Controlling Fusarium Head Blight in oat

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Abstract:

Oats (*Avena sativa*) is a versatile crop grown worldwide for animal feed and human consumption. Human oat consumption has recently risen due to its various health benefits. However, oats are susceptible to Fusarium head blight (FHB) caused by various Fusarium fungi. FHB reduces yield and leads to mycotoxin accumulation. The most commonly reported mycotoxins in oat are trichothecenes deoxynivalenol (DON) and T-2/HT-2 toxins. Trichothecenes inhibit eukaryotic protein biosynthesis and cause acute and chronic toxicoses in human and animals. Effective control of FHB is important for ensuring safety and quality of oats. This thesis examines various aspects of FHB in oats, relevant to the development of better FHB control strategies.

Accurate FHB symptom identification is crucial for breeding resistant oats, but the symptoms of FHB are cryptic, causing errors in scoring the disease during trials. This work presents an affordable method for assessing FHB symptoms in oats by de-hulling mature seeds. Symptoms of blackening and discoloration of the oat kernels significantly correlate with Fusarium DNA and mycotoxin accumulation and thus can be used as quantitative disease indicators.

To enhance pathogen resistance, identifying and characterizing plant resistance genes is key. In this work two oat genes coding for DON-detoxifying UDP-glucosyltransferases (UGTs) were identified and characterised. Transcripts of two oat UGTs were highly upregulated in response to DON treatment and *F.graminearum* infection. The genes conferred resistance to several trichothecenes when expressed in yeast. Both UGTs, recombinantly expressed in *E.coli* were confirmed for their ability to detoxify DON. These genes could potentially be used for developing genetic markers for FHB resistance in oat.

Further in this thesis, biocontrol possibilities for FHB in oats are investigated. The fungal BCA *Clonostachys rosea*'s potential against FHB is examined. Treating oat spikelets with *C. rosea* reduced Fusarium DNA and DON content in mature kernels. *C.rosea* enhanced both rate of DON detoxification and expression of DON-detoxifying UGTs. Furthermore, there was significant upregulation of markers of induced resistance, including PR proteins and the WRKY23 transcription factor, indicating that the biocontrol effect of *C. rosea* is attributed to the induction of plant defences.

Additionally, oats' own endophytes were explored for FHB biocontrol. Fungal endophytes from oat spikelets were isolated and tested for reducing FHB in greenhouse trials. The most successful isolate *Pseudozyma flocculosa* significantly reduced FHB symptoms, *F. graminearum* biomass, and DON accumulation in oat. Treatment of oat with *P. flocculosa* induced expression of genes encoding for PR proteins, known to be involved in FHB resistance.

Key words: Oat, Avena sativa, Fusarium head blight, Fusarium graminearum, biocontrol, BCA, Clonostachys rosea, endophytes, mycotoxins, thrichothecenes, deoxynivalenol.

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Table of Contents

Popular summary

Oats (*Avena sativa*) are a versatile cereal crop cultivated worldwide. Accounting for 2% of global grain production, they rank as the seventh most important cereal. Approximately 23 million tonnes of oat grain is produced annually, with the majority stemming from spring-sown cultivars in Canada, Russia, and Northern Europe. Although used mostly for animal feed, oats have increased in their popularity for human consumption over the past 25 years due to their numerous health benefits. High in protein, unsaturated lipids and soluble fibre beta-glucan, oats promote cholesterol reduction, improved glycaemic control, and gastrointestinal health. They are also safer than, *e.g.*, wheat for individuals with celiac disease. In addition, oats have high nitrogen use efficiency, making them suitable for organic crop systems.

However, oats are vulnerable to Fusarium head blight (FHB), a disease caused by various fungal species in the genus Fusarium. FHB not only reduces crop yields but also results in accumulation of harmful mycotoxins in grains. The most common toxins produced by Fusarium fungi belong to a class of compounds called trichothecenes, including deoxynivalenol (DON) and T-2/HT-2 toxins. These mycotoxins can cause vomiting, diarrhoea, and other gastrointestinal symptoms in humans and animals upon ingestion. Trichothecenes exert their harmful effects by interfering with protein biosynthesis in eukaryotic cells. Mycotoxin levels in food and feed commodities are regulated by governing bodies, including European Food Safety Authority and the US Food and Drug Administration. As mycotoxin contamination due to FHB poses a significant public health concern, effective FHB disease control is crucial for farmers and food producers.

Managing FHB in cereals is challenging, with integrated pest management (IPM) strategies offering the best approach for controlling FHB. IPM combines multiple control methods, such as use of disease-resistant cultivars, agronomic practices, and chemical or biological control strategies, tailored to the specific needs and environmental conditions of each growing region. However, no oat varieties are completely resistant to FHB. Genetic resistance to FHB disease is a complex trait, influenced by multiple genes and environmental factors, making breeding for FHB resistance challenging. The absence of a complete oat genome sequence had further impeded progress in this area. Fortunately, in 2022, two high-resolution genome sequences were made public, offering invaluable tools for researchers and breeders

to understand the genetic basis of important oat traits and develop new technologies, such as gene editing, to enhance resistance of oat against diseases.

When it comes to breeding disease-resistant oats, correctly identifying FHB symptoms is essential. However, unlike wheat and barley, in oat FHB symptoms are hidden beneath thick hulls, and to complicate matters further, these symptoms are easily confused with signs of natural ripening of oats. This often leads to guesswork and errors in disease scoring, resorting to costly chemical and molecular biological analyses. Consequently, the quest continues for a quicker, affordable, and dependable method to reveal these elusive FHB symptoms in oat.

To improve genetic resistance against pathogens, it is crucial to identify plant resistance genes and study their functions. One example is the genes encoding UDP-dependent glucosyltransferases (UGTs), which detoxify mycotoxins and other harmful compounds. UGTs bind DON and other trichothecenes with a glucose molecule, transforming the resulting compound into a much less toxic substance. Enhanced activity of such enzymes was directly linked to FHB resistance. In cereal crops like barley, wheat, and rice, researchers have identified several genes, including UGTs, that contribute to FHB resistance. However, the genetic foundation of oats' resistance to FHB has yet to be explored thoroughly.

Together with utilizing moderately resistant cultivars, agronomic practices such as crop rotation and soil tillage can effectively control FHB. In addition, fungicides can reduce FHB severity and prevent mycotoxin production in wheat and barley, but they are largely ineffective against FHB in oats. Even if effective fungicides against FHB in oat could be developed, there is a considerable risk of pathogens developing resistance against fungicides as well as growing societal concerns of negative environmental impacts of excessive fungicide use.

Sustainable and eco-friendly alternatives to fungicides include microbial biological control agents (BCAs), which have been found to reduce FHB symptoms and mycotoxin accumulation in wheat and barley. BCAs encompass bacteria, fungi, and other microorganisms that protect plants through various modes of action, including direct pathogen destruction, competition for nutrients and space, production of antifungal compounds, and induction of plant resistance mechanisms. Recently, endophytic microorganisms, have been gaining interest as potential BCAs. Endophytes, a mix of fungi, bacteria, and other microorganisms, reside within living plant tissues without causing harm. Instead, these quiet inhabitants often boost plant growth and help their hosts fend off pathogen attacks and abiotic stress, while using the plant as a shelter and food source. Although research on BCAs and endophytes against FHB in oats is scarce, studies have shown promising results in wheat.

This work examines different aspects of controlling FHB in oats, such as more accurate disease symptom assessment, identification and functional characterization of DON-detoxifying UGT genes in oats, and the use of BCAs and endophytes for FHB control.

List of Papers

Paper I

Khairullina, A., Jørgensen, H. J. L., Bülow, L., Collinge, D. B., & Jensen, B. Visual signs of kernel damage in oat correlate with Fusarium biomass and mycotoxin content (manuscript).

Paper II

Khairullina, A., Tsardakas Renhuldt, N., Wiesenberger, G., Bentzer, J., Collinge, D. B., Adam, G., & Bülow, L. (2022). Identification and Functional Characterisation of Two Oat UDP-Glucosyltransferases Involved in Deoxynivalenol Detoxification. *Toxins*, 14(7), 446.

Paper III

Khairullina, A., Micic, N., Jørgensen, H. J. L., Bjarnholt, N., Bülow, L., Collinge, D. B., & Jensen, B. (2023). Biocontrol effect of *Clonostachys rosea* on *Fusarium graminearum infection* and mycotoxin detoxification in oat (*Avena sativa*). *Plants*, 12(3), 500.

Paper IV

Khairullina, A., Jørgensen, H. J. L., Collinge, D. B., & Jensen, B. Oat spike endophyte (*Pseudozyma flocculosa*) acts as a BCA against *F. graminearum* in oat (manuscript).

Papers not included in the thesis:

Petrucci, A, Khairullina, A., Sarrocco, S. Jensen, D.F. Jensen, B., Jørgensen, H.J.L., & Collinge, D.B. Understanding the mechanisms underlying plant-mediated control of *Fusarium* diseases in cereals. (Submitted to *European Journal of Plant Pathology*).

My contribution to the papers

Paper I

I planned experiments, designed primers, optimized qPCR for Fusarium identification, prepared samples for LC-MS/MS, assisted in data analysis, and co-wrote and edited the manuscript with co-authors.

Paper II

I took a major part experiment planning. I performed greenhouse cultivation, plant inoculation, RNA preparation, primer design, and gene expression analysis. I cloned genes in yeast and *E. coli* vectors, expressed and purified recombinant oat UGTs, conducted DON-detoxification assays, assisted in LC-MS/MS analysis, and drafted and finalized the manuscript, incorporating co-author revisions.

Paper III

I took major part in planning the experiments. I performed greenhouse cultivation, plant inoculation, sample preparations, primer design, and gene expression experiments and assisted with data analysis. I drafted the manuscript and finalized it together with the co-authors.

Paper IV

I planned the experiments, isolated and identified oat fungal endophytes, performed greenhouse cultivation, plant inoculation, sample preparations, primer design, and gene expression experiments and assisted with data analysis. I drafted and edited the manuscript together with the co-authors.

Abbreviations

3-ADON	3-acetyldeoxynivalenol
15-ADON	15-acetyldeoxynivalenol
ABA	abscisic acid
ABC	ATP-binding cassette
AE	anther extrusion
BCA	biological control agent
BEA	beauvericin
BLAST	Basic Local Alignment Search Tool
cAMP-PKA	cyclic AMP/protein kinase A
CRISPR-Cas9	clustered regularly interspaced short palindrome repeats, CRISPR associated protein 9
CUL	culmorin
DAS	Diacetoxyscirpenol
DON	deoxynivalenol
DON-3G	deoxinivalenol-3-glucoside
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
ENNs	Enniatins
ET	ethylene
ETI	Effector triggered immunity
FAO	Food and Agriculture Organization
FCR	Fusarium crown rot
FDK	Fusarium damaged kernels
FGSC	Fusarium graminearum species complex
FHB	Fusarium head blight
FSAMSC	Fusarium sambucinum species complex
GMO	genetically modified organism
GPCR	G-protein-coupled receptors
GSH	L-glutathione
GST	glutathione-S-transferases

GWAS	genome wide association study
HIGS	Host-Induced Gene Silencing
HPLC	High-Performance Liquid Chromatography
HRMS	high-resolution mass spectrometry
IPM	integrated pest management
ITS	internal transcribed spacer
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LFIA	lateral flow immunoassay
MAMP	Microbe-Associated Molecular Patterns
MAPK	mitogen-activated protein kinases
MAS	marker-assisted selection
MDR	multiple drug resistance (transporters)
MFS	major facilitator superfamily (transporters)
MLST	Multi-Locus Sequence Typing
MON	moniliformin
MRL	maximum residue levels
NCBI	National Centre for Biotechnology Information
NIV	nivalenol
NO	nitric oxide
PAMP	Pathogen-Associated Molecular Patterns
PCD	programmed cell death
PR	pathogenesis related (proteins)
PTI	pathogen triggered immunity
QTL	quantitative trait locus
RGB	Red, Green, Blue (colours used in digital display)
ROS	reactive oxygen species
SA	salicylic acid
SIGS	Spray-Induced Gene Silencing
TAFC	triacetylfusarinine C
TDI	tolerable daily intake
TEF1α	translational elongation factor 1α
TF	transcription factor
UGT	UDP-dependent glucosyltransferases
USDA	United States Department of Agriculture
WRKY	conserved amino acid sequence WRKYGQK
ZEA	zearalenone

Introduction

Oats (*Avena sativa*), a member of the Poaceae family, are a versatile crop grown worldwide for animal feed, human consumption, and non-feed applications. While 74% of oats are used for animal feed, the crop has experienced a shift in usage over the last 25 years [1,2]. Particularly, human consumption of oats has surged due to its array of health benefits, with a standout feature being its high content of the unique soluble fibre, beta-glucan. Oat beta-glucan is believed to actively contribute to reducing cholesterol levels, enhancing glycaemic control, and promoting better gastrointestinal health [3–6]. Moreover, oats are safer for individuals with coeliac disease due to their lower prolamin (gluten) protein content [3,7]. Oats' nitrogen efficiency make them useful in low-input crop rotation [1,8]. The oat genome has been sequenced recently, providing valuable insights into the genetic basis of important traits such as yield, disease resistance and nutritional content [9,10].

Oat is susceptible to Fusarium head blight (FHB) which not only affects yield but also leads to the accumulation of mycotoxins. These mycotoxins are harmful to both human and animal health and can result in reduced feed quality, food safety issues, and economic losses for farmers [11,12]. Therefore, controlling FHB is crucial to ensure the safety and quality of oat-based products, and several strategies have been developed, including crop rotation, breeding for resistance, fungicide applications and biocontrol [13,14].

FHB in oat can be caused by several species of Fusarium, with *F. graminearum, F. culmorum, F. poae, F .avenaceum* and *F. langsethiae* identified as the main causal agents in recent years [15–20]. Composition of the Fusarium species involved in FHB outbreaks varies based on factors such as geographic location, crop type and cultivar, weather conditions, agricultural practices, and niche-competition with other pathogens [21,22].

Symptoms of FHB in oats are rarely apparent and can be easily confused with senescence of glumes and hulls covering kernels, leading to errors and biases during the greenhouse and field trials. [23]. **Paper I** in the current work describes how symptoms of Fusarium infection of oat can be more accurately accessed when de-hulling the mature seeds upon harvest. The symptoms of blackening and discoloration of the kernels correlate with high amounts of Fusarium DNA and accumulated mycotoxins and thus can be used in scoring of the FHB disease.

The most prevalent mycotoxins produced by FHB-causing fungi in oat are group A and B trichothecenes [7–9]. In oat grains, the commonly reported group A trichothecenes are T-2 and HT-2 toxins, while the group B trichothecenes include deoxynivalenol (DON) and nivalenol (NIV). Apart from most common mycotoxins, several others such as Zearalenone (ZEA), Beauvericin (BEA), moniliformin (MON) and Enniatins (ENNs) are often reported in oat grains [10–13]. As mycotoxins cause acute and chronic toxicoses in human and animals, specific regulations dictating permitted mycotoxin levels are proclaimed by the authorities, such as European Commission, the Food and Drug Administration of United States (U.S. FDA) and others [14,15].

Trichothecenes exert their toxicity by inhibiting eukaryotic protein biosynthesis through their interaction with ribosomes [16–18]. In *F. graminearum* pathogenicity, DON plays an important role by acting as a virulence factor [19–21]. Plants have different molecular mechanisms to detoxify mycotoxins, of which, conjugation of trichothecenes with glucose seems to be the principal detoxification mechanism[22–25]. Conjugation of DON into DON-3-glucoside (DON-3G) is catalysed by uridine diphosphate-glucosyltransferases (UGTs), a large superfamily of enzymes involved in specialised metabolism in plants [26,27]. DON-detoxifying UGTs were characterized in different cereal species, such as *Brachypodium*, wheat, barley and rice [28–31]. The increased glycosylation of DON with the help of UGTs has been directly linked to resistance of plants to *F.graminearum* infection. It was shown that the transgenic expression of barley *HvUGT13248* gene in wheat conferred resistance to both DON and NIV and decreased disease severity of FHB and Fusarium crown rot (FCR).

In the present work, **paper II** describes identification and characterization of two oat UGT genes, *AsUGT1* and *AsUGT2*, orthologous to barley *HvUGT12348*. Both UGT genes were strongly upregulated following treatment with DON and *F.graminearum* infection and conferred a high level of resistance to trichothecenes DON, NIV and HT-2 in yeast. Both enzymes, expressed recombinantly in *E.coli*, showed the ability to convert DON into DON-3G. *AsUGT1* and *AsUGT2* aid in effective mycotoxin detoxification and thus potentially could serve as markers for the selection of FHB resistant lines and cultivars.

Spraying fungicides at anthesis is another control method for FHB in cereals. However, this carries the risk of fungicide resistance in the pathogen[32,33]. The effectiveness of fungicides against FHB in oats varies widely, often proving inefficient, and greatly relies on the specific oat cultivars and Fusarium species involved [34–37]. Recently, there has been a growing focus on using biological control agents (BCAs) as sustainable alternatives to chemical fungicides [38]. BCAs demonstrate diverse modes of action to control pathogens, including the activation of the plant's own defence genes, which hinders pathogen infection [39]. **Paper III** describes the ability of the fungal BCA *Clonostachys rosea* IK726 to substantially reduce Fusarium DNA (79%) and DON accumulation (80%) in oat kernels. *C.*

rosea-treatment resulted in higher conversion of DON to DON-3G and in a significant enhancement of expression of two oat UGT-glycosyltransferase genes. Furthermore, the treatment with *C. rosea* activated the expression of genes encoding four PR-proteins and a WRKY23-like transcription factor, signifying that *C. rosea* induces resistance in oat. The paper proposes that *C. rosea* IK726 has a strong potential to be used as a BCA against FHB in oat.

In the ongoing search for new and effective BCAs, endophytic microorganisms have emerged as a promising source. Endophytes reside within living plant tissues, promoting plant growth and stress resistance while utilizing the plant as a habitat and nutrient provider [40]. Many endophytes have been found to offer protection against plant pathogens. **Paper IV** focuses on isolating fungal endophytes from oat spikelets and examining their effect on reducing FHB and mycotoxin content in mature oat grain. Furthermore, the most promising BCA candidate, *Pseudozyma flocculosa,* is studied for its ability to induce resistance in oat spikelets.

The thesis is organized as follows: a comprehensive literature review covering various facets of managing FHB in oats is compiled. Overview of resent studies are provided into the causal agents of FHB and the mycotoxins they produce, along with the mechanisms underlying their toxicity. A particular focus is directed towards elucidating the detoxification of mycotoxins within plants. Aspects of breeding of oats that are resistant to FHB are included, as well as other FHB controlling strategies (agricultural practices, use of fungicides, etc). The thesis further explores aspects of biological control. The modes of action of different BCAs are discussed, highlighting the most effective ones within the context of FHB. Finally, the utilization of endophytes as BCAs is examined, focusing on essential practices during their isolation and testing. The experimental findings presented in papers I-IV are integrated into the corresponding chapters, aligned with the relevant topic.

Oat

Oats (Avena spp.) are annual grasses belonging to the Poaceae family, which includes economically important cereal crops such as wheat, rice, barley, and maize. Oats are found in nature as diploids, tetraploids, and hexaploids [1]. The greatest genetic diversity of Avena species is observed in regions around the Mediterranean, Middle East, Canary Islands, and Himalayas. The oat plant exhibits a panicle-type inflorescence, which enables it to regulate the number of grains during the grain filling stage [41]. The primary cultivated oat species is hulled oat *Avena sativa*. Other agricultural species, such as *A. strigosa*, *A. byzantina* and *A. abyssinica* are also grown in some regions for animal feed and fodder [4]. The oat spikelet of a hulled oat (**Figure 1**) typically comprises one to three florets arranged in a primary, secondary, and tertiary hierarchy according to their position within the spikelet [42, 43]. Each floret is confined within two overlapping protective bracts - lemma and palea and the whole spikelet is protected by two outer glumes [1, 46].

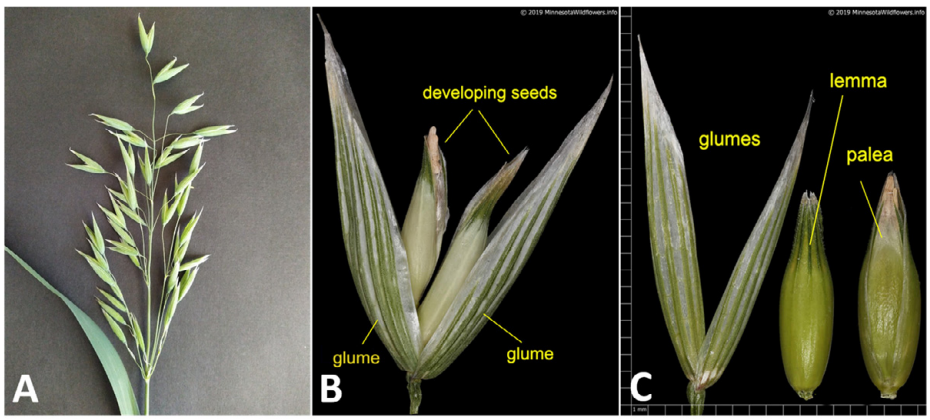

Figure 1. Oat (*Avena sativa*) panicle (A), a spikelet (B), a dissasembled spikelet (C). Images B and C are by K. Chayka and by P. M. Dziuk, modified (https://www.minnesotawildflowers.info/).

Oats are versatile crop that are grown worldwide for animal feed, human consumption, and non-feed applications [2]. Historically, oats had great importance due to its traditional use as animal feed, especially for workhorses and on-farm animals. Farm mechanizations, which required less horsepower and a shift for high-

input crops such as maize, wheat, and soybean led to decrease of oat cultivation from 46.9 to 26.3 million metric tons from early 1970s and on (**Figure 2**). Currently, worldwide production of oat grain is approximately 24 million metric tons per year, grown over 9.5 million hectares [44]. Oats account for a mere 1.3% of global grain production and thus represent the seventh most important cereal [1,44]. Of this, 77% of the world's oat is produced in Europe, Canada, Russia and Australia. Most oat production comes from spring-sown cultivars in Northern Europe, Russia and Canada, while autumn-sown cultivars are grown in the UK, Southern USA, and Australia [1]. Poland, Finland, UK, Spain and Sweden are the leading oat producers in Europe [44]. In Sweden over last decade between 363.5 thousand tons and 815.5 thousand tonnes oats had been harvested yearly (**Figure 3)** (data from *Statistikmyndigheten* SCB). Sweden is the third largest oat exporter in the world after Russia and Finland [44].

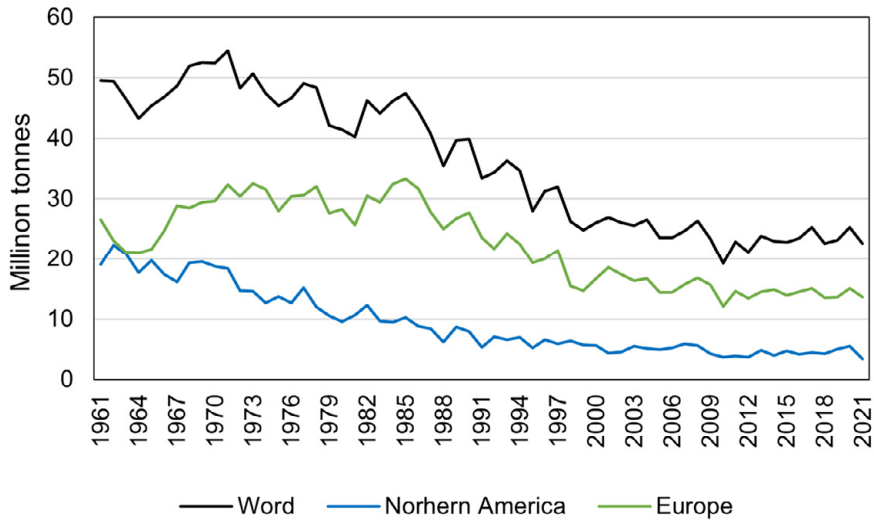

Figure 2. Production of oat in the word, Northen America and Europe from 1961 to 2021 (FAOSTAT)

Approximately 75% of the oat crop is utilized as animal feed for cattle, sheep, horses, and to a lesser extent, poultry [1,45,46]. The majority of oats in the world are used as feed on the same farms where they are grown [1]. Hulled oats are utilized as a feed source for ruminant animals, as the hulls contribute valuable fibre content to their diet [47]. To be used as feed for pigs, oats need to be de-hulled (or naked varieties can be used) as pigs do not have the digestive enzymatic system to process the tough fibres. The same is true for specialized feed for racehorses, which require a high-energy diet [47]. Additionally, oats are used as a forage crop, particularly in subtropical regions [48,49].

While usage of oat for feed has decreased worldwide during last 30 years, its usage for human consumption has been increasing due to the discovery of many health benefits [2]. Oats contain 7–14% of dietary fibre including 3–5% of β-glucan ((1→3), (1→4)-β-D-glucan), which is one of the important functional components of oats. β-glucan is a water-soluble fibre with high viscosity and located in the outer layer (aleurone) of an oat grain. Four approved European Food Safety Authority (EFSA) health claims apply to oat beta-glucans. These include the ability to reduce cholesterol levels, as well as to delay carbohydrate absorption, leading to improved glycaemic control and reduced risk of diabetes type II [2,3,6,50-52]. Moreover, beta-glucans act as a prebiotic, promoting gastrointestinal health through the stimulation of beneficial bacteria growth in the gut and improved bowel regularity[6]. Another type of oats soluble fibres, arabinoxylans, similarly, contribute to good digestive health, controlled blood sugar levels and a favourable gut microbiome [53].

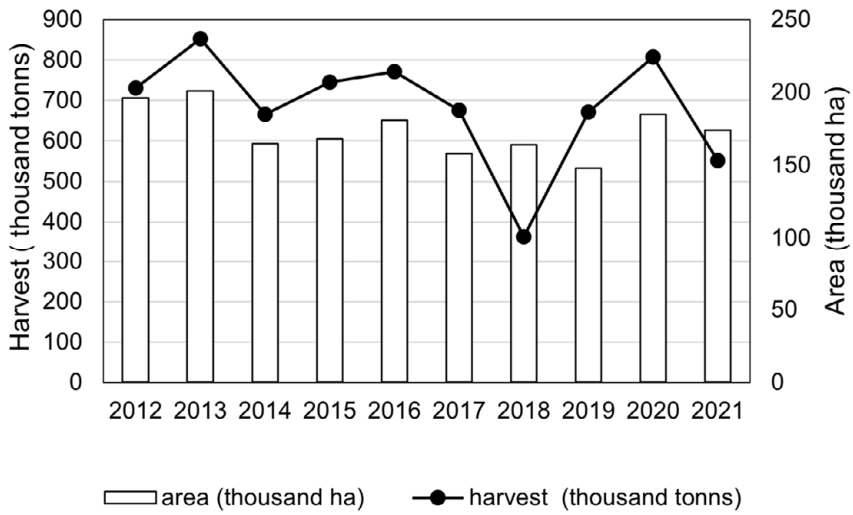

Figure 3. Crop production and cultivated area for oat in Sweden during 2012-2021.

One more notable attribute of oats is their high unsaturated lipid content, which varies between 6-12% across different varieties [54]. One more EFSA approved health claim is related to the high content of unsaturated fatty acids in oat [3, 55].

Oats have a high protein content compared to other cereals, ranging from 12-22% and the best amino-acid balance among cereals [4,56,57]. In fact, oats are known to contain the highest levels of lysine compared to other grains, making them an important source of this essential amino acid in vegetarian and vegan diets [2,57].

Additionally, oats are abundant in antioxidants such as vitamin E, avenanthramides (exclusive to oats), phenolic acids, flavonoids, sterols, and phytic acid [50,52].

Oats are safer for the majority of people affected by coeliac disease, as they have much less prolamins (gluten), which trigger coeliac disease and food allergies (15% compared to 40% in wheat)[3,57]. Recently, a number of new value-added food products have been emerging in the market, such as oat milk, oat yogurt etc.[55,58]. These products are often marketed as healthy and sustainable alternatives to dairy-based products and are suitable for individuals with lactose intolerance or dairy allergies. In terms of environmental impact, oat milk has a lower carbon footprint than cow's milk, as it requires less water and land to produce [59]. Oats are a common ingredient in the cosmetics industry due to their natural soothing and moisturizing properties [2].

Agronomically, oats have greater nitrogen use efficiency, therefore they require relatively low fertilizer input and are productive in a wide range of soils [1,59]. Oats are often grown in minimal land area thereby inhibiting the soil from eroding. They can be used in crop rotations and as a winter cover crop, as sustainable agriculture practices for soil conservation [1,59]. The ability of oat roots to synthesize saponins, possessing strong fungicidal activity against a range of soil-borne fungal pathogens make oat an attractive crop rotation plant [60].

The genome of oat has been sequenced and assembled recently, providing a valuable resource for studying the genetic basis of important traits such as yield, disease resistance, and nutritional characteristics [61,62]. The genome of hexaploid oat has an estimated size of 12.5 Gb, which is more than three times larger than the human genome. It contains seven chromosome pairs originating from each of its three diploid progenitors, which harbour at least 120,000 protein-coding genes [61,62].

Although oats are considered to be more resistant to pests and diseases compared with other cereals, they are prone to a number of diseases, mostly fungal, that can impact yield and quality significantly. Some of the most common fungal diseases affecting oat include Crown rust (*Puccinia coronata f.sp. avenea*), Powdery mildew (*Blumeria avenea*), Leaf spot (*Pyrenophora avenea*), Leaf blotch (*Parastagonospora avenae*) and Fusarium head blight (FHB) [1,4,63,64]. While the first four affect yield, FHB leads not only not only to yield reduction but also accumulation of mycotoxins. These mycotoxins are harmful to both human and animal health and can result in reduced feed quality, food safety issues, and subsequent economic losses for farmers [8,65]. Therefore, controlling FHB is crucial to ensure the safety and quality of oat-based products. The best way to control FHB, both economically and ecologically, is via integrated disease management, which combines several strategies such as use of resistance genotypes, crop rotations, chemical and biological protection, monitoring and predicting disease outbreaks and others.

FHB epidemiology

Causal agents of FHB in small cereals

The genus Fusarium belongs to the phylum Ascomycota, class Sordariomycetes, order Hypocreales, family Nectriaceae. It is one of the most frequently occurring genera of plant-pathogenic fungi in the world and one of the most important mycotoxin-producing genus [66]. Fusarium species cause disease losses in cereals worldwide, such as Fusarium head blight (FHB) and Fusarium crown rot (FCR) [33,67,68]. FHB is a particularly devastating disease of small cereal crops, including bread wheat (*Triticum aestivum*), durum wheat (*Triticum turgidum subsp. durum*), oat (*Avena sativa*), barley (*Hordeum vulgare*) and triticale (x *Triticosecale*), and can result in significant yield losses, reduced grain quality and contamination of grain with mycotoxins [33,69–71].

Since its initial description in the beginning of 1800s, the taxonomic classification of the genus Fusarium has undergone numerous revisions and refinements [66]. This genus is a challenging group to classify due to the high level of morphological and genetic diversity among its species and the fact that many are only known from the anamorph (asexual) phase [76]. Currently, there are at least 300 phylogenetically distinct species recognized, of which almost half are not formally described [76]. Additionally, new species of Fusarium are continuously being discovered, particularly in regions with high levels of crop diversity and fungal activity [10]. Recent progress in molecular biology and genetics has led to a more profound comprehension of the evolutionary connections and genetic variability present within the genus Fusarium [72,73] [74]. Thus, the genus Fusarium has been reclassified into 23 species complexes, which comprise closely related lineages of multiple species, grouped based on common phenotypic characteristics and toxin production [76, 81, 82] [74]. According to this classification, causal agents of FHB in small cereals belong to *Fusarium sambucinum* species complex (FSAMSC) [75]. The new classification has not been widely accepted yet, and this could cause some confusion especially in the case of several species under broad term *Fusarium graminearum sensu lato*, which have been previously grouped to *Fusarium graminearum* species complex (FGSC). Many species among FSAMSC produce a wide range of mycotoxins, varying in their chemical structure and degree of toxicity for plants and animals [76].

FHB was first documented, in England, by W.G. Smith in 1884, and since then numerous severe epidemics have occurred worldwide, resulting in extensive crop damage and yield losses [65,77–79]. FHB in cereals can be attributed to a minimum of 17 Fusarium species [80]. Two non-toxicogenic fungi belonging to genus Microdochium (*M. nivale* and *M. majus*) are often co-occur with Fusarium pathogens [70,81,82]. Prevalent Fusarium species and accumulated mycotoxins in oat worldwide over last twenty years are listed in **Table 2**. *F. graminearum, F. langsethiae*, *F. poae* and *F. avenaceum* have been dominating in oat FHB. The mycotoxins most often found in oat are deoxynivalenol (DON) and T-2/HT-2 toxins, produced by *F. graminearum* and *F. langsethiae*, respectively. Oat is more susceptible to *F. langsethiae* infection compared to wheat and barley and infections by *F. langsethiae* in oat are symptomless, even when they cause accumulation of high level of T-2/HT-2 toxins produced by this fungus [83–86].

F. graminearum is regarded as the most virulent causative agent of FHB, while *F. langsethiae* is considered a weak pathogen [87], although the strain of a Fusarium species may also play a role [88–90]. Some Fusarium species, such as *F. poae*, are known to act as opportunistic pathogens, as the presence of other Fusarium species can enhance their ability to infect plants, and they may work synergistically to undermine the plant's defences [82,91]. The specific composition of Fusarium species involved in FHB outbreaks can vary based on geographic location, crop type and cultivar, weather conditions, agricultural practices and niche-competition with other pathogens [82,92–95]. Main FHB causing agents have been shifting over the last 20 years depending on environmental conditions and agricultural practices used in cultivation [94,96]. The severity of the FHB disease for a given cereal depends on the aggressiveness of the main causal agent, cultivar resistance and the environmental factors, specially the relative humidity around anthesis of the cereal crops [71,95,97]. *F. poae* and *F.avenaceum* are found to co-occur with *F.graminearum* [91], while *F. graminearum* and *F. langsethiae* occurrences are negatively correlated [90].

It is worth noting that Fusarium species can colonize plants as endophytes without inducing any disease. Notably, several mycotoxin-producing Fusarium species, including *F. graminearum*, *F.poae* and *F.avenaceum* were found in asymptomatic grasses in North American prairies [98].

Table 2. Fusarium species and mycotoxins, reported in oat worldwide during the period from 2004 to 2020 (NA - not analysed in this work). Symbols equal = and more > are used to indicate relative quantitative differences.

Years of survey	Country	Prevalent Fusarium spp.	Prevalent Mycotoxins	Refs
2004-2009	Norway	*F. graminearum* = *F. langsethiae* = *F. avenaceum* > *F. poae* > *F. culmorum*	DON, ZEA, ENNs, T-2/HT-2, MON	Hofgaard et al. 2016 [99]
2007	Denmark	*F. langsethiae* > *F.graminearum* > *F. avenaceum* > *F.poae*	T-2/HT-2 > DON > NIV	Nielsen et al. 2011 [70]
2010-2011	Sweden	*F. poae, F. langsethiae, F. avenaceum*	DON, NIV, BEA, ENNs	Fredlund et al. 2013 [100]
2004-2018	Sweden	*F. graminearum, F.poae, F.langsethiae, F.culmorum*	DON, NIV, T-2/HT-2, ZEA	Karlsson et al 2022 [101]
2007-2008	Belgium	*F. poae* and *F. graminearum*	NA	Audenaert et al. 2009 [102]
2005–2014	Finland	*In 2005: F. avenaceum; in 2006: F. langsethiae = F.poae > F. graminearum > F.culmorum*	In 2006: T-2/ HT-2, DON; In 2012-13: DON	Hietaniemi et al. 2016 [103]
2002-2005 2006-2008	UK	NA	T-2/HT-2 > DON T-2/HT-2	Edwards 2009, Edwards 2017 [35,104]
2013-2014	Switzerland	*F. poae > F. graminearum, F. langsethiae, F. avenaceum, F. culmorum.*	T-2/HT-2, NIV, DON	Schöneberg et al. 2018 [105]
2016-2019	Spain	NA	T-2/HT-2, ZEA, DON	Tarazona[106]
2001-2017	Ontario, Canada	*F. poae > F. graminearum, F. sporotrichioides, F. avenaceum, F. equiseti*	NA	Xue et al. 2019 [107]
2014-2017	Canada	NA	BEA, DON, CUL	Tittlemier et al. 2020 [108]
2016-2018	Manitoba, Canada	*F. poae > F. graminearum, F. sporotrichioides, F. avenaceum, F. culmorum*	DON, NIV, BEA	Islam et al. 2021 [109]
2015-2016	Ireland	NA	T-2/HT-2, ENNs, BEA	Colli et al. 2021 [10]
2020	Spain UK	*F.poae, F.langsethiae* *F.poae, F.lansethiae, F.cerealis, F.tricinctum species complex*	DON, T-2 BEA, NIV, T-2	Gil-Serna et al. 2022 [110]

FHB disease cycle

Fusarium fungi survive the winter by colonizing on the crop debris from the preceding season (**Figure 4**). In spring, they grow saprophytically on plant residues [69]. Under warm and moist conditions, fungi produce abundant sexual ascospores or asexual conidia. Of prevalent pathogen species only *F. graminearum* and *F. avenaceum* are known to possess the sexual stage [111]. Depending on the species, three types of asexual spores can be produced, namely macroconidia in sporodochia, microconidia on conidiophores, and chlamydospores within hyphae [112,113]. The existence of a sexual stage in *F. graminearum* promotes genetic recombination, thereby increasing the possibility of emergence of new strains with enhanced virulence [67].

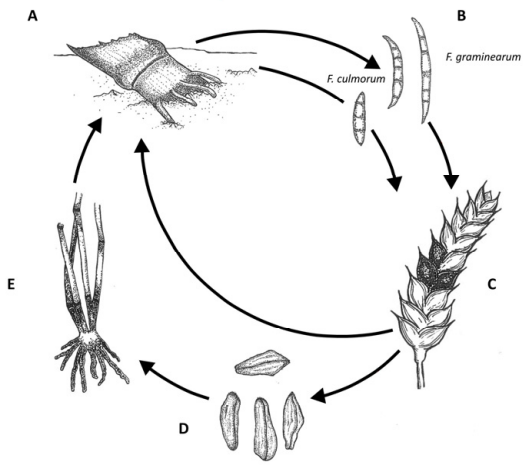

Figure 4. Disease cycle of Fusarium on oat (adopted from Rojas et al., 2018 and modified).
A) Saprophytic phase on crop residues, B) Production of macroconidia or ascospores, C) Infection of flowering cereal spikelets, D) Fusarium damaged kernels with mycotoxin contamination, E) Infected grains can be used as seeds and may produce Fusarium crown rot on seedlings.

Ascospores and conidia are spread by wind and water splashing during the rain. Fusarium species primarily infect small cereal heads during the period spanning from anthesis to the soft dough stage. In oats, the most severe infections occur during anthesis [114,115]. The development and progression of fungal growth, infection, and disease in head tissues are highly favoured by a warm and moist environment. In oat, germinated fungal mycelium enters floret cavity via floret mouth. An alternative pathway for infection occurs through the crevices located between the palea and lemma [114]. It is worth noting that the spread of infection in oat is restricted to a single spikelet and does not spread between separate spikelets [116].

Symptoms of FHB in oat

In wheat and barley, FHB damage to spikes, *i.e.*, "blighting", is normally distinct and can be quantified [117–119]. Mature wheat and barley kernels affected by FHB often exhibit discoloration and abnormal, shrunken form [71,120,121]. In contrast, symptoms of FHB in oat are not apparent due to thick oat hulls concealing the kernel. In addition, bleaching of glumes and hulls of oat can be easily confused with the appearance of natural senescence. This makes the assessment of disease less reliable and prone to error. Current methods to access FHB in oat are rather elaborate and costly. As visual methods, the examination of germination capacity of the seeds or the of appearance of Fusarium growth on the seeds placed on agar plates are used. More exact methods such as LC-MS/MS and immunoassays to quantify mycotoxins [132,133, 134], or qPCR to quantify Fusarium fungal biomass [130,131] need specialized equipment and/or expensive chemicals.

In the present work, **paper I** describes how symptoms of Fusarium infection of oat can be more accurately accessed when de-hulling the mature seeds upon harvest. FHB in oat manifests itself as blackening and discoloration of the kernels (**Figure 5**).

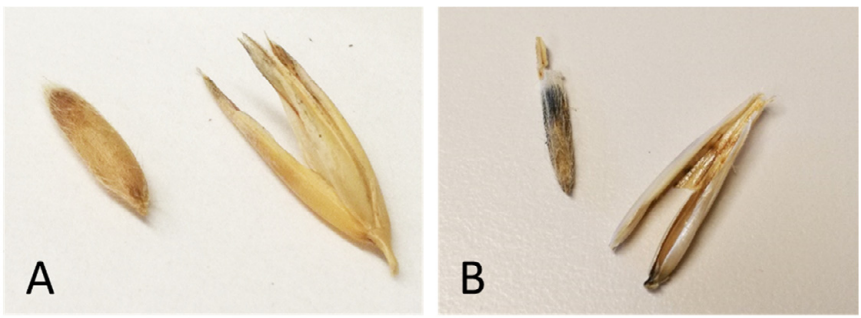

Figure 5. Dee-hulled oat symptoms with (A) no FHB symptoms, (B) with clear FHB symptoms (paper I).

We have accessed Fusarium damaged kernels by dehulling samples obtained from 3 different sources: greenhouse trials, artificial field trials and naturally contaminated farmers' fields. Seeds with visible symptoms contained considerably larger quantities of both Fusarium DNA and corresponding mycotoxins compared to the symptomless seeds (**Figures 6 and 7**).

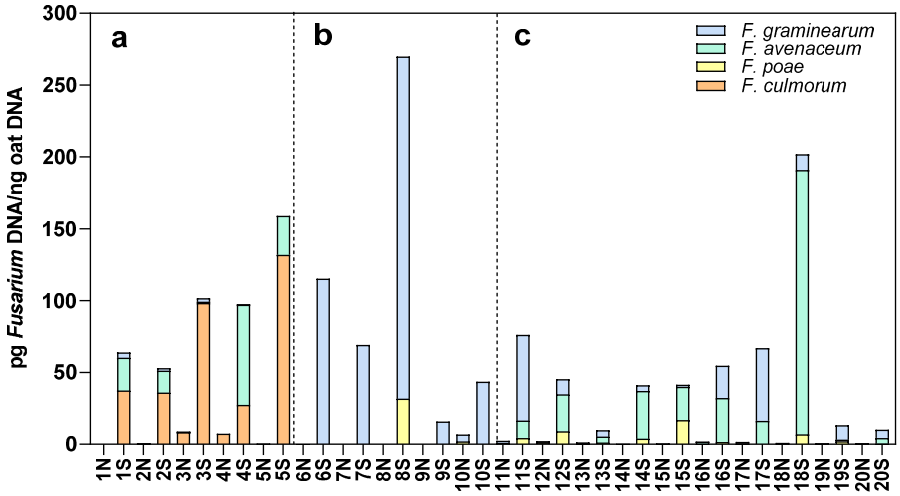

Figure 6. Amount of DNA of four different Fusarium species in 20 sorted samples: (a) greenhouse trial, (b) field pawn inoculation trial, (c) farmers samples. Fractions with no symptoms is marked with letter N, fraction with visible symptoms is marked with letter S (paper I).

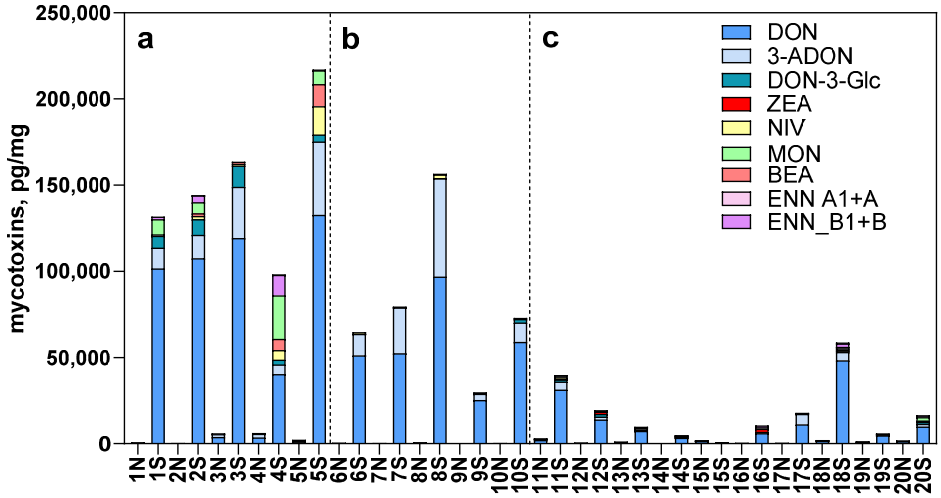

Figure 7. Levels of mycotoxins in 20 sorted samples: (a) greenhouse trial, (b) field spawn inoculation trial, (c) farmers samples. Fractions with no symptoms is marked with letter N, fraction with visible symptoms is marked with letter S (Paper I).

The correlations between the symptoms and the amount of Fusarium DNA and mycotoxins accumulated in such damaged kernels were significant (paper I). In the samples analysed, *F. graminearum, F. culmorum, F. avenaceum* and *F. poae*

contributed to the appearance of symptoms. Analysis of Fusarium DNA content in single kernels confirmed these observations (**Figure 8**). Most of the kernels with symptoms contained high amounts of Fusarium DNA. The symptoms of the seeds where Fusarium DNA was not detected were most probably caused by other fungi (Microdochium spp) or insect infestation (Paper I).

Fusarium damaged kernels (FDK) of oat have much lower density compared to healthy ones, due to the amount of air trapped under the lemma and palea. This allows separation of damaged kernels from healthy ones by density, as described in **paper I**. This difference in density of fusarium-damaged and healthy seeds potentially could be used to develop inexpensive industrial application.

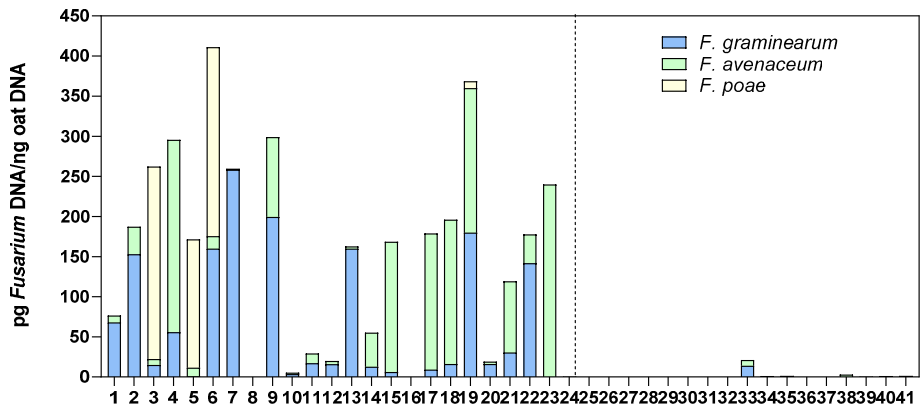

Figure 8. Amount Fusarium DNA in individual oat kernels with damage symptoms (kernel No. 1-24) and kernels without visible symptoms of damage (kernel No. 25-41). Kernels were de-hulled for assessment of symptoms. Paper I.

Assessment of FDK by dehulling oat kernels is a rapid and reliable *ad-hoc* tool to evaluate Fusarium infections prior to using more precise but expensive and time-consuming methods. In **paper IV**, we have successfully used de-hulling to evaluate the effect of biocontrol agents on *F. graminearum* infection in oat.

Mycotoxins, produced by Fusarium species causing FHB

Structure and toxicity of Fusarium mycotoxins

Mycotoxins are low-molecular-weight specialised (also known as secondary) metabolites produced by fungi and toxic to other organisms [23]. During seasons when Fusarium fungi do not actively colonize living plants, they have to survive saprophytically on crop debris from previous seasons. In such environments mycotoxins significantly aid mycotoxigenic fungi's defensive strategies against resident microbes, often serving as essential chemical mediators in these competitive or cooperative interactions [22,137–139]. Fungi also potentially can use mycotoxins as signalling molecules to modulate host responses and enhance colonization. Moreover, mycotoxins often make fungi more effective in their role as plant pathogens by increasing pathogenicity in plants [22,137,139].

The most prevalent mycotoxins found in oat grains during recent years are trichothecenes, which are produced by several causal agents of FHB. Trichothecenes are heterocyclic sesquiterpene molecules, containing epoxy-group which is crucial for the stability of the molecule and its toxicity (**Figures 9 and 10**)[140] [60]. More than 200 trichothecenes have been described and they are divided into four types (A–D) according to their chemical structure. Trichothecenes produced by Fusarium fungi belong to Types A and B, while Type C and D trichothecenes are not associated with FHB [8,141][23,60].

A and B types differ by the presence of different functional groups in the C-8 position of the molecule backbone (**Figure 10**). Most important Type A trichothecenes include T-2 and its hydrolysed form HT-2, produced mainly by *F. langsethiae* and *F. sporotrichoides* [9,141,142]. As was mentioned earlier, oat is more susceptible to infection by *F. langsethiae* and *F. sporotrichoides*, therefore T-2/HT-2 toxins have been found to accumulate in oat in high quantities [83–85,143]. Main type B trichothecenes found in oat are deoxynivalenol (DON) and nivalenol (NIV). DON is produced by *F. graminearum* and *F. culmorum* [7,22,76,144], while the main producers of NIV are *F. culmorum* and *F. poae* [140,145]. DON has been the most prevalent trichothecene accumulating in cereals, including oat, and is also one of the most studied mycotoxins in regard to its toxic effects in plants and animals [9,22].

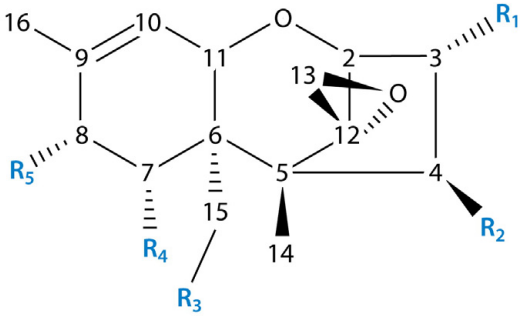

Figure 9. Backbone structure of trichothecenes (Adapted and modified from Chen et al., 2019 [7])

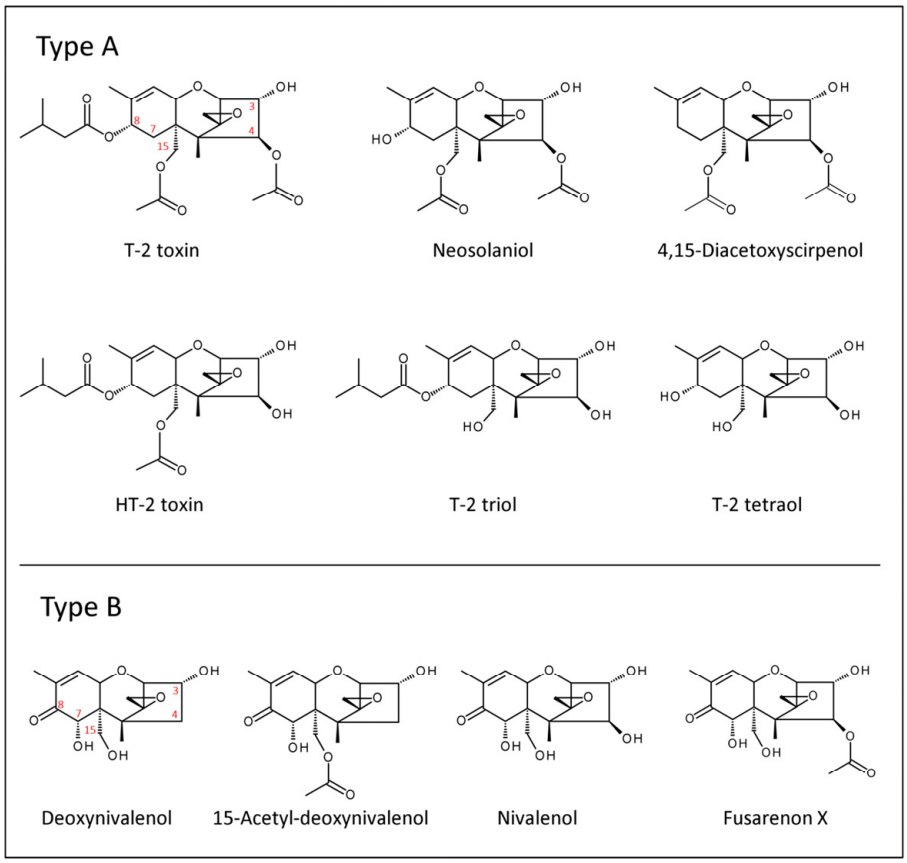

Figure 10. Chemical structures of some of the type A and B trichothecenes (Adapted from Michlmayr et al. 2018 [30]) .

The genetics and biochemistry underlying trichothecenes, as well as the genetic bases for chemotype variation among strains have been elucidated over the recent decade [146]. The synthesis of trichothecenes involves a series of up to 15 enzymatic modifications of the primary metabolite, farnesyl diphosphate. Trichothecene-producing Fusarium species harbour a cluster of TRI-genes, which participate in the enzymatic stages of this process. The presence or absence of specific genes within this cluster results in a diverse array of structurally distinct trichothecenes [146]. In addition, differences in function of allelic variants of the same TRI gene produce trichothecene chemotype variation. For example, different alleles of TRI-gene clusters are responsible for producing different chemotypes of *F. graminearum*: 3-acetyl-deoxynivalenol (3-ADON) or 15-acetyl-deoxynivalenol (15-ADON) and *F. culmorum:* 3-ADON or NIV. Recently, *F. graminearum* isolates possessing the novel NX-2 chemotype (type A trichothecene), were found in USA and Canada. Such toxin diversification on the molecular evolution of trichothecene genes may be important in niche adaptation of the fungus [147].

Fusarium strains initially synthesize acetylated forms of trichothecenes. As was mentioned above, in the case of DON-producing *F. graminearum,* 3-ADON or 15-ADON is synthesized. Similarly, NIV-producing Fusaria excrete 3-,15-diacetyl nivalenol into plants tissues. These acetylated forms are later deacetylated into DON or NIV in the plant by either plants own or fungal carboxylesterases [146,148]. Producing acetylated form of trichothecenes is a self-protection mechanism in Fusarium, as acetylated (at C3 or C15 positions) forms are much less toxic to the eukaryote cells [149]. Efflux of mycotoxins out of the cells is an additional self-protection mechanism utilized by toxin-producing microorganisms. Integral membrane proteins belonging to the ATP-binding cassette (ABC) superfamily or the major facilitator superfamily (MFS) transporter classes are involved in the efflux process [150]. Production of mycotoxins by Fusarium fungi is very much affected by environmental conditions such as temperature, pH and light [144].

Trichothecenes hinder eukaryotic protein synthesis by binding to the peptidyl transferase centre of the ribosome, thereby disrupting the elongation process of protein translation and causing ribotoxic stress [16–18]. This interaction with the ribosome ultimately leads to a reduction in protein production and can result in severe cellular dysfunction [149]. On an organism level, this translates to both acute and chronic toxicoses of digestive, immune and central neural systems of vertebrates [149]. According to toxicological studies, among the Fusarium mycotoxins, T-2 is found to be the most toxic in humans [142,151]. This is followed by NIV [152], and then DON [153].

In wheat, DON production by *F.graminearum* is found to be crucial for the efficient spreading of the disease across the tissues and therefore DON is considered a virulence factor [19,154]. DON non-producing *F.graminearum* disruption mutant in the TRI5 gene could establish infection in the plant cells but was not able to spread across the spike [19–21,154]. Plants with a panicle structure of the spike, like

barley and oat, display natural high resistance against the spread of the disease, as it is hindered by the narrow and fibrous rachis separating the spikelets in a panicle [116,155]. DON does not seem to enhance fungal spread in barley, however infecting barley with DON-nonproducing mutant of *F. graminearum*, resulted in lower disease severity and decreased fungal biomass accumulation compared to infection with a wild type [156,157]. Therefore, DON can be assumed as a factor increasing pathogenicity not only in wheat but in barley too.

Another important mycotoxin produced by FHB-causative agents is zearalenone (ZEA) (**Figure 11**), a mycoestrogen (oestrogen mimic) causing reproductive disorders in mammals leading to infertility in livestock [13]. In oat, ZEA is mainly produced by *F. graminearum* and *F. culmorum,* and the contamination often co-occurs with DON. ZEA is produced rather late during the infection compared to DON, a problem in cereals if cool and humid weather delays harvesting [158]. This toxin has a worldwide distribution with various levels of contamination, which are generally lower compared to DON. ZEA is not essential for disease development on wheat [76]. As phytotoxicity of zearalenone is low, its virulence during the infection seems questionable [137]. Nevertheless, there is a hypothesis that ZEA could inhibit the activity of plant HSP90, an agent with prominent role in stress resistance [137].

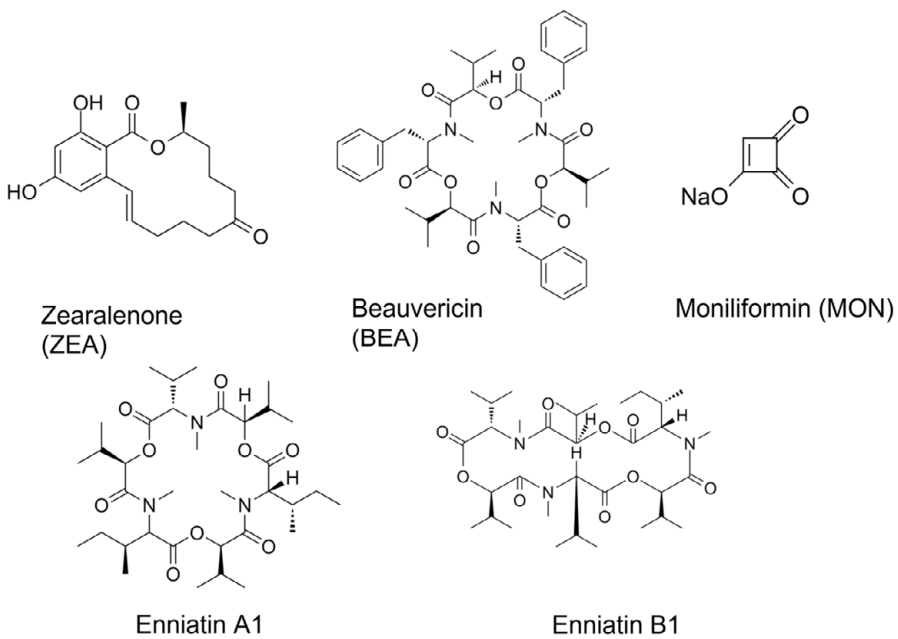

Figure 11. Chemical structures of some mycotoxins, frequently found in oats.

Apart from most common mycotoxins, several others have been reported in oat [10,12] and in large quantities they pose potential health threat (**Figure 11**). Beauvericin (BEA) and Enniatins (ENNs) are cyclic depsipeptides and their toxic effects caused by their ionophoric properties. More than 20 ENNs compounds are found, but the most frequently occurring are ENNs A1 and B1 [161]. BEA and ENNs can incorporate into cell membranes and create increased transport of several metal cations (Ca^{+2} and others), altering normal physiological concentrations of these cations [12,161,162]. Increased efflux of Ca^{+2} due to BEA and ENNs was shown to impair cell cycle, cause mitochondrial dysfunction and to induce apoptosis in many cell lines [12,161]. *In vivo* studies of these mycotoxins have been limited, and although no acute human toxicoses been reported, the possible hazards from long-term exposure to these mycotoxins are unknown. BEA and ENNs are phytotoxic and induce necroses of plant tissues [161][162]. Accumulation of BEA in oat is attributed mainly to *F. poae*, but also to *F. avenaceum* and *F. tricinctum* [100,109]. Strongest producers of ENNs are *F. avenaceum* and *F. tricinctum* [12,76,161].

Moniliformin (MON), a cyclobutane compound, a toxic action of which is considered to be due to the inhibition of tricarboxylic acid cycle, namely the incorporation of pyruvate. MON is phytotoxic, cytotoxic and causes acute toxicoses [11,161,163]. Bird cardiomyocytes are highly sensitive to MON, therefore high exposure to MON causes heart failure leading to death [164]. Frequently found in cereals, Fusarium species such as *F. avenaceum* and *F. tricinctum* appear to be the cause of contamination of these crops with MON [14,76,161].

Methods of identification of Fusarium species and analysing mycotoxins

The accurate identification of Fusarium species is essential for effective disease management, as different species can exhibit varying levels of virulence and produce diverse mycotoxins. The best methods to identify Fusarium species combine both morphological and molecular techniques. Morphological examination involves studying the macroscopic and microscopic features of fungal cultures, such as colony appearance, growth rates, and spore characteristics [66]. However, morphological identification can be challenging due to the high degree of variability and overlapping traits among Fusarium species. Molecular methods, on the other hand, provide a more reliable and precise identification. Polymerase chain reaction (PCR) and DNA sequencing, targeting phylogenetically informative genes regions like the translation elongation factor 1 (TEF-1) and DNA-directed RNA polymerase II largest (RPB1) and second largest subunit (RPB2) genes can resolve Fusarium identification at the species level [74]. In addition to the National Centre for

Biotechnology Information (NCBI) non-redundant nucleotide collection, two specialized Fusarium databases are available: FUSARIUM-ID v.3.0 at Pennsylvania state university and Fusarium MLST database at the Westerdijk Institute [73,165]. BLAST queries with sequences of TEF1, RPB1, RPB2 and other genes fragments can be executed through these regularly updated databases.

When it comes to identification to Fusarium mycotoxins, it is noteworthy that various mycotoxins frequently coexist within the same matrix. This complexity necessitates the development of analytical techniques designed for simultaneous detection of multiple mycotoxins. Targeted methods based on high-performance liquid chromatography (HPLC) and gas chromatography (GC) coupled with tandem mass spectrometry (MS/MS) and High-Resolution Mass Spectrometry (HRMS) [108,166] offer sensitive multi-mycotoxin analysis, and their selectivity allows for the development of simpler extraction protocols. As most mycotoxins undergo modifications in plant tissues by various plant detoxification mechanisms, it is important to accurately identify such modified (so called “masked”) forms [159]. Moreover, as causative agents of FHB shift and adapt to changing climate and new hosts, new previously unknown mycotoxin groups are continuously emerging [8,96,147,167]. Non-targeted methods of HRMS are handy for finding unknown and various masked forms of mycotoxins. These methods do not require previous knowledge about the compounds in the sample [166,168]. In situations where rapid mycotoxin analysis is essential, particularly for grain farmers and traders, immunoassay methods such as enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay (LFIA) are popular, offering affordable and sensitive analyses [166,169]. Commercially manufactured ready-use ELISA plates and LFIA stripes are widely used for analysing DON, ZEA and T-2/HT-2.[167]. Recently, biosensors have emerged as a promising tool for mycotoxin detection, in particular aptasensors, which are utilized for identifying T-2 toxin and ZEA [166,170]. Aptasensors are based on aptamers, synthetic nucleic acids or peptides, selected through a combinatorial screening process for their affinity and specificity for a molecular target [166].

Regulations of mycotoxins

To safeguard public health and livestock production from the potential damage caused by mycotoxins, specific regulations both at global, multinational and single country levels are proclaimed by corresponding authoritative bodies, such as Word Health organization (WHO), Food and Agriculture Organization (FAO), The European Commission, Food and Drug Administration of United States (U.S. FDA) and others. The European Union (EU) regulation comprises the most extended list of commodities and mycotoxins, compared to other regulations, and specifically includes oat. Thus, the established maximum residue levels (MRL) for DON in

unprocessed oats is 1750 µg/kg [171]. MRL for ZEA is 100 µg/kg [172]. For the combined levels of T-2 and HT-2 toxins in unprocessed oats, an indicative limit of 1000 µg/kg has been established [173]. For NIV, only tolerable daily intake (TDI) value is determined at 1.2 µg/kg b.w. per day [175].

According to the published reports by European Food Safety Authority (EFSA) on exposure assessment and hazard characterizations of BEA, ENNs, MON [164,174], as well as modified forms of ZEA and T-2 toxins [175,176] these mycotoxins are not of concern, because of their low occurrence. Thus, these food and feed contaminants are not regulated at the moment. However, attention should be given to the fact that emerging mycotoxins and numerous modified mycotoxins often are not included in routine analyses set-ups because of the unavailability of commercial standards.

At the moment, regulations throughout the world concern only toxicities of single mycotoxins, while numerous surveys report co-occurrence of several mycotoxins in crops and commodities [143,177,178]. Frequently occurring are combinations of DON with ZEA, NIV, ENNs, BEA and MON as well as mycotoxins produced by fungi other than Fusarium [143,178,179]. Predicting the toxicity arising from the interplay of various mycotoxins is a complex task. Yet, scenarios may emerge where the cumulative effects of low levels of individual mycotoxins manifest as a significant health risk [177,178].

Detoxification of mycotoxins in plants

As was noted earlier, mycotoxins produced by fungi may provide protection from abiotic stress and competitive advantage among other microbes co-occurring in soil and on plant debris. Some mycotoxins are shown to act as virulence factors in various plant diseases [139]. Regardless of the impact of mycotoxins on the pathogen infection, most mycotoxins are phytotoxic, and it is beneficial for plants to be able to neutralize the toxicity of these compounds.

Plants have different molecular mechanisms to detoxify mycotoxins. As an initial protective reaction, an active efflux of mycotoxins could reduce the concentration of mycotoxins at a given site. To perform this task, plants may employ various membrane-bound Multiple Drug Resistance (MDR) transporters [180]. However, the most effective detoxification of mycotoxins in plants occurs via chemical modification of mycotoxins and their subsequent compartmentation. Over recent years, numerous products of plant biotransformation of the Fusarium mycotoxins such as DON, ZEA and T-2/HT-2 toxins have been analysed [181]. Thus, 9 DON-metabolites were identified in DON-treated wheat heads [39], 16 HT2 and 17 T2 metabolites were annotated in oat [25], 18 putative ZEA metabolites were reported in micropropagated durum wheat [182]. Reported metabolites of mycotoxins consist of their conjugates with sugars, malonic acid, glutathione (GSH), cysteine, sulphate, ferulic acid and other chemical groups. The conjugation of mycotoxin molecules with hydrophilic groups dramatically diminishes their toxicity and increases their water solubility [15, 181]. Consequently, water soluble conjugates can be transported through various MDR transporter systems, *e.g.*, ATP-binding cassette (ABC) superfamily transporters [137,159]. As a result, conjugated mycotoxins, transported to vacuoles, apoplasts, and bound within the plant matrix become spatially isolated from vital cellular processes.

Glucosides of DON, NIV, ZEA, T-2 and HT-2 toxins in cereals are found to a significantly greater extent compared to other conjugates, therefore glycosylation is considered the primary detoxification mechanism [23–25,183–185]. Glycosylation of DON in cereals has been studied extensively during recent years, both because DON has been most frequently found mycotoxin and because DON adds to the virulence of *F.graminearum* in wheat. DON-3-O-glucoside (DON-3G) is the major DON glycosylation product found in cereals [23]. Oligoglycosylated DON is

reported in cereal products ([186]. In **paper III** of this work di- and tri-glucosides of DON were detected, although in minor quantities.

In plants, the conversion of DON into DON-3G (**Figure 12**) is catalysed by uridine diphosphate-glucosyltransferases (UGTs), a large superfamily of enzymes involved in specialised metabolism [26,187]. Numerous UGTs are upregulated in responses to biotic and abiotic stresses. They are involved in glycosylation of phytohormones, secondary metabolites and exogenous substances, including microbial toxins or pesticides [27]. UGTs catalyse the transfer of a glucose molecule to the C3 hydroxyl group of DON, which dramatically reduces its toxicity. Large numbers of family-1 UGTs have been identified in plant genomes, including 159 in *Brachypodium distachyon*, 179 in wheat, and 147 in maize [29,188,189]. Multiple UGT genes are expressed in response to both Fusarium infection and DON treatment as was demonstrated by transcriptomic studies in wheat and barley [156,190,191]. Treatment of wheat with DON-producing *F. graminearum* resulted in expression of twice as many UGTs compared to the treatment with DON non-producing strain [188].

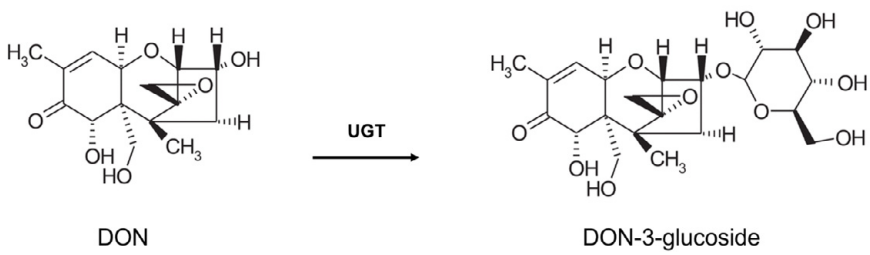

Figure 12. Conversion of DON into DON-3-glucoside by UDP-glucosyltransferase (UGT).

The first DON-detoxifying UGT was identified in *Arabidopsis thaliana* [192] and later similar enzymes where found in *B. distachyon* [29], barley, rice [193] and wheat [189]. Barley glucosyltransferase *HvUGT13248* is the most studied UGT, especially with respect to its effect on conferring FHB resistance. When transgenically expressed in wheat, this gene provided resistance to both DON and NIV, reducing the severity of FHB and FCR [194–196]. Furthermore, mutations in UDP-binding site of HvUGT13248 resulted in diminished glycosylation of DON in roots and spikes of barley. On the contrary, the constitutive expression of *HvUGT13248* in susceptible barley lines provided resistance to DON [157].

Although a number of homologous UGT genes in plants reported to be highly induced by DON application or during *F. graminearum* infection, not all these genes are potentially involved in conjugation of DON into DON-3G. In barley, only HvUGT13248 conferred resistance to DON of total four genes induced during *F. graminearum infection* [44]. In *B. distachyon*, of six genes, highly upregulated in

response to DON, only *Bradi5g03300* conferred tolerance to DON in yeast [45]. In wheat, several UGT genes were found to be activated during the *F. graminearum* infection and while some of them contributed to FHB resistance, such as *TaUGT3, TaUGT5, TaUGT6* , only the latter has been reported to [188,197–200] conjugate DON into DON-3G. Recently an orthologous to barley *HvUGT13248* gene *AET5Gv20385300* from *Aegilops tauschii*, the diploid progenitor of the wheat, was shown to detoxify DON and confer DON resistance to *Ae. tauchii*. Wheat orthologues of *AET5Gv20385300* were found to be involved in DON-detoxification [201].

The substrate specificities and the kinetic properties of recombinant enzymes from barley (HvUGT13248), *B. distachyon* (Bradi5g03300) and rice (OsUGT79) have been recently studied [30], and the crystal structure is determined for the latter [202]. All three enzymes conjugate both DON and NIV, although HvUGT13248 enzyme preferers NIV over DON. Furthermore, while HvUGT13248 can glycosylate low quantities of C-4 acetylated trichothecene, like T-2 toxin, neither OsUGT79 nor Bradi5g03300 exhibit this capability. Interestingly, the hydroxylated forms of T-2 toxins, such as HT-2 toxin and T-2 triol were kinetically preferred substrates for HvUGT13248 and Bradi5g03300. It has been shown that T-2 toxin in cereals is promptly metabolized into HT-2, which could be conjugated by UGTs to form HT-2-3-O-β-glucoside [25,185].

In the present work, **paper II** describes identification and characterization of two oat UGT genes, *AsUGT1* and *AsUGT2*, orthologous to barley *HvUGT12348*. (**Figure 13**). Both UGT genes were strongly upregulated when DON was inoculated to oat spikelets and during *F. graminearum* infection (**Figure 14**). Similar to their barley orthologue, *AsUGT1* or *AsUGT2* provided effective resistance in yeast to DON, NIV and HT-2, but not to T-2 toxin and DAS (diacetoxyscirpenol) (**Figure 15**).

Preliminary study showed that two more genes (AVESA.00010b.r2.5DG0945080.1, AVESA.00010b.r2.4CG1260040.1) from the neighbouring branch from the same orthogroup provided resistance to DON in yeast cells (**Figure 16**) (unpublished data).

The recombinant enzymes expressed in *E. coli* very rapidly lost their activity upon purification, thus we could not study their kinetic properties. Yet, when DON was added to intact *E. coli* cells, expressing either AsUGT1 or AsUGT2, approximately 25% of DON was converted to DON-3G, which clearly shows that both enzymes are active with DON as a substrate [203].

To explore the role of these genes in oat during Fusarium infection, we attempted to identify potential polymorphisms in these genes among FHB-tolerant and susceptible oat cultivars using Sanger sequencing. Unfortunately, due to the hexaploid nature of the oat genome and the presence of the multitude of highly homologous UGTs, we could not reliably identify differences in the sequences. The

upcoming oat pangenome (https://wheat.pw.usda.gov/GG3/PanOat) that will encompass 29 genomes of hexaploid oat cultivars as well as tetraploid and diploid oat relatives, might provide a potential solution to this issue.

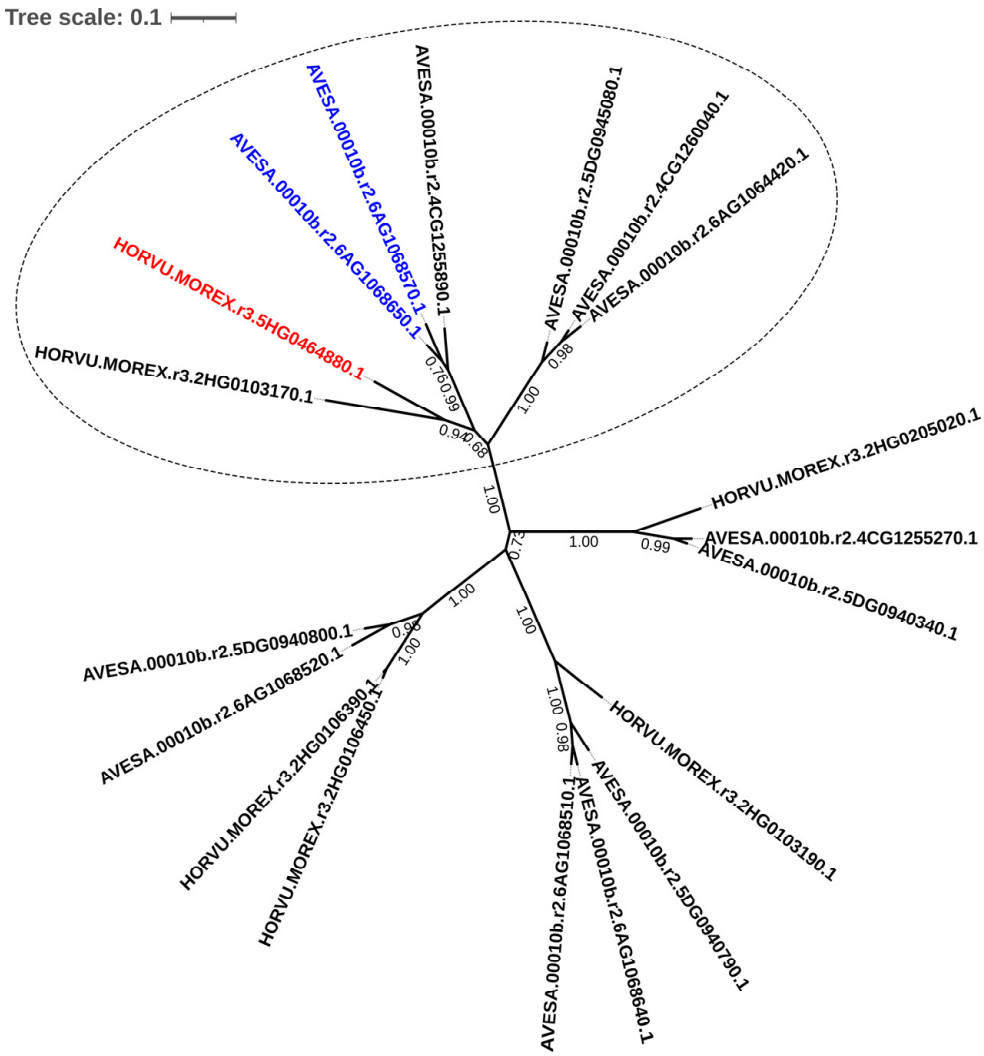

Figure 13. Phylogenetic tree of OG0000783 orthogroup. Barley HvUGT13248 (HORVU.MOREX.r3.5HG0464880.1) is marked in red. The two oat proteins, AsUGT1 (AVESA.00010b.r2.6AG1068650.1) and AsUGT2 (AVESA.00010b.r2.6AG1068570.1) are marked in blue. The clade formed by the proteins phylogenetically closest to HvUGT13248 is marked with a dotted line. (Khairullina et al 2022 [203])

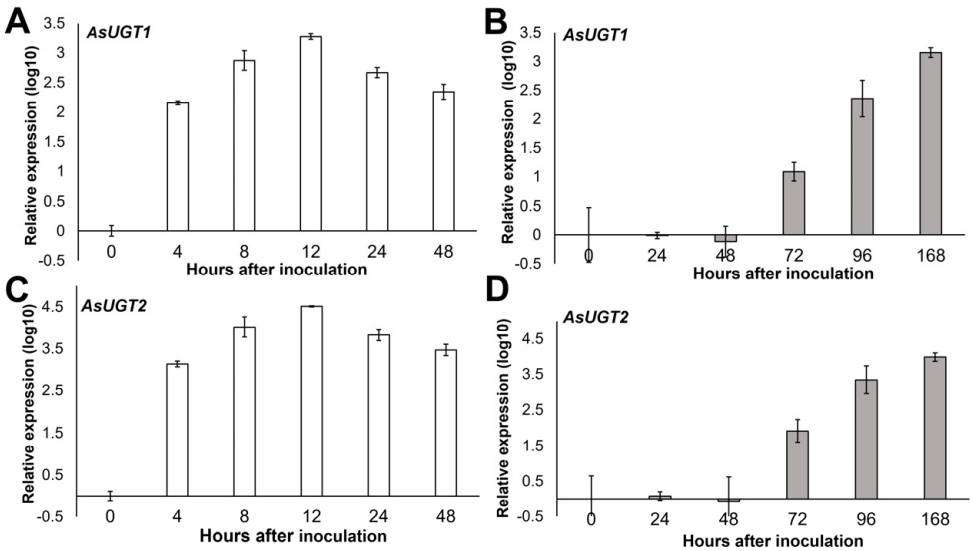

Figure 14. Relative expression of *AsUGT1* and *AsUGT2* genes in oat spikelets after inoculation with either DON (A,C) or *F. graminearum* (B,D) (Khairullina et al. 2022 [203]).

The reasons behind the apparent redundancy of UGTs remain to be elucidated. The study of substrate specificities of UGTs from the named orthogroup, could shed some light on this issue. Structurally different plant UGTs could have developed as an adaptation against constantly evolving mycotoxins as well as fungal inhibitors of these enzymes. Recently, a synergistic phytotoxic effect has been demonstrated for mycotoxins culmorin and DON in wheat [204,205], frequently co-occurring in Fusarium-infected grains. Interestingly, culmorin was found to selectively inhibit several plant UGTs, but not others (G.Adam, personal communication).

Although glycosylation of mycotoxins dramatically reduces its toxicity for plants, there is growing evidence that most commonly observed modifications of DON (DON-3G) and ZEA (ZEA-glucosides, ZEA-14-sulfate), to various degrees, are reconverted by the microbiota of the intestinal tract of humans and animals [149,206]. As modified forms of mycotoxins are difficult to screen for in food and feed samples, they are often termed “masked” mycotoxins [159,207]. Identification and quantification of these compounds are challenging both due to the unavailability of commercial standards and decreased extraction efficiency [206,208]. Various modified forms of DON, ZEA, NIV and HT-2 toxin have been detected in cereals and cereal-based foods [13,206,209,210], although most toxicological studies are limited to DON-3G and ZEA-14G [149,206,211]. Modified mycotoxins are considered relevant for risk assessment for human and animal health by EFSA. There are published scientific opinions on food and feed regarding the derivatives of DON, ZEA, NIV and T-2/HT-2 toxins [175, 176]. These documents provide

guidelines on how to calculate the toxicity of the modified forms of certain mycotoxins in relation to the toxicity of their parent forms.

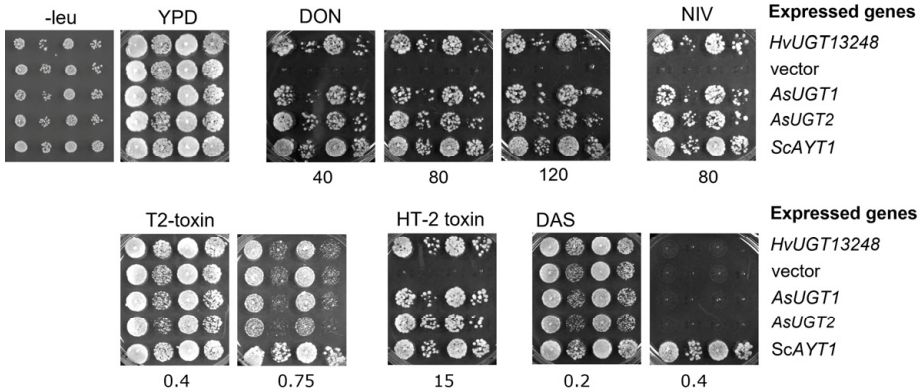

Figure 15. Yeast transformants expressing oat glucosyltransferases *AsUGT1* and *AsUGT2* on plates with indicated concentrations of five different mycotoxins. Strains carrying barley *HvUGT13248*, yeast acetyltransferase *ScAYT1* and the empty vector were used as controls. Control plates without toxin are SC-leu, where only transformed yeast cells can grow, and the rich medium YPD, which allows for growth of strains without a plasmid. Two independent transformants of each construct were spotted in two different dilutions. (Khairullina et al. 2022 [203])

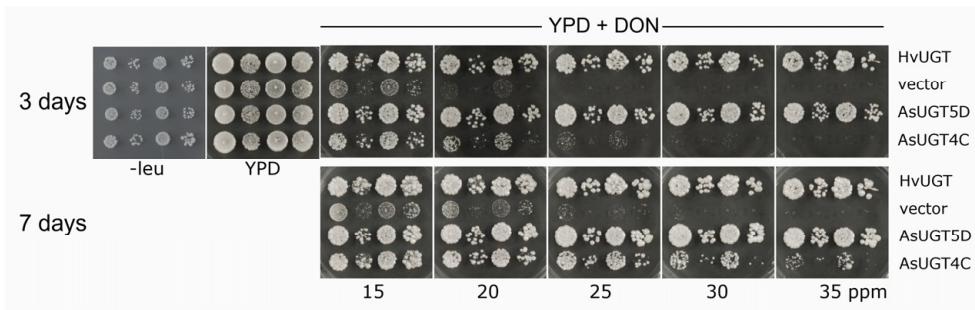

Figure 16. Yeast transformants expressing oat glucosyltransferases *AsUGT5D* (AVESA.00010b.r2.5DG0945080.1) and *AsUGT4C* (AVESA.00010b.r2.4CG1260040.1) on plates with indicated concentrations of DON. Strains carrying barley *HvUGT13248* and the empty vector were used as controls. Experiment is performed in similar way as one on Figure 15.

In the context of masked mycotoxins, a distinct mechanism of mycotoxin detoxification in plants merits mention, namely, conjugation of DON with L-glutathione (GSH). A number of adducts resulting from the reaction of DON with GSH have been identified in naturally contaminated grains [24]. This conjugation occurs primarily at the epoxide group (the 13-position of DON) and is irreversible, thus GSH conjugates are unlikely to be degraded in the digestive tract of animals to release the original trichothecenes mycotoxins. Discovery of several upregulated glutathione-S-transferases (GST) genes in response to DON suggests the occurrence

of an enzymatic GSH conjugation process [212,213]. Recently, *Fhb7* gene coding for GST was found in wheatgrass *Thinopyrum elongatum* and is believed to be acquired by this grass through horizontal gene transfer from an endophytic Epichloë species. *Fhb7* is detoxifies trichothecenes through de-epoxidation and its transgenic expression in wheat is shown to confer FHB resistance [214].

Despite the potential attractiveness of GSH-conjugation over glycosylation, the latter mechanism is employed for the detoxification of the larger bulk of the trichothecenes and characterized by much efficient metabolism kinetics [23]. There are several studies showing that FHB-tolerant cultivars are more efficient in converting DON into DON-3G [23,184,215]. According to Li et al. 2017 [196], rapid trichothecene detoxification is key to FHB resistance. Prompt and effective neutralization of mycotoxins plays a crucial role in preserving the plant ribosomes from translation inhibition, which in turn empowers the plant to restrain the pathogen. **Paper 3** of this work shows how increasing the effectiveness of DON-glycosylation in oat spikelets could be achieved by application of fungal biocontrol agent. Details are described in the chapter dedicated to the biological control of FHB.

Host-pathogen interactions

The interaction between Fusarium species and cereals has been studied extensively. Transcriptomic analyses examining gene expression during *F. graminearum* interactions with wheat and barley have unveiled distinct patterns at various infection stages [216–218].

FHB infection occurs in two phases: a biotrophic phase during which the fungus colonizes the intercellular space in the tissues and a necrotrophic phase where the fungus breaks down the infected tissues [217]. In wheat, a biotrophic phase is symptomless, while necrotrophic is characterized by symptoms such as bleaching and necrosis [217]. To initiate the infection, *F. graminearum* ascospores attach to the host surface using hydrophobin proteins [219] and once attached, they develop into specialized unbranched hyphae called runner hyphae. These hyphae develop multicellular infection cushions, which penetrate plant cuticles [220]. In the infection cushions, numerous genes such as carbohydrate-active enzymes (CAZymes), candidate effectors, and specialised metabolism gene clusters [220] are upregulated. During the colonization, fungal signalling pathways are actively involved in switching between different stages of plant infection [217].

ROS generated by plants can cause damage to fungal cells, prompting fungi to produce iron-scavenging molecules, called siderophores. Siderophores, such TAFC (triacetylfusarinine C) and malonichrome are essential for both the establishment of symptomless infection and the expansion of infection throughout the wheat head [217].

Additionally, many small cysteine-rich effectors are induced during both biotrophic and necrotrophic phases of infections. Effectors suppress host defence responses by manipulating plant physiology [221]. At least 357 secreted effectors of *F. graminearum* were identified [222].

TRI genes are highly induced in symptomless tissues, indicating a crucial role of DON in modulating host defence and the establishment of infection [217]. Interestingly, *F. graminearum* can hijack biosynthetic pathways in plants to facilitate DON production . One such pathway is polyamine biosynthesis, employed by plants to generate defence-related compounds such as hydrogen peroxide and hydroxy-cinnamic acid amides . Another defence-related plant response, generating hydrogen peroxide (H_2O_2) also promotes the biosynthesis of DON [216,223].

The necrotrophic phase of infection is characterized by predominant expression of cell wall-degrading enzymes (CWDEs) [217], which liberate nutrients from plant tissues for the growth of the pathogen. In wheat, this phase is characterized by the appearance of bleached tissues. During the transition from the biotrophic to the necrotrophic phase, the fungus undergoes transcriptional reprogramming in response to host signals [217].

When a pathogen invades a plant, pattern recognition receptors (PRRs) located at cellular membranes recognise conserved pathogen patterns (such as fungal chitin, mannans, glucans etc). These patterns are termed microbe- or pathogen associated molecular patterns (MAMPs or PAMPs). Upon this recognition, PAMP- triggered immunity (PTI) is induced [217,221]. It stimulates various immune responses, including the influx of calcium ions, reactive oxygen species (ROS) burst, transcriptional reprogramming, antimicrobial substance production, stomata closure and deposition of callose [224,225]. Unsurprisingly, highly susceptible and moderately resistant cultivars exhibit differential expression of various defence genes, encoding the functional agents of processes mentioned above [224,226].

To withstand the penetration of fungus, plant reacts by enhanced lignification of the secondary cell wall. Metabolites synthesized via the phenylpropanoid pathway, including lignin, flavonoids, lignans, phenylpropanoid esters, and hydroxycinnamic acid amides (HCAAs), are known to contribute to FHB resistance in plants [227,228]. These metabolites play a critical role in both fortifying the cell wall and impeding the activity of plant cell wall degrading enzymes. Lignin aggregates at the site of pathogen infection to form a physical barrier to prevent the spread of pathogens. Production of lignin, flavonoids and HCAAs induced in higher degree in resistant cultivars [226,227]. Cytochrome P450 (CYP450) family enzymes which participate in the pathways of biosynthesis of lignin, plant hormones and anti-fungal metabolites are produced in in cereals higher quantities under Fusarium infection [229].

To mount a robust defence against Fusarium infection, plants must enhance their primary carbon and nitric metabolism. Consequently, the genes involved in these processes are among the most highly expressed in wheat plants following Fusarium infection [230]. The rapid ROS production in response to pathogen infection is thought to regulate programmed cell death (PCD). As excessive ROS at the later stages has a strong toxic effect on plant cells by inducing DON, involvement of antioxidant enzymes in ROS removal is especially important in resistance to pathogen infection [226,229,230]. Plant defence against microbial attack involves a complex signalling network, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET)[226].

In the early stage of pathogen infection, pathogenesis related (PR) proteins, including PR-2 (β-1-3-glucanase), PR-3 (chitinase), involved in degrading the cell wall components of pathogens are pronounced in plant response [226,230]. Most

PR proteins have higher abundance in resistant wheat genotypes than in susceptible wheat genotypes [226,229]. Numerous transcription factors associated with pathogen interactions such as WRKYs and bZIPs, and protein kinases, such as leucine-rich receptor-like kinases, involved in signal transduction are also expressed during FHB [226,229].

As was described earlier, to counteract the cytotoxic effects of mycotoxins during *F. graminearum* infection, cereals upregulate production of detoxifying enzymes, such as UGTs and glutathione S-transferases (GSTs) [225][137]. Additionally, ATP-binding cassette transporters (ABC transporters) are strongly upregulated in resistant cultivars upon both DON treatment and *F. graminearum* infection and involved in active transportation of toxic compounds out of the cell .

FHB disease management

The progression and severity of FHB infection are fundamentally influenced by three primary factors: (a) the abundance and aggressiveness of inoculum present during the anthesis, (b) the environmental conditions encountered during this pivotal period, and (c) the inherent susceptibility or resistance of the plant. Integrated disease management which allows combination of several pre-harvest disease controlling strategies is the most effective way for preventing the FHB in cereals. These strategies include a combination of agronomic practices, planting resistant or tolerant cultivars, chemical control, biological control and use of forecasting systems [33,112].

Agronomical practices

Previous crop residues serve as primary sources of inoculum for pathogenic Fusarium spp., therefore, properly designed crop rotation can significantly reduce the incidence of FHB and grain contamination with mycotoxins [65]. As the same Fusarium species are found in wheat, barley and oats, any of these cereal species may make the Fusarium inoculum accessible for the subsequent year's crop. Several studies showed that the cultivation of cereals, particularly maize, increases the risk of FHB and mycotoxin contamination in subsequent cereal crops. On the contrary, crop rotations that include non-cereal crops (oilseed rape, potatoes, legumes, vegetables) have been shown to reduce Fusarium infection and after 2–3 years of growing a non-Fusarium host plant species, they are thought to effectively remove Fusarium pathogen inoculum from agricultural soils [36,231–233].

A majority of studies investigating the impact of crop rotation have concentrated on its efficiency in reducing mycotoxins in wheat and barley. Predictably, crop rotation has also demonstrated a reduction in the accumulation of mycotoxins in oat, underlining its universal application across various grains. Oat crops grown in the fields with the previous cereal crop showed increased concentrations of T-2/HT-2, compared to oat grown in rotation with non-cereal crops [35,36,88]. In a recent study by Kolawole 2021, higher levels of DON, ZEA, T-2 and HT-2 mycotoxins were found in oat crops grown after oat, while oats grown after grass and non-cereal crops were lowest in mycotoxin accumulation [89].

Deep tillage practices, such as inversion ploughing, are often regarded as effective methods to reduce FHB incidence, severity, and mycotoxin levels, due to their ability to incorporate cereal stubbles and crop residues – major sources of Fusarium inoculum – into the soil. In several studies, deep tillage has been found potent in reducing levels of *F. graminearum* inoculum and DON in wheat, barley, and oat [33,233,234], as well as *F. langsethiae* and T-2/HT-2 toxins in oat [105,235].

However, the picture is more complex. Despite the evident benefits in controlling Fusarium inoculum, heavy tillage practices can compromise soil structure and moisture content and consequently, adversely affect the balance of microbial communities [236]. Soil with high biological activity or antagonistic microbial communities may exhibit disease suppressiveness and can reduce Fusarium inoculum and disease development [14,36]. Interestingly, higher levels of *F. poae* and NIV have been discovered in inversion ploughed fields when compared to minimally tilled ones in wheat [232] and barley [233,237]. It has been suggested that agricultural strategies that aim to target specific Fusarium species could inadvertently create vacant ecological niches, which potentially could be occupied by other FHB species [232,233,237].

Organic and conventional cereal production methods differ in a range of agronomic practices. Organic farming practices typically feature more active use of crop rotation, reduced reliance on mineral nitrogen, less intensive tillage and lesser usage of fungicides compared to conventional farming. Several studies indicate that organically grown cereals tend to exhibit lower incidences and concentrations of Fusarium mycotoxins than cereals grown via conventional methods. As was reviewed by Bernhoft et al., 2022 [238], in 24 studies, mycotoxin levels were lower in organic production, in 16, differences were not significant and only in two cases were mycotoxin levels higher. On average, conventionally produced cereals had significantly higher levels of DON, ZEA, and T-2/HT-2 than organic cereals. For oats specifically, no significant reduction in levels of DON was found associated with organic cultivation [240] [239] but statistically significant decreases were observed in the accumulation of T-2/HT-2 toxin in organically grown oat [35,89,239,240].

Utilisation of FHB-resistant cultivars.

Resistance to FHB in wheat and barley

Regarded as the most cost-effective strategy, genetic resistance plays a pivotal role in managing diseases in cereals. However, embedding a robust resistance to FHB within these crops is challenging, due to the complex and quantitative nature of such resistance. Elements contributing to FHB resistance can be categorized into two

groups. The first involves factors causing avoidance of infection (passive resistance), such as, for example, earliness (of flowering or plant development) and plant height. The second group includes aspects of partial resistance. This partial resistance to FHB, which was initially modelled in wheat, is typically broken down into five key components: resistance to initial infection (Type 1), resistance to the spread of infection across the spike (Type 2), resistance to kernel infection (Type 3), resistance to mycotoxins (Type 4) and tolerance, *i.e.*, the ability of the plant to endure the effects of pathogen infection (Type 5) [241]. Type 1 resistance (FHB incidence) is assessed by counting numbers of infected florets after spraying a spore suspension on flowering spikes, Type 2 resistance (FHB spread) quantified as the percentage of symptom spread within a spike after point inoculation of a spike, Type 3 is measured as the percentage of infected mature kernels FHB within a spike. To evaluate Type 4 resistance, mycotoxin concentrations are analysed in infected spikes. Lastly, rarely used Type 5 resistance is represented by relative yield decline when infected and uninfected plants of the same cultivar are compared [241]. As a measure for overall FHB resistance types are used either singly or in combination.

Morphological traits in wheat and barley, associated with avoidance mechanism /passive resistance to FHB include plant height, lodging, spike form and length, flowering time and flowering type [243]. Spikes of upright and tall plants are less exposed to the humidity and the source of inoculum in the soil, which slows the disease progression [242,244]. Given that FHB infections predominantly occur during the flowering stage, the duration of flowering and the type of flowering can significantly influence the susceptibility to this disease. A lengthy flowering period, which extends the exposure to pathogen spores, increases chances of infection [33]. As to the feature of flowering of cereals termed as anther retention /extrusion, multiple studies agree that anther retention is often accompanied by increased FHB severity [242,244].

Resistance to FHB in cereals is quantitative and is controlled by multiple genes with individual alleles responsible for small levels of increased resistance. In wheat, these genes are scattered across chromosomes and exhibit strong genotype-by-environment interactions. More than 500 QTLs (quantitative trait loci) associated with FHB have been reported in literature, but most require validation through reverse genetics experiments [245]. A handful of QTLs have been mapped in wheat in detail so far, of which only two, *Fhb1* and *Qfhb.mgb-2A* have been cloned [113,242]. *Fhb1*, conferring type 2 FHB resistance is wheat, is derived from the Chinese wheat cultivar Sumai-3 and is considered to be the most durable and effective source of FHB resistance [113,242,245]. Various functional mechanisms of *Fhb1* have been proposed, of which DON-detoxification is the most claimed, as this QTL is known to simultaneously reduce FHB severity in the spikes and DON content in the kernels [246]. Few studies to elucidate functional agents within Fhb1 have shown contradictory results. Nevertheless, research on the functional components of *Fhb1* is ongoing and recently gene *WFhb1-1* encoding putative

membrane protein is proposed as key agent constituting to FHB resistance [242, 246].

Similar to wheat, barley's FHB resistance QTL regions have been mapped and studied for gene expression. Significant QTLs have been identified on all seven chromosomes of barley, with the largest effect QTLs associated with FHB and DON, showing correlations with plant height, spike length, spike density, and flowering traits [113,218]. Moreover, several metabolomics studies have analysed specific compounds in resistant and susceptible barley genotypes. Metabolites associated with resistance are primarily represented by various phenylpropanoids, flavonoids, and terpenoids [218,228]. The combination of metabolomics data with transcriptomics has helped to identify several individual genes contributing to reduced FHB severity, including the transcription factor WRKY23, involved in modulating the expression of defence genes [247], and isochorismate synthase ICS, responsible for SA-based immunity in barley [248].

While QTLs with large FHB resistance effects are used in marker-assisted selection (MAS), Genomic selection (GS) is an alternative method of selection of multiple QTLs, which have small effects, but cumulatively contribute to FHB resistance [249]. Breeding efforts employing both MAS and GS have resulted in the development of wheat and barley cultivars with moderate resistance to FHB and DON [113,218,242].

Oat resistance to FHB

In terms of morphology, oat panicles differ from wheat and barley heads by their long rachis. Infection has been rarely found to move from spikelet to spikelet, due to longer distance through rachilla, pedicles and rachis compared to the more compact heads [114]. Thus, oat exhibits high Type 2 resistance. On the other hand, oat flowering endures for a longer time, up to 10 days for a single panicle and up to one month for the whole plant [250], which prolongs the time of susceptibility.

Similar to other wheat and barley, the most important passive resistance traits (avoidance mechanisms) related to FHB in oats in field conditions are the plant height, lodging and earliness [251–253]. Hulled oat genotypes exhibit greater resistance than hulled ones [254]. Anther extrusion (AE) in oat, similar to wheat and barley, results in slower Fusarium infection rate [114,115,122] However, the impact of AE on mycotoxin accumulation is ambiguous, with other factors such as plant height, earliness, lodging, or hull content having greater effects [253].

To evaluate resistance to FHB in oats, several rankings of oat genotypes have been performed recently. In one of rankings, performed in Norway, 543 Nordic breeding lines and cultivars from spawn-inoculated nursery experiments (resembling natural infection) where tested, relying on DON accumulations and germination capacities [122]. DON content showed a highly significant negative correlation with

germination capacity after Fusarium infection and with plant height. Later-flowering lines showed a tendency for higher mycotoxin accumulation. In a study from Finland, 406 oat genotypes consisting of Nordic cultivars, breeding lines and gene bank accessions were analysed for both DON and Fusarium infected kernels (FDK) [123]. As with the previous study, days to maturity and the plant height of the genotypes both significantly affected the Fusarium infection and DON in the field. This study led to selection of a set of both highly susceptible and moderately resistant 30 oat genotypes [123].

Compared to number of studies reporting resistance against DON and its producers, there are fewer studies of T-2/HT-2 resistance in cereals. As was pointed out earlier, oat is particularly susceptible to *F. langsethiae* infection and consequently accumulation of T-2/HT-2 toxins. Only few studies featuring oat resistance to T-2/HT-2 managed to identify clear varietal differences. In an analysis of several trials of UK varieties [255], naked varieties had lower T-2/HT-2 compared with hulled oats and short oat varieties were more susceptible than tall varieties. In addition, winter oats showed to be more susceptible to T-2/HT-2 contamination than spring oats. Supported by several studies, the resistance against pathogenic Fusarium fungi in cereals is proclaimed to be non-species-specific [241,253]. A resent cultivar trial of Nordic spring oat varieties and breeding lines was performed based on their levels of both *F. graminearum* and *F. langsethiae* DNA, as well as the presence of their respective mycotoxins, DON and T-2/HT-2 [256]. Importantly, the ranking of oat varieties based on the analysis of *F. langsethiae* DNA/T2-HT+2 differed from the ranking based on the analysis of *F. graminearum*/ DNA /DON. This implies that separate tests are necessary to determine resistance towards T-2/HT-2 and DON producers.

While numerous genomic studies have been conducted on FHB resistance in wheat, very few such studies have been reported for oats. One of the reasons is the absence, until very recently, of a genome sequence for oats' highly complex hexaploid genome [61,62]. Genomic studies can reveal important associations between measured traits and genetic markers and require availability of accurate measurements for FHB resistance and genotypic information on the tested lines. In a recent study, 424 spring oat lines from North America and Nordic countries were phenotyped [251]. Significant negative correlations were found between FHB and DON with phenological traits, among which earliness had the biggest impact on FHB and DON, followed by plant height . Subsequent genotyping with nearly 3000 SNP (single nucleotide polymorphism) markers and GWAS (genome wide association study) identified multiple QTLs associated with FHB and DON. According to this study, DON accumulation in oat appears to be a heritable trait. Furthermore, the study provides a list of lines and cultivars with consistently low DON accumulation [251].

Regarding resistance of oat to *F. langsethiae* and T-/HT2 accumulation, a study reporting results of genotyping by sequencing of 190 spring oat varieties has been

published recently [257]. A genomic loci was identified, linked to the biomass of *F. langsethiae* DNA and T-2/HT-2 accumulation. GWAS has associated T-2 + HT-2 mycotoxin accumulation with five SNPs in a linkage group. A single QTL was identified, and one of the markers mapped within genes similar to a lipase-like or lipase precursor mRNA sequences and zinc finger proteins, which have previously been linked to increased resistance to Fusarium species [257].

In conclusion, breeding FHB resistant cereals represents a complex challenge, especially in the case of oats. While there are oat cultivars that display reduced mycotoxin accumulation, only a few genetic markers associated with resistance against Fusarium infection are identified, and the genetic basis of FHB resistance in oat is largely unknown. Incorporation of marker assisted selection is essential for effective breeding programs. Identification of major resistance QTLs together with their constituent genes are of great importance in creating robust genetic markers. As presented in **paper II**, identification of two DON-detoxifying genes could be a first small step in unravelling mechanisms behind complex FHB resistance in oat.

Fungicidal control of FHB

Fungicides, known as demethylation inhibitors, like propiconazole, prothioconazole, and tebuconazole (or a combination like Prosaro®), are commonly used to combat FHB in wheat and barley during the early or mid-anthesis [32,33]. These fungicides target the fungal enzyme cytochrome P450, crucial for the biosynthesis of ergosterol a primary component of the fungal cell membrane. While applications of these fungicides can reduce infection and mycotoxin contamination with a single application, their efficacy is typically less than 50% [32, 33]. The efficacy of Fusarium control is heavily dependent on the timing of application, spray coverage, weather conditions and cultivar susceptibility [258, 259]. Quinone inhibitors and succinate dehydrogenase inhibitors have low efficacy for controlling Fusarium and may even stimulate mycotoxin production in the infected plant [33]. They may also increase mycotoxin levels by inhibiting the colonization of cereal plants by commensal or other pathogenic fungal species [232,236].

The use of fungicides for FHB is even more questionable in oat. As mentioned above, it takes up to ten days for all florets of an oat panicle to complete flowering. Moreover, it can take up to a month for all tillers of a single oat plant to go through anthesis. Thus, it is difficult to find optimal fungicide application times for the treatment of FHB in oat. The use of fungicides in oats has proven to be mostly unsuccessful, with inconsistent effects that depend on oat cultivars and the specific Fusarium species involved [34,36,37,260]. Few field experiments have shown that commonly used fungicides have very little or no effect on reducing HT2+T2 levels in harvested oat grains [260,261].

Regardless of the efficiency of fungicides, their indiscriminate use will inevitably lead to the development of fungicide-resistant strains of Fusarium. Natural resistance to fungicides has been observed in wild populations of Fusarium in China, Europe, and the USA [262] . Multiple genes associated with reduced sensitivity to fungicides and increased pathogenicity have been identified in Fusarium species [262]. Selective pressure from fungicides used to control FHB in the field is believed to have contributed to recent shifts within the FHB complex. *F. poae* and *F. avenaceum* have demonstrated lower sensitivity to fungicides, with *F. avenaceum* showing reduced sensitivity to metconazole in particular [93,262].

Predictive tools for FHB outbreaks

Forecasting systems have become an important instrument in the practical management of FHB as they provide near-real-time estimates of FHB disease risks throughout the growing season. Built primarily on local meteorological data, such as temperature, rainfall, and humidity, together with the information on history of FHB epidemics in the growing region, these web-based systems have been developed and implemented in several countries worldwide [33,113,263,264]. These systems require input from farmers regarding the resistance of the cultivar and the timing of flowering as well information about previous crops and soil/debris management. By combining this information with the local weather data, the forecasting system generates online estimates of the anticipated infection. These estimates are useful for making informed decisions about the necessity and optimal timing for potential fungicide or BCA application. The accuracy of FHB severity and DON contamination predictions varies depending on the specific country and local farming site. However, they generally claim to be around 80% accurate [263,264] .

Airborne inoculum plays a crucial role in FHB epidemics and the accumulation of mycotoxins in grains. The use of spore traps makes it possible to identify major sources of Fusarium spore inoculum and to investigate the relationship between inoculum quantities and factors such as weather conditions, cropping systems, and plant developmental stage. Research focused on quantifying airborne inoculum has confirmed a strong correlation between high airborne inoculum quantities at the anthesis stage and *F. graminearum* infection, as well as DON production [265–267]. The primary sources of inoculum appear to be local and farm-based, influenced by a variety of cropping factors including crop rotation and tillage. The results derived from inoculum trap analysis can be used both in *ad-hoc* forecasting of an immediate FHB threat and as well as provide an indication for developing and refining sustainable cropping systems [265-267].

Novel approaches in manipulating active FHB resistance in plants

Genetic engineering and biotechnologies are consistently expanding our toolbox for improving plant disease resistance. One of such instruments is the creation of genetically modified (GM), or transgenic, plants, which enables isolating and transferring strong functional genes to crops from sexually incompatible plants and other organisms [268]. In the past 25 years, GM crop production has increased over 100-fold, although most GM crop products are typically not grown for human consumption [269]. Importantly, adoption of GM technology has shown to reduce pesticide use, cutting down environmental pollution and fuel consumption, leading to a significant drop in greenhouse gas emissions from GM cultivated areas [270]. Despite these advantages, GMO use still sparks great public concern due to perceived risks to human health (despite lack of evidence for this) and the environment, leading to strict regulatory controls around the world [269,271]. Compared to transgenic plants, the *cis*-genic approach—where crops are modified using genes isolated exclusively from sexually compatible plants, thus more closely resembling traditional breeding – is gaining more positive consensus from both the public and farmers [268].

Recently, gene editing, using, *e.g.,* CRISPR-Cas9 (clustered regularly interspaced short palindrome repeats, CRISPR associated protein 9) technology have been attracting researchers' attention as the most versatile and powerful new breading technique. CRISPR-Cas9 allows precise genetic modifications of single or multiple gene targets without altering other regions [268,272,273]. Regarding crop protection, the most common and relevant use of this technique is producing knock-out mutants of plants' susceptibility genes [272]. In the context of FHB in wheat, CRISPR-Cas9 was used to produce loss of function mutants of *TaLpx-1*, which encodes for 9-lipoxygenase [274], and the transcription factor [275] *TaNFXL1* are shown to confer resistance to *F. graminearum* in wheat. Despite the potential and promises of CRISPR/Cas for practical applications in plant pathology and disease management, The European Court of Justice has imposed the same strict regulations for genetically edited plants as for conventional GM crops, which limits full benefit CRISPR/Cas technique for agriculture [276], though there are prospects that this might be lifted in the foreseeable future.

Simultaneously, advancements in genomic tools targeted on the silencing of pathogen genes have opened new avenues for FHB control in cereals. RNA interference (RNAi) is an inherent mechanism in all eukaryotic organisms that modulates post-transcriptional gene expression [277]. This mechanism involves the action of small interfering RNA (siRNA) molecules, leading to the sequence-specific degradation of mRNA. Through the use of transgenic technology, siRNA can be synthesized in plant cells, a process termed Host-Induced Gene Silencing

(HIGS). Alternatively, the non-transgenic RNAi approach known as Spray-Induced Gene Silencing (SIGS) utilizes the same process but involves the direct application of RNA molecules on the surface of the plants [268,278]. Both HIGS and SIGS have proven effective in controlling *F. graminearum* [279]. Several studies, featuring HIGS and SIGS, targeting sets of *F. graminearum* genes have shown to effectively reduce FHB infection in wheat [278,280].

Biological control

The recently published by European Commission Farm to Fork Strategy [281], sets a target of reducing pesticide usage and risks from chemical pesticides by 50% by the year 2030. While fungicides commonly employed for FHB control may not be the most acutely hazardous, their documented toxic effects extend to all classes of organisms ranging from mammals to soil microbiota [282].

Growing concerns about adverse effects of fungicides on the environment and the health of humans and animals along with fungicide resistance, have prompted a shift towards more sustainable disease management practices. Biological control is a prominent alternative or complement to chemical measures in integrated disease management plans [38]. Biological control utilizes various microorganisms, including bacteria, filamentous fungi, yeasts, and mycoviruses, that possess the potential to hinder the growth and proliferation of pathogens and safeguard plants against infection [38,283]. Such microorganisms are termed biocontrol agents (BCAs). Over the last decades numerous promising bacterial and fungal BCAs have been shown to significantly reduce FHB and mycotoxin content in cereals under field conditions [284,285]. A separate chapter is dedicated to the use BCA in management of FHB.

Biological control: modes of action of BCAs and examples of BCAs used against FHB

An in-depth understanding the mechanisms of the tripartite interaction between BCA, host plant, and pathogen are important in order to optimise the selection and utilisation of biocontrol microorganisms. Four types of action modes are generally recognised for BCAs: (1) direct inhibition through antibiosis, where BCA inhibits the pathogen via effects of toxic secondary metabolites (2) hyperparasitism, where the antagonist acts as a predator and exploits the pathogen as a prey, (3) competition for space and essential resources (oxygen, carbon, nitrogen and others) and (4) induced resistance in the plant by activation of its own defence system, such as the oxidative burst, accumulation of PR-proteins, and enzymes involved in the phenylpropanoid pathway among others. Additionally, growth promotion through nutrient acquisition (nitrogen, phosphorous and essential minerals) or modulating plant hormone levels can also improve general plant health and thereby protection against diseases [38,39]. Several mechanisms could be activated simultaneously. It is assumed that the most efficient BCA will employ a combination of different modes of action for pathogen control at any given time [38,39,286, 375].

Main mechanism of inhibition of *F. graminearum* by of most bacterial BCAs is attributed to be their ability to produce antibiotic compounds, as demonstrated for numerous strains of Bacillus, Pseudomonas, Lactobacillus and Streptomyces [284,287]. For example, strains of *B. amyloliquefaciens* are shown to produce fengycin and iturin, which have strong antagonistic effects against *F. graminearum* (Gong et al., 2015a). In addition to antibiosis, bacterial BCAs antagonistic to Fusarium spp., may potentially employ several mode of actions [287]. Competition with Fusarium spp. for essential minor elements, such as iron is reported for several bacterial isolates, particularly for fluorescent Pseudomonas spp. [284]. Siderophores, which are expressed by most microorganisms, help acquire iron due to its low bioavailability in aerobic environments. This competition for essential nutrients is partly responsible for the Fusarium disease suppressive nature of certain soils [38]. Finally, there has been an increasing body of evidence regarding bacterial BCAs being able to induce active resistance mechanisms in plants [284,287] .

When it comes to fungal BCA isolates, suppressing *Fusarium spp.,* the most effective studied antagonists are found among fungi of the order Hypocreales, exhibiting mycoparasitic lifestyle. In particular, the genera Trichoderma and Clonostachys offer many effective BCA candidates [284,288,289, 375]. *In vitro* studies showed that these fungi make direct contact with the hyphae of their prey and during these contacts, the mycoparasite produces compounds for nutrient release and acquisition, including cell wall degrading enzymes (CWDEs), antibiotics, and toxins [288,290,291]. However, the biocontrol mode of action of these fungi are not limited to mycoparasitism.

Trichoderma spp. are shown to produce specialized metabolite 6-pentylalpha-pyrone which suppressed perithecial production and ascospore discharge and completely impeded germination of conidia of *F. graminearum* [292]. A case exemplifying nutrient competition, *T. gamsii*, when co-cultured with *F. graminearum*, was observed to increase the expression of a ferric reductase, a key player in iron acquisition [293]. *T. gamsii* are also reported to reduce DON production in *F. graminearum* and *F. culmorum* [290]. Finally, Trichoderma spp. are reported to induce systemic and local resistance in plants as well as promote plant growth [294,295, 375]. Importantly, Trichoderma spp. possess the ability to grow and multiply as saprophytes and endophytes [289] which enhances their competitive edge in various ecological niches. In fact, *T. gamsii* can be used effectively to outcompete *F. graminearum* when grown on cereal residues [296,297]. Another highly valuable aspect of several Trichoderma strains concerning disease control is their potential for utilization in combination with other fungal and bacterial isolates, and this was demonstrated successfully in several studies [298–300, 375].

Similarly to Trichoderma spp., several strains of *Clonostachys rosea* are known to degrade Fusarium mycotoxins, as well as minimise the effect of FHB in wheat [301–303]. As **Paper III** in current work exclusively focuses on the biocontrol effect of *C. rosea* to reduce FHB in oat, a separate chapter further below is dedicated to this versatile fungus.

Another mycoparasite that has shown good BCA potential in controlling FHB, is *Sphaerodes mycoparasitica* [304]. Not only it is reported as specific mycoparasite of multiple Fusarium spp., but numerous studies showed its ability to downregulate expression of TRI (trichothecene) and AUS (aurofusarin) genes in Fusarium spp. as well as degrade mycotoxins DON, 3ADON, 15ADON and ZEN [304].

In addition to filamentous mycoparasites, yeast BCAs of the genera Cryptococcus, Kluyveromyces and Saccharomyces have exhibited antagonistic activities against Fusarium spp., which are associated mainly with an arsenal of various antifungal metabolites which target the pathogen [284,305].

Despite significant progress in the development of BCAs, the number of commercial products for suppression of FHB is limited. There are two bacterial

BCA products against Fusarium fungi available on the market: Cerall® based on *Pseudomonas chlororaphis* and Mycostop® based on *Streptomyces griseoviridis*. Both have been available for many years. Other products include Polyversum® based on the oomycete *Pythium oligandrum,* and a formulation of *T. asperellum* named Xedavir®. Additionally, patents for a few BCAs have been registered, including TrigoCor strain of *Bacillus amyloliquefaciens* and ACM941 strain of *C. rosea* [38,284].

It is important to point out that BCAs are subject to specific regulations depending on their application. For instance, an organism can be marketed and sold as a biofertilizer or biostimulant when its BCA ability is not specified. However, all products using microorganisms with claimed BCA activity undergo an extensive registration process, which hinders the smooth development of such products [306].

Lately, the trend of harnessing endophytic microorganisms as a source of novel BCAs has been growing. Potential of employment of endophytes as BCAs in controlling FHB in cereals are reviewed in separate chapter, together with summary of the **paper IV** focusing on isolation of fungal endophytes antagonistic to *F. graminearum* from oat.

Clonostachys rosea as BCA against FHB in cereals

Clonostachys rosea, an ecological generalist, can colonize both phyllosphere and rhizosphere of plants, and exhibits mycoparasitism of multiple host species. This traits are significant in the context of BCAs, as it allows them to adapt and survive in various ecological niches and environments thereby enhancing its efficacy in controlling plant diseases [307].

Numerous strains of *C. rosea* have been isolated from of various sources, such as soil, fungi, living plants and plant debris, as well as nematodes and insects [307]. Currently, genome sequence data exists for 56 strains of *C. rosea* [291,308].

Mycoparasitism by *C. rosea* was demonstrated against multiple plant pathogens, including *F. oxysporum* [291] and *F. graminearum* [303]. During interactions with *F. graminearum*, *C. rosea* responds by expressing an array of genes actively involved in mycoparasitism and competition. Several studies highlight expression of multiple polyketide synthase genes in response to *F. graminearum* [308–310]. These genes are involved in synthesis of polyketides Clonorosein A and B, secreted by *C. rosea* and are shown to inhibit germ tube formation in *F. graminearum* [309]. High tolerance of *C. rosea* towards mycotoxins produced by *F. graminearum* could be due to the active efflux via numerous membrane transporters [291,310]. In addition, direct enzymatic detoxification of DON and ZEA by *C. rosea* has been reported [302,308,311]. High number of genes of cell wall-degrading enzymes, such as proteases, glucanases and chitinases [307,312] explains *C. rosea*'s ability to degrade the cell walls of many plant pathogens including Fusarium spp. Recent study by Piombo 2023 [313] found that effectors comprised 35–37% of the *C. rosea* secretome, and genes for several effectors were induced during the *C. rosea* response to *F. graminearum*. Furthermore, the active sRNA interference mechanisms observed in the mycoparasitism of *C. rosea* contribute to the effective suppression of *F. graminearum* [314].

The ability of *C. rosea* to exist as an endophyte in plants is significant for biocontrol, as it allows the BCA to be systemically present within a plant before the arrival of the pathogen. Hydrophobin and chitin-scavenging LysM protein genes found in *C. rosea* play roles during root colonization and plant-fungus interactions [315]. To

support its saprophytic lifestyle, *C. rosea* possesses high number of genes encoding carbohydrate-active enzymes [291].

C. rosea is known to induce resistance responses in plants. For instance, the colonization of wheat seedlings by *C. rosea* triggers the expression of PR genes, which encode pathogenesis-related (PR) proteins in both wheat [316] and tomato [317]. *C. rosea* has been found to activate various transcription factors (TFs) involved in defence responses in plants, such as WRKY family TFs [318] in plants.

In addition to all above mentioned mechanisms of biocontrol, *C. rosea* can contribute to increased plant growth response [319]. A plant that exhibits robust growth and vitality is likely to possess greater resilience against pathogen attacks.

C. rosea spores germinate relatively rapidly (up to 6 h) even at colder temperatures and dried spores can be stored during several months or even several years with preserved biocontrol efficacy [307, 320]. Another feature of *C. rosea*, adding to its flexibility as BCA in integrated pest management context, is its relative tolerance towards fungicides [307]. Several strains of *C. rosea* are used as commercial products worldwide, including available in Europe, strain J1446 (in products LALSTOP G46 WG®, Prestop® and Gliomix®).

Regarding *C. rosea*'s specific use for FHB control in cereals, various strains have been shown to be effective in reducing this disease. In different experiments, when *C. rosea* was either directly sprayed to wheat heads or applied to the overwintering maize stalk pieces before the season of wheat cultivation, it considerably reduced both incidences of FHB and mycotoxin accumulation. [301,321,324]. *C. rosea* has been shown to inhibit the development of perithecia and ascospore formation of *F. graminearum* on maize stalks [323]. Interestingly, treatment of Fusarium infected maize roots with *C. rosea* increased the conversion of DON into DON-3G [322].

Until now, no reports have been published regarding the use of BCAs to combat FHB in oats. Since oats are commonly grown in the same regions as wheat, often in succession after wheat cultivation, it is practical to consider that BCAs proven to be effective in wheat could also be applicable to oat production.

Paper III in current work examines the ability of *C. rosea* IK726 to reduce FHB and mycotoxin accumulation in oat. This strain of *C. rosea* was previously isolated from barley roots and reported to act as a strong BCA of several plant pathogens [291,307]. IK726 demonstrated effective control of Fusarium seedling blight in wheat and barley, as well as a reduction of FHB symptoms and DON content in wheat, observed in both greenhouse tests and field trials [307].

In our three separate greenhouse trials *C. rosea* reduced the biomass of *F. graminearum* by 79% and the amount of DON (sum of DON, 3-ADON and DON-3G) by 78%, in average) (**Figure 17**).

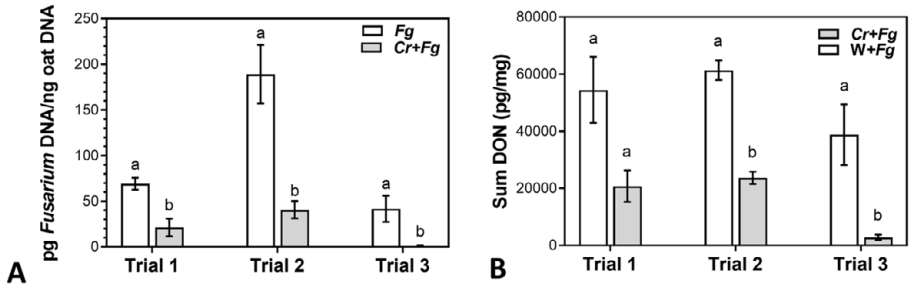

Figure 17. *F. graminearum* biomass and mycotoxin levels in three independent experiments. (A) *F. graminearum* DNA, (B) sum of DON, 3-ADON, and DON-3G. (Khairullina et al., 2023 [367])

Remarkably, *C. rosea* dramatically enhanced conjugation of DON applied to oat spikelets into DON-3G (**Figure 18**). Moreover, the accumulation of transcripts of two DON-detoxifying UGT genes (*AsUGT1 and AsUGT2*) significantly increased upon *C. rosea*-treatment (**Figure 19**). As *C. rosea* alone (without DON) did not directly activate expression of UGTs, this enhancement probably occurs due to *C. rosea*-mediated activation of certain transcription factors, couple with the transcription of UGT genes.

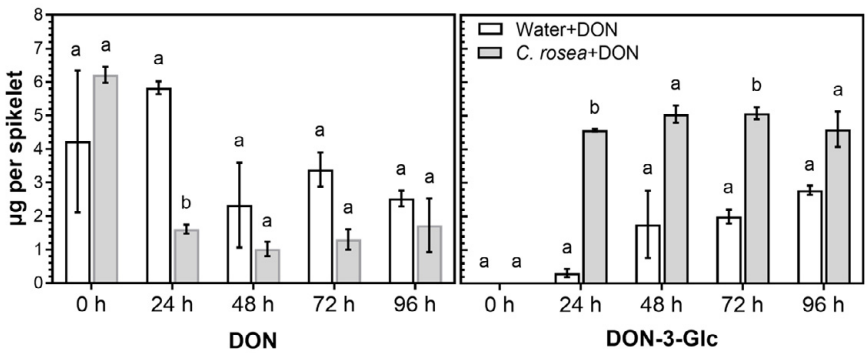

Figure 18. DON and DON-3G in *C. rosea*-treated and mock treated oat spikelets. Spikelets were treated either with *C. rosea* followed by DON (*C. rosea* + DON), water+DON, C. rosea+water or water + water. Error bars represent standard error of the mean. Within each part of the figure, means marked with different letters are significantly different ($p < 0.05$). (Khairullina et al 2023 [367])

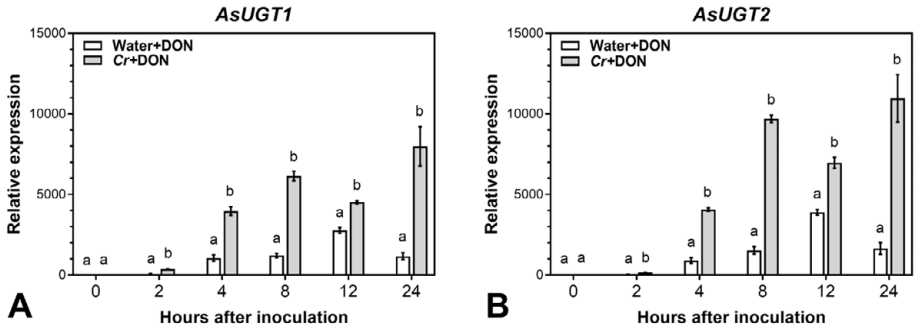

Figure 19. Relative expression of DON-induced (A) *AsUGT1* and (B) *AsUGT2* transcripts in *C. rosea*-treated and mock-treated oat spikelets. Error bars represent standard error of the mean. Bars marked with same letter are not significantly different ($p \leq 0.05$). Khairullina et al 2023

Furthermore, the expression of four PR proteins and the WRKY-23-like transcription factor was significantly upregulated in oat spikelets in response to *C. rosea* treatment (**Figure 20**). The selected proteins PR1, PR3, PR4, and PR5 have well-established roles in increasing the *F. graminearum* resistance in cereals [325–328]. Recently, WRKY23 was found to be involved in defence responses against FHB in barley [247].

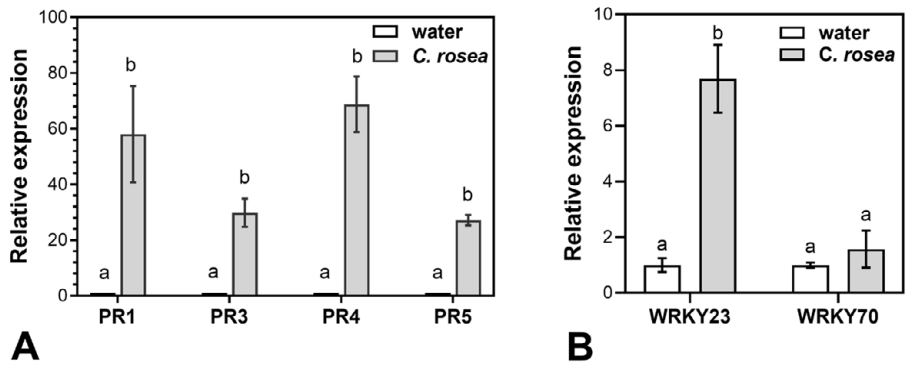

Figure 20. Relative expression of genes of four PR-proteins (A) and two WRKY transcription factors (B) in *C. rosea*-treated and water-treated oat spikelets 3 days after the inoculation. Error bars represent stand-ard error of the mean. Bars marked with same letter are not significantly different ($p \leq 0.05$ (Khairullina et al 2023)

In conclusion, *C. rosea* IK726 has good potential for utilization in combatting FHB in oats. It exhibits strong efficiency in inhibiting *F. graminearum* infection and facilitates the rapid detoxification of DON mycotoxin. The capacity of this strain to reduce FHB in oat in field conditions remains to be investigated.

Endophytes in FHB disease control

The plant hosts a diverse microbial community known as the phytobiome [329]. Chemical and physical exchanges contribute to the phytobiome's complex network, largely controlled by the plant itself but also influenced by environmental factors. Endophytic communities, particularly serve as a valuable reservoir of beneficial isolates for potential use as BCAs [39,330].

Endophytes, according to their recent definition, are microorganisms that colonize the internal tissues of plants without causing disease symptoms or harm to their host[39,40]. The close association and coevolution of endophytes with plants have led to their significant contribution to various plant benefits, such as growth enhancement and improved nutrient acquisition, as well as increased tolerance to abiotic and biotic stresses [40,331–333]. Endophytes encompass a broad spectrum of microorganisms, such as archaea, bacteria, and various fungi [39,332,334]. Despite this abundance and diversity, their roles in plant defence remain remarkably understudied.

Importantly, several microorganisms among already established effective BCAs display endophytic lifestyle. A few examples are bacteria *B. subtilis* [332], root-colonizing fungus *Serendipita indica* [335,336] and several Trichoderma fungi [336]. Since endophytes colonizing internal plant tissues are better protected from the external environment, they hold the potential to be more dependable BCAs, offering improved consistency in their effectiveness [39,40].

A microorganism might be endophytic in certain conditions and saprotrophic, epiphytic or pathogenic in others. For example, strains of *F. graminearum* were found as symptomless endophytes in grasses, upon isolation and inoculation of wheat behaved as pathogens [98]. As a means of distinguishing between latent pathogens, beneficial symbionts, and neutral microorganisms within plants, the concept of "mutualism-parasitism continuum" is employed [38].

In respect to their symbiotic relationship with plants, endophytes exhibit different biological behaviours. The majority of endophytes reported as potential BCAs exhibit facultative symbiotic relationships with their host plants, i.e., they can be associated with different host plants, include a stage within insects, abide within the rhizosphere or live as saprophytes [332]. Fungus *Serendipita indica*, is an example a truly facultative endophyte, as it can colonize roots of a wide range of monocot

and dicot plants and by doing so promotes plant growth, induces resistance against phytopathogens and protects its host from abiotic stresses [332].

Regarding plant colonization mode, endophytes can occupy their host systemically (entire plant) or locally (certain tissues or sites) [331]. Prevailing number of reported microorganisms documented for their beneficial properties are non-systemic and are often located either in roots or phyllosphere of the plants [332]. For example, many endophytic species in the genus of Trichoderma exhibit their broad anti-pathogen properties by colonizing roots of various plants [336], while mutualistic Epichloë fungi abide exclusively in the aboveground parts of grasses [337].

Plants response to colonization by endophytes and reaction to infection by pathogens, seems to be very similar, especially during the initial stages of the interaction [40,331]. Endophytes, like pathogens, could use effectors to suppress their detection by the host, which is exemplified by *Serendipita indica* colonization of the roots [338]. Moreover, endophytes are known to modulate levels of plant hormones, such as abscisic acid (ABA), auxins, gibberellins, jasmonates (JA), salicylic acid (SA) among others, which could help to tune plant's initial defence reaction towards symbiotic mode [40,331].

In the context of their application as BCAs, endophytes display the same mechanisms as general BCAs in promoting plant resistance against pathogens. However, since endophytes reside within the plant and do not have direct contact with the pathogens, mechanisms such as direct parasitism and nutrient competition are expected to be less effective. Instead, antibiosis and host-induced resistance are particularly viable mechanisms for an endophytic BCA [331,332].

Isolation and selection of endophytes for biocontrol

When searching for specific endophytic BCA, adopting an ecological approach was found to be efficient, as it focuses on isolating of microorganisms from plants that thrive under high disease pressure. The idea is to identify healthy plants in these habitats, as they may possess a biocontrol microorganism in their microbiome that contributes to their improved performance. Endophytes obtained from the target environment are more likely to be well-adapted to the specific conditions of that environment [40,339].

An entirely novel, culture-independent approach is used by (Deroo et al., 2022) [340]where they first extracted whole bacterial communities from wheat spikes and tested them against FHB. After identifying the most effective community, the individual members were isolated and tested *in vitro* and *in planta* assays.

Another aspect of isolation of endophytes is using crop wild relatives as sources for better adapted microorganisms. Wild species are known to harbour greater diversity

of plant-associated microbes compared to cultivated varieties [334,341]. A long history of crop breeding may have unintentionally resulted in the loss of crops' ability to attract and host many beneficial microorganisms. Introducing endophytes from wild species to their cultivated relatives, could augment their defence against pathogens [334,339].

When endophytes are isolated from plant surface-sterilized tissues, a particular concern is that the growth requirements of certain microorganisms may vary significantly. Additionally, some strains may face inhibition due to antimicrobial compounds released by the host tissues upon wounding, or they might be outcompeted by faster-growing species [40]. These issues can be addressed, at least partially, by adjusting the nutritional contents of the media and/or incorporating compounds that favour the growth of specific microorganisms over others, such as antibiotics [40].

Once endophytes are isolated in pure culture, their identification can be achieved by the assessment of morphological features and sequencing various DNA loci. For bacterial identification, regions encoding 16S rRNA are commonly used, while for fungal identification, sequencing ITS (internal transcribed spacer), large ribosomal subunit rDNA, and, additionally, genes like beta-tubulin and translation elongation factor is routinely performed [342–344]. Upon identification, any known or potential plant and animal pathogens are excluded from further assessment.

After the identification of the microorganisms, the next step involves designing assays for biological control. While high-throughput *in vitro* screenings, known as confrontation assays, are commonly used, recent research indicates that well-designed biocontrol assays conducted *in planta* are much more to be preferred [40,345,346]. Efficacy of an *in vitro* assay disease often does not correlate with the results obtained from *in planta* assay [347,348]. Most importantly, the mechanism of induced resistance cannot be discerned in the absence of the plant.

Conditions during the screenings of endophytes should ideally replicate the actual conditions where the potential BCAs will be employed [40,349]. Initially, *in planta* screenings are conducted under controlled conditions to determine the optimal timing and application amounts of the BCA. Subsequently, field trials are carried out at different locations with larger plots, as BCAs often display varying efficiency depending on environmental conditions [40,349]. For trials targeting biocontrol against FHB, artificial irrigation is typically applied to maintain a high level of pathogen infection.

An essential aspect of developing an endophytic BCA is identifying the most dependable delivery systems for introducing the microorganism to the plant. These systems might involve seed coating, spray-inoculation of the crop or crop residues from the previous harvest, or even employing insects to deliver BCA spores to the plant's flowers [40,349]. Selecting microorganisms which can be stored for

prolonged periods and developing formulations that extend shelf-life of the BCAs greatly enhances the practicality and convenience of their industrial application.

Endophytes with antagonistic effect against FHB

Several studies have focused on isolating endophytes exhibiting biocontrol activity against FHB in cereals, with the most recent findings presented in **Table 4**. However, only a limited number of the listed microorganisms have been subjected to evaluation through field trials. Furthermore, the possible mechanisms of action have been explored for only a few endophytic BCA candidates. For instance, *Penicillium olsonii* ML37, during its colonization of wheat spikes, was found to trigger the expression of various WRKY transcription factors, PR proteins, and other defence metabolites within the first 24 hours after pathogen inoculation [350]. Another fungal endophyte, *Sarocladium zeae*, initially isolated from maize, not only demonstrated systemic colonization in wheat but also exhibited vertical transmission to the progeny. The mechanism underlying its capacity to reduce the spread of FHB and the accumulation of DON appears to be related to *S. zeae*'s ability to influence the host plant's hormonal defence responses and induce the expression of defence signalling pathways [351]. The anti-FHB activity of two bacterial endophytes, belonging to *Streptomyces* and *Bacillus*, is inferred to be a combination of plant growth promotion, synthesis of auxin (indole acetic acid, IAA), and induced resistance [352,353]. A remarkable antagonistic mechanism against *F. graminearum* is observed for an *Enterobacter sp*., [354] which forms biofilm-mediated microcolonies, building a root-hair endophyte stack (RHESt). This multilayered RHESt acts as a physical barrier for *F. graminearum* hyphae, leading to subsequent trapping and killing of the pathogen.

It is unsurprising that the majority of the endophytes listed in Table 4 are intended for the protection of wheat crops. Studies on the microbiome of oats have been largely ignored. **Paper IV** in current work describes isolation and identification of fungal strains, associated with green oat spikelets and exhibiting biocontrol activity against *F. graminearum* infection in oat.

In total, 88 fungal strains were isolated from oat spikelets collected at early anthesis, milk, and dough stages of grain development. The isolates underwent morphological examination and identification by sequencing of several gene loci, including ITS, actin, and TEF-1α. To evaluate their potential as BCAs, 10 selected isolates were subjected to two separate greenhouse trials using both spray inoculation and point inoculation of oat spikelets.

Table 4. Fungal and bacterial endophytes with antagonistic effect against FHB and mycotoxin accumulation:

Microorganisms	Isolation source	Assay type	References
Fungal endophytes			
Fungi of genera Phoma, Alternaria, Fusarium	Wheat leaves, steams and roots	Confrontation assay, Seedling assay (wheat)	Gdanetz and Trail, 2017 [355]
Phoma glomerata, Aureobasidium proteae, Sarocladium kiliense	Wheat aerial parts and roots	Confrontation assay, wheat detached spikelet assay	Comby et al. 2017 [348]
Simplicillium lamellicola	Wheat roots	In planta (inoculation of wheat heads) Field trials	Abaya et al. 2021 [356]
Sarocladium zeae	Maize roots, aerial tissues, and seeds	in planta assay (wheat) inoculation of steams and spikes	Kemp et al. 2020 [351]
Alternaria destruens, Fusarium commune, Fusarium oxysporum.	wheat	In planta (wheat)	Noel et al. 2022 [357]
Sarocladium strictum Anthracocystis floculossa Penicillium olsonii	Wheat leaves and spikelets	Detached wheat spikelet assay In planta (inoculation of wheat heads)	Rojas et al. 2020, 2022 [330,350]
Metarhizium anisopliae	entomopathogenic fungus, able to colonize roots	Coating of wheat seeds for in planta assay	Hao et al. 2021 [358]
Pseudozyma sp, Papiliotrema flavescens	Leaf, flower, anther and/or stem samples of cereals and weed plants	In planta (inoculation of wheat heads)	Shude et al. 2022 [359]
Bacterial endophytes			
Bacillus subtilis	Roots of *Salicornia brachiata*	Coating of durum wheat seeds for in planta assay	Brahim et al. 2022 [353]
Bacillus amyloliquefaciens	Wheat stems, leaves, panicles, and roots of wheat	Detached wheat heads	Zhang et al. 2019 [360]
Streptomyces sp.	Wheat roots	In planta assay, inoculation of heads, Seed treatment (spring wheat and durum wheat) Field trials	Colombo et al. 2019, Mattei et al. 2022 [352,361]
Enterobacter sp. (M6)	Roots of finger millet (*Eleusine coracana*)	In planta, wheat and maize	Mousa et al. 2016 [354]
Pseudomonas piscium	Wheat head	Confrontation assay, in planta assay (inoculation of wheat heads)	Chen et al. 2018 [362]
Pantoea ananatis, Erwinia persicina	Whole bacterial communities from wheat heads	In planta (wheat heads)	Deroo et al. 2022 [340]

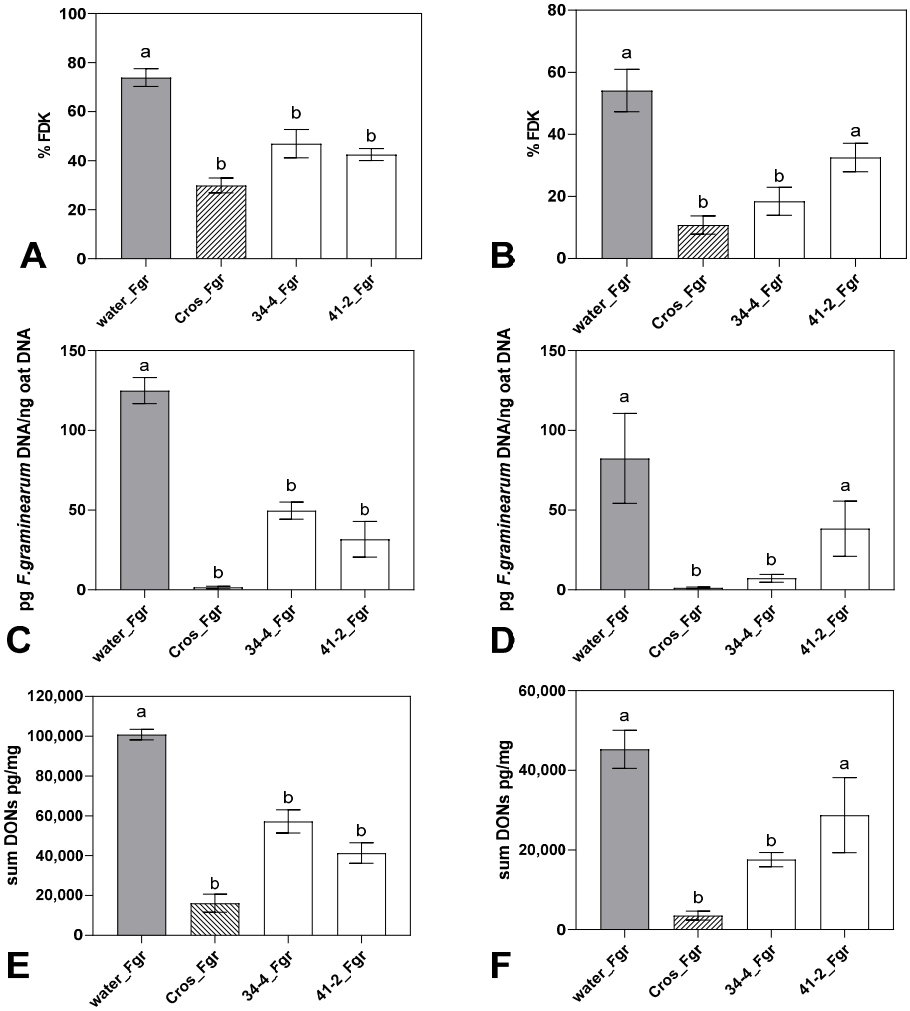

Figure 21. Comparison of mock treatments (water) and treatments with *P.flocculosa* 34-4, *P.flocculosa* 41-2 and *C.rosea* IK726 (used as a control). Percentage of FDK in spray (A) and point inoculated (B) samples. Amount of F.graminearum in spray (C) and point (D) inoculated samples. Sum DON (DON+3ADON+DON-3G) in in spray (E) and point (F) inoculated samples. Paper IV

Among the tested isolates, a strain 34.4, identified as *Pseudozyma flocculosa*, demonstrated effectiveness when point inoculated or sprayed three days before *F. graminearum* treatment. *P. flocculosa* 34.4 significantly reduced the number of damaged kernels, *F. graminearum* DNA load, and DON content in mature oat kernels (**Figure 21**).

P. flocculosa a basidiomycetous yeast, not pathogenic to plants or animals [363]. Several previous studies have shown its strong biocontrol ability against powdery mildew pathogens in various plants, including cereals [363–367]. Recently, two *P. flocculosa* strains isolated from green wheat spikes, demonstrated good biocontrol action against *F. graminearum* in wheat plants [330].

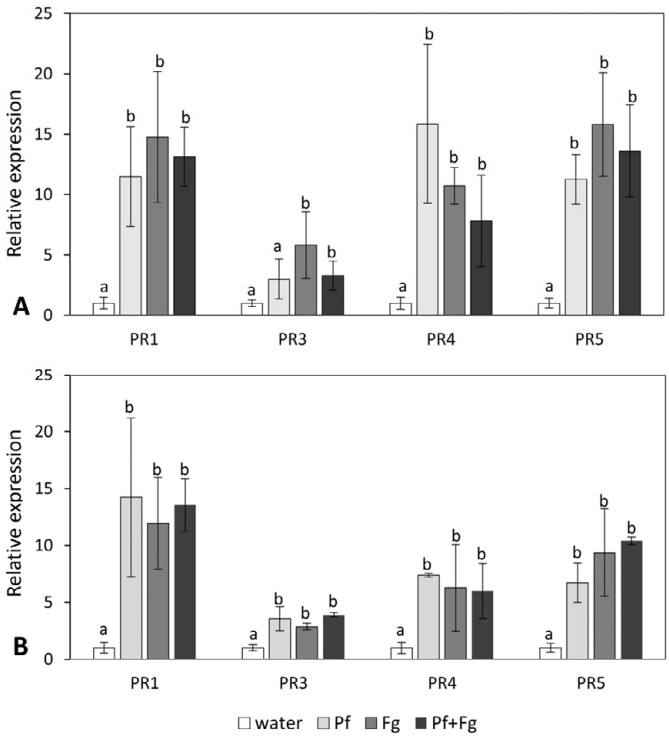

Figure 22. Expression of genes of four PR-proteins in P.f locculosa-treated oat spikelets with subsequent challenge with *F. graminearum* after 48h (**A**) and 72h (**B**). Designations of treatments: water = mock treatment with water; Pf = *P. flocculosa* 34.4 control, Fg = *F. graminearum* control; Pf+Fg = *P. flocculosa* 34.4 subsequently challenged with *F. graminearum*. Error bars represent standard deviation. Bars marked with same letter are not significantly different ($p \leq 0.05$).Paper IV

P. flocculosa's biocontrol mode of action against powdery mildews has been studied in detail. *P flocculosa* was reported to induce PR proteins in barley [367]. In tripartite interaction of barley, its pathogen *Blumeria hordei* and *P. flocculosa*, the effector of the latter was found to interact with barley PR1 protein and chitinase as well as with an effector protein from *B.hordei* [369]. The haustoria of *B. hordei* seems to be the main point where this interaction occurs. Previously it has also been shown that *P.flocculosa* draws essential microelements from powdery mildew without penetrating the pathogens hyphae [366,367].

In our further experiments, *P. flocculosa* 34.4 inoculated into oat spikelets resulted in accumulation of transcripts of genes encoding PR-proteins PR1, PR3, PR4, and PR5, associated with enhance resistance against FHB in cereals [368] (**Figure 22**). The results suggest that *P. flocculosa*'s biocontrol effect against *F. graminearum* could be attributed to its capacity to induce resistance in oat spikelets. Hypothetically, *P. flocculosa* could also weaken *F. graminearum* via drawing nutrients from the pathogen during the initial stages of infection.

In our greenhouse experiments, the biocontrol efficiency of *P. flocculosa* was lower compared to the efficacy of the strong biocontrol agent *C. rosea*. However, *P. flocculosa's* BCA efficiency in field conditions could be different. *P. flocculosa* seems to frequently inhabit cereal heads, thus it is well-adapted both to the hosts and to other common microorganisms inhabiting these hosts. Its' growth shown to be mostly epiphytic [364,367], suggesting that it can withstand harsh environmental conditions. The capability of *P. flocculosa* to control not only powdery mildew pathogens but potentially also such pathogen like *F. graminearum,* certainly makes this BCA worth to explore further. Studies of its interaction with Fusarium pathogens and other BCAs, as well as field FHB biocontrol trials could help to elucidate its true potential.

Conclusions and future prospects

Effective control of FHB in oats is important for ensuring the safety and quality of oats used for both animal feed and human consumption. FHB is a complex disease, influenced by various factors such as environmental conditions, the amount and composition of causal agents, cultivar resistance, and soil and plant microbiota. Integrated pest management (IPM), incorporating diverse control methods, aligns with these parameters [33,112]. This study explores multiple facets of management of FHB in oats, relevant to the establishment of a thorough IPM strategy.

Accurately identifying FHB symptoms is crucial for breeding disease-resistant oats and assessing the effectiveness of chemical or biological control methods for controlling FHB. However, FHB symptoms in oats are often not apparent, leading to possible errors and biases in scoring the disease. In **Paper I**, we present an affordable and rapid method for assessing FHB symptoms in oats by de-hulling mature seeds upon harvest. Paper demonstrates that symptoms of blackening and discoloration of the kernels correlate with high amounts of Fusarium DNA and mycotoxins accumulated in the affected kernel. Thus, these symptoms could be used as indicators by breeders, farmers, and researchers for fast quantification of FHB in oats.

To improve genetic resistance against pathogens, it is crucial to identify plant resistance genes and study their functions. In the present work, **paper II** describes identification and characterization of two oat UGT genes, involved in detoxification of several trichothecenes including DON, most prevalent mycotoxin contaminating oat. In other cereals, the increased glycosylation of DON has been directly linked to resistance to *F.graminearum* infection. Both identified UGT genes aid in effective mycotoxin detoxification and thus potentially could be used for developing genetic markers for FHB resistance in oat lines and cultivars.

The use of fungicides not only poses health risks to animals and contributes to environmental pollution but also increases the risk of pathogen resistance. Moreover, fungicides have shown limited effectiveness against FHB in oats, making biological control an attractive sustainable alternative. Recently, there has been growing interest in harnessing endophytic microorganisms as effective BCAs.

In wheat, both conventional BCAs and endophyte-based BCAs have been found to effectively reduce FHB symptoms and mycotoxin accumulation. However, research on BCAs and endophytes against FHB in oats has been scarce. In **Paper III**, we

investigated the biocontrol potential of the fungal BCA *Clonostachys rosea* against FHB in oats. Our results showed that treating oat spikelets with *C. rosea* significantly reduced Fusarium DNA and DON accumulation, enhanced DON detoxification, while upregulating several markers of induced resistance. We propose that *C. rosea* IK726 holds strong potential as a BCA against FHB in oats.

Furthermore, **Paper IV** focused on exploring the possibility of utilizing oats' own endophytes. We isolated fungal endophytes from oat spikelets and examined their effects on reducing FHB pathogen load and mycotoxin content in mature oat grain. Among the candidates, *Pseudozyma flocculosa* showed promising results, significantly reducing FHB symptoms, *F. graminearum* biomass, and DON in mature oat spikelets. *P. flocculosa* treatment was found to induce expression of PR proteins, involved in FHB resistance.

Together, **Papers III** and **IV** highlight the potential of fungal BCAs as effective means to combat FHB in oats, offering promising alternatives to traditional fungicides and contributing to sustainable disease management practices.

Considering the continuous threat of FHB in oat cultivation, innovative and effective control strategies become imperative. The future of FHB research is expected to be shaped by advancements in agricultural methods, breeding techniques, biological control, and genetic engineering, all fuelled by a deeper understanding of the disease's molecular mechanisms. The following short outline presents the potential avenues for exploration.

The recent availability of the oat genome has opened up significant possibilities for identifying disease-resistance genes in oat breeding [61]. Additionally, the forthcoming oat pangenome (https://wheat.pw.usda.gov/GG3/PanOat), capturing the greater genetic diversity within the oat species, promises to reveal novel resistance alleles. Leveraging metabolomics and transcriptomics methods can uncover key defence compounds and gene expression patterns during disease progression, aiding in the discovery of novel resistant genes in oats [221,228]. Through marker-assisted selection and genomic selection based on these resources, the development of disease-resistant oat varieties can be accelerated.

Deciphering molecular crosstalk between pathogenic Fusarium species and plants could help to discover susceptibility genes, which could be targeted to improve durability of FHB resistance. Host-, spray- or virus-induced gene silencing holds promise in silencing genes involved in the virulence processes of Fusarium fungi, hindering infection [221].

Agroecological strategies such as crop rotation and mixed cultivation should be further developed and adapted for commercial farming conditions to reduce inoculum build-up by integrating different plant species in the same field. Automated disease symptom diagnostics using sensors, image analysis, and machine learning can greatly benefit FHB trials in both greenhouses and the field,

enabling rapid and accurate identification of disease symptoms and facilitating breeding decisions for disease-resistant lines [113,166].

Deeper insights into the interactions between Fusarium species involved in FHB and the environment can shed light on shifts in various Fusarium species and expose interconnected disease dynamics. Continuous monitoring of Fusarium species and mycotoxins is crucial, as shifts in causal agents may alter disease severity, resistance effectiveness, and mycotoxin profiles.

BCAs employed for controlling FHB can be enhanced by combining different species of microorganisms or integrating with synthetic fungicides. Considering the combined modes of action and quality of interaction of BCAs is crucial, as synergistic or antagonistic effects impact disease control. Customizing commercial BCAs based on geographic and environmental conditions could provide tailored solutions [38,370, 375]. Use of BCAs that are directed towards different stages of the FHB disease cycle, such as reducing Fusarium inoculum in the soil and crop residues, could be widely utilized [371].

Understanding the interaction between Fusarium species and the plant microbiome is crucial for FHB management, and microbiome manipulation holds alluring potential [370,372]. Metabarcoding approaches are used exceedingly to study the co-occurrence patterns of various microorganisms in plants and are of a great help in searching for most efficient Fusarium suppressive microorganisms and microbial communities [371]. Synthetic microbial communities, designed to mimic the natural microbiome, offer a promising strategy against biotic stress [373]. However, further work is needed to ensure that the synthetic consortia can be integrated into the natural microbiome equilibrium. Furthermore, plant breeding to produce plants better adapted to attract and host most beneficial microbiome communities is another important way to improve disease resistance [374].

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