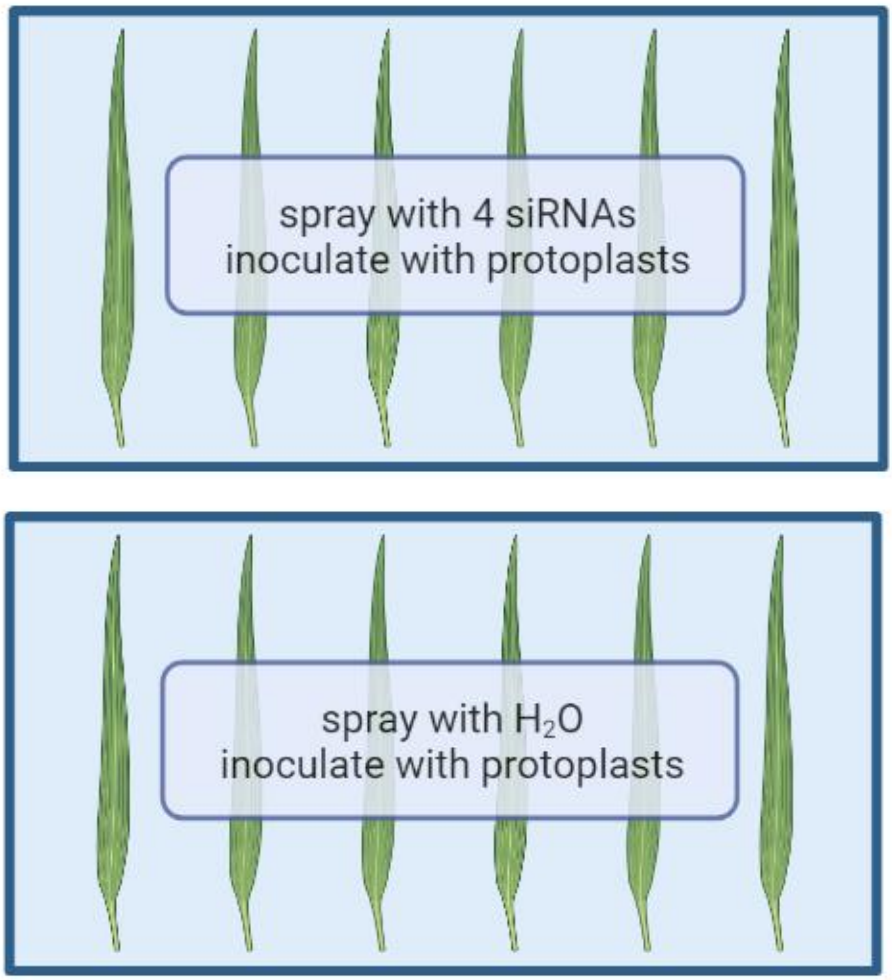
Experimental setup of protoplast + siRNA treatments (pp = protoplasts) on PDA-plates:



*1A - 5B: siRNA complexed to Lipofectamine carriers
(1 siRNA : 2 P300 reagent : 3 Lipofectamine)

SUPPLEMENT IV.

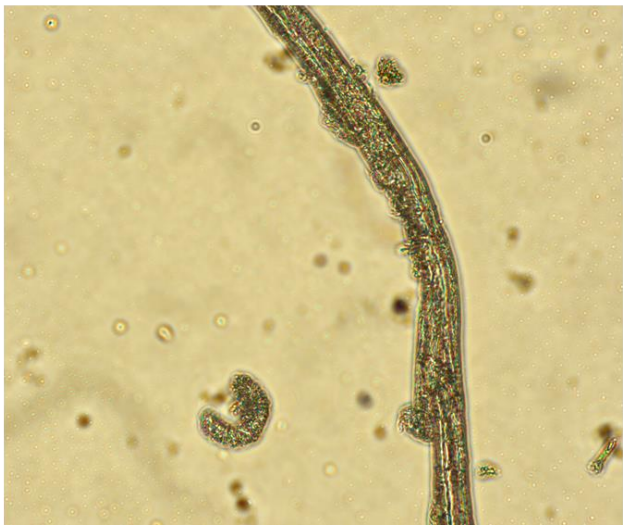
Detached leaf assay for spray-induced gene silencing in wheat:



SUPPLEMENT V.

Fungal mycelium present in the protoplast samples

Widefield



GFP signal (Green)