"***Intelligence is based on how efficient a species became at doing the things they need to survive***."

Charles Darwin (1809-1882)

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Interactions between the cereal aphids *Sitobion avenae* and the mycotoxin-producing fungal pathogen *Fusarium graminearum* on wheat

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) of Applied Biological Sciences: Agricultural Sciences

Dutch translation of the title:

Interacties tussen de graanluizen *Sitobion avenae* en de mycotoxine-producerende schimmelpathogeen *Fusarium graminearum* in tarwe

Illustrations on the cover:

Fusarium head blight symptoms and *Sitobion avenae* aphids (in frame) on wheat ears

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Nathalie De Zutter

September 2016, Brakel

Table of contents

List of abbreviations

A

3A-DON	3-acetyl-deoxynivalenol
15A-DON	15-acetyl-deoxynivalenol
AA	amino acid
ABA	abscisic acid
AFL	aflatoxin
AOS	allene oxide synthase
Avr	avirulence gene

B

Bt	*Bacillus thuringiensis*
BVOC	biogenic volatile organic compounds
BYDV	barley yellow dwarf virus

C

CAD1	cinnamyl alcohol dehydrogenase 1
CCR3	cinnamoyl CoA reductase 3

D

dai	days after infection
dbi	days before infection
DOM	deepoxydeoxynivalenol
DON	deoxynivalenol
DON-3G	deoxynivalenol-3-glucoside
DON-GSH	deoxynivalenol-gluthatione

E

ESI	electrospray ionization
ET	ethylene
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility

F

FDR	false discovery rate
FHB	Fusarium head blight
FPS	farnesyl pyrophosphate synthase

G

GA	giberellic acid
GLV	green leaf volatiles
GST	glutathion-S-transferase

H

HR	hypersensitive response
HRMS	high resolution mass spectrometry

I

ICS	isochorismate synthase

J

JA	jasmonic acid

L

LC-MS/MS	liquid chromatography tandem mass spectrometry
LOX	lipoxygenase
LSD	least significant difference

M

MRM	multiple reaction monitoring

N

NADPHOX	NADPH oxidase
NIV	nivalenol
NO	nitric oxide

O

OWBM	orange wheat blossom midge

P

P450s	cytochrome P450 mono-oxygenases
PAL	phenylalanine ammonia lyase
PAMPS	pathogen-associated molecular patterns
PBO	piperonyl butoxide
PCD	programmed cell death
PEROX	peroxidase
PR	pathogenesis related
PTI	PAMP-triggered immunity

R

r_m	intrinsic rate of increase
R	resistance gene
ROS	reactive oxygen species
RPL3	ribosomal protein L3
RT-qPCR	quantitative real time polymerase chain reaction

S

SA	salicylic acid

T

TOF	time of flight

U

UGT	UDP-glucosyltransferase

Thesis outline and research objectives

Introduction to organisms involved in current study

When pathogens and herbivores inhabit the same niche on a plant they can interact. These interactions are called tripartite as they involve three parties. This thesis will focus on cereal aphids *Sitobion avenae* and the toxigenic phytopathogen *Fusarium graminearum,* who both live on the ears of wheat *Triticum aestivum*. The interactions of the three organisms are represented in Fig. 1. This interaction triangle is expanded with the mycotoxin deoxynivalenol (DON) and the parasitic wasp *Aphidius ervi*. It is imperative to investigate every interaction of the fungus or aphid with other organisms inhabiting the same plant tissue (in this case the wheat ears), in order to learn more about the infection process of *F. graminearum* and to learn more about grain aphid epidemiology. These interactions are continuously changing throughout the developmental stages of wheat.

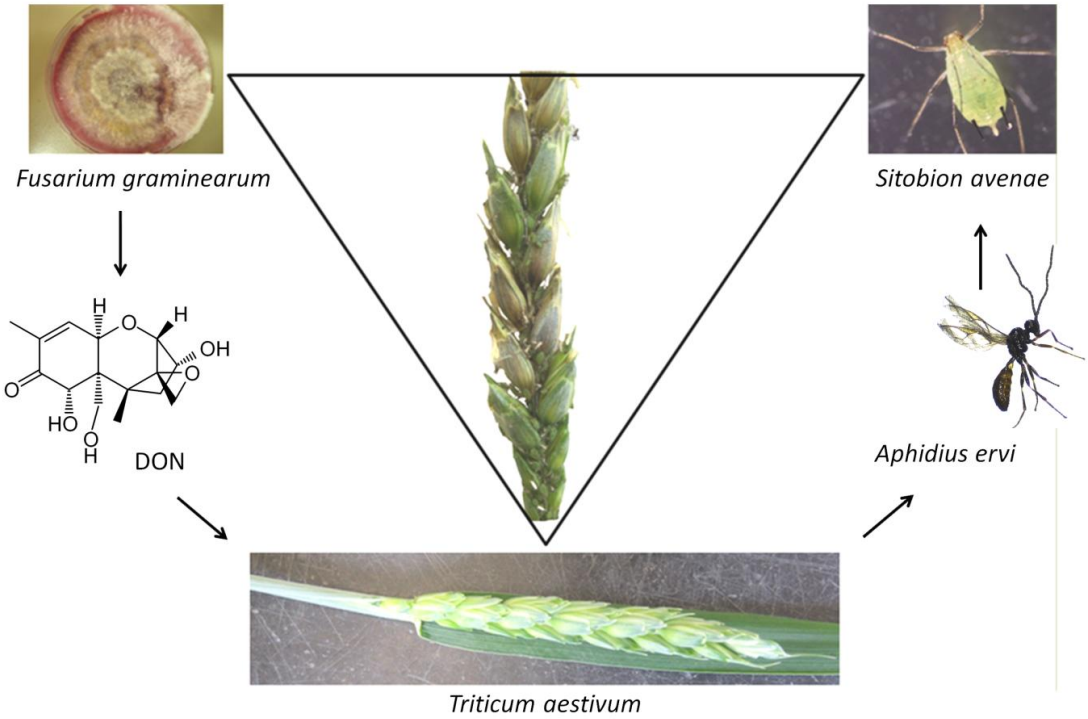


Fig. 1 Tripartite interactions between the grain aphid *Sitobion avenae* (and its parasitoid *Aphidius ervi*) and the pathogen *Fusarium graminearum* (with main focus on mycotoxin deoxynivalenol (DON)) both colonizing the ears of wheat.

Cereal crops are a very important food source in the world. With the human population increasing rapidly, a higher demand for cereals arises to satisfy the human nutritional needs. One of the world's most important food grains is wheat *Triticum aestivum* L. (Poales: Poaceae). This crop is especially desired because of its nutritional value and its adaptation to different growing conditions (Bushuk, 1998, Cassman *et al.*, 2003). Looking at data from 2010-2014 (FAOSTAT, 2016, consulted 14-03-2016) Europe produces yearly approximately 219,492,952.40 tonnes which represents 31.8 % of the production worldwide (Asia: 44.6% and America: 16.3 %). With an average yield of 8.8 tonnes/Ha Belgium is number two in the top five European countries delivering the highest yields.

Wheat is prone to many diseases ranging from root rot (*e.g. Pythium* and *Rhizoctonia*) and leaf diseases (*e.g.* powdery mildew and *Puccinia* rusts) to ear diseases like head blight (*Fusarium* and *Microdochium*). Fusarium Head Blight (FHB) is an economically important fungal disease that affects several cereal crops. This disease is characterized by typical symptoms like bleached spikelets, white to pink mycelium colonizing the ears and shriveled grain kernels (Goswami & Kistler, 2004). FHB is caused by a complex of fungi belonging to the *Fusarium* and *Microdochium* genus. This complex is not fixed but depends on climatic conditions, geography, fungicide application, etc. In Europe, *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Microdochium nivale* (former name *F. nivale*) are the most prevalent species (Parry *et al.*, 1995, Brennan *et al.*, 2007). The most studied and prevalent *Fusarium* species in Europe is *F. graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch.) (Hypocreales: Nectriaceae). Wheat is most susceptible for FHB during anthesis. The primary inoculum of FHB in the soil or on crop residues can affect an entire ear due to splashing rain drops (Trail, 2009). Infection of only a few spikelets can result in necrosis of the whole ear (Wang *et al.*, 2005) causing yield losses up to 40 percent (Bai & Shaner, 1994, Parry *et al.*, 1995). In addition, quality losses leads to difficulties during downstream processing such as brewing or baking (McMullen *et al.*, 1997).

FHB is not only a serious concern for the farmer but also for authorities and the end-consumer. On top of yield losses induced by all plant pathogens, toxigenic plant pathogenic fungi like *Fusarium* species produce a highly specific blend of mycotoxins which might cause acute or chronic health problems for animals and humans (Table 1) (Bottalico & Perrone, 2002, Goswami & Kistler, 2004).

Table 1 Several mycotoxins produced by *Fusarium* species (Bottalico & Perrone, 2002).

Species	Geographical incidence		Mycotoxin
	North/Centre	South	
F. graminearum	+++	+++	**DON**, **NIV**, **ZEN**, AcDON, FUS
F. avenaceum	+++	++	**MON**, BEA, ENS
F. culmorum	+++	++	**DON**, **ZEN**, ZOH,NIV
F. poae	++	+	**NIV**, **BEA**, DAS, FUS, ENS
F. equiseti	++	+	**DAS**, ZEN, ZOH
F. tricinctum	+	+	**MON**
F. cerealis	+	±	**NIV**, **FUS**, **ZEN**, ZOH
F. sporotrichioides	+	±	**T2**, **HT2**, **T2ol**, NEO
F. acuminatum	±	±	**T2**, NEO
F. subglutinans	±	—	**MON**
F. solani	±	—	—
F. oxysporum	±	—	—

AcDON = Monoacetyl-deoxynivalenols (3-AcDON, 15-AcDON); BEA = Beauvericin; DAS = Diacetoxyscirpenol; DON = Deoxynivalenol (Vomitoxin); ENS = Enniatins; FUS = Fusarenone-X (4-Acetyl-NIV); HT2 = HT-2 toxin; MON = Moniliformin; NEO = Neosolaniol; NIV = Nivalenol; T2 = T-2 toxin; T2ol = T-2 tetraol; ZEN = Zearalenone; ZOH = zearalenols (α and β isomers).

Deoxynivalenol (DON) is one of the most prevalent mycotoxins encountered in grain fields and is mainly produced by *F. graminearum* and *F. culmorum*. DON causes vomiting and food refusal in non-ruminants when exposed to high concentrations, but also in other animals and humans DON can pose a serious health threat (McMullen *et al.*, 1997). In animals, the toxic effects of DON range from diarrhea, vomiting, gastro-intestinal inflammation, necrosis of the intestinal tract, the bone marrow and the lymphoid tissues. In eukaryotic cells, it causes inhibition of mitochondrial function and has effects on cell division and membrane integrity and induces apoptosis (Pestka, 2010). Finally, it also inhibits protein, DNA- and RNA synthesis (Rocha *et al.*, 2005). Trichothecenes inhibit protein synthesis by binding to the 60S subunit of eukaryotic ribosomes. They impair peptidyltransferase by either inhibiting initiation of the peptide chain or, as DON does, inhibiting elongation (Goyarts *et al.*, 2006).

To tackle problem of toxic DON in grains, the European Commission introduced a legislation in 2006 (EG 1881/2006) (European Commission, 2006) regarding maximum threshold concentrations of mycotoxins allowed in grains and grain products with food purposes. While the maximum concentration of DON allowed in unprocessed grains is 1.25

mg kg^{-1}, grains for human consumption, like flour, can only reach 0.75 mg kg^{-1}. For bread a limit of 0.5 mg kg^{-1} is set. DON contamination in animal feed is regulated through directives imposed by the EU (2006/576/EC). For example, while pig feed can only contain 0.9 mg kg^{-1}, the directive applied for ruminants is 5 mg kg^{-1}. In comparison, concentrations found in wheat samples from fields all over Flanders (Belgium) fluctuate around 0.1-10 mg kg^{-1} DON (Audenaert *et al.*, 2009, Isebaert *et al.*, 2009, Landschoot *et al.*, 2013).

In order to tackle the mycotoxin issue, insights into the physiological function of these metabolites for the fungus can be a first important step. Nevertheless, the role of these mycotoxins in the pathogens life- and infection cycle remains largely unknown. One exception is the mycotoxin DON. DON is an important metabolite throughout the life cycle of the pathogen. It is an important metabolite involved in saprophytic survival in soil and crop residues and in the formation of spores. More importantly, DON is a virulence factor which interferes with the production of reactive oxygen species and with the plant's primary N-metabolism (Audenaert *et al.*, 2014).

As wheat is a crop grown in an agro-ecosystem, anthropogenic influences such as the use of agrochemicals also influences the behavior of *Fusarium* spp. Although crop pesticides are available to fight the *Fusarium* pathogens, chemical control remains difficult. Treatment of the fungus is only efficient around the short flowering period of the crop. Furthermore, not all species are equally sensitive to fungicides, and it was demonstrated that suboptimal fungicides application may lead to increased mycotoxin production. For example, treatments of wheat infected with *F. graminearum* with sublethal azole (*e.g.* propiconazole) concentrations led to an inducing effect of several mycotoxins (DON, 3-acetyl deoxynivalenol and nivalenol (NIV)) (Kulik *et al.*, 2012). Also higher levels of NIV were retrieved in wheat heads sprayed with sublethal tebuconazole (Becher *et al.*, 2010). Audenaert *et al.* (2010) suggested that hydrogen peroxide (H_2O_2) is induced by a sublethal dose of triazole prothioconazole and that this could trigger DON biosynthesis by *F. graminearum*. Changes in agricultural practices may drive the *Fusarium* pathogen populations to shift to those with greater aggressiveness and DON production (Al-Taweel *et al.*, 2014).

Finally, it is important to highlight that in wheat ears, *Fusarium* spp. also encounter other pests and diseases. Whereas the information on the role of insects in spread of viral plant diseases is present, the role of vectors in spread of FHB pathogens remains enigmatic due to a lack of research into the role of vectors, such as insects, transporting spores within

and between plants. Therefore, research on interaction between these pathogens and insects is particularly interesting. One of the major insects residing on wheat ears are aphids, more specifically the grain aphid (*Sitobion avenae*).

English grain aphids *S. avenae* Fabricius (Hemiptera: Aphididae) are an important pest in wheat fields of western Europe. It is known to migrate from leaves to the emerging ears and feed like many piercing-sucking insects on the phloem sap stream. Due to their high reproductive capacities on ears, they rapidly become a problem, leading to considerable yield losses (Wratten, 1975, Watt, 1979, Larsson, 2005, Kehr, 2006). Aphid populations vary greatly between years and population peaks occur at different crop stages. The economic threshold for high yielding wheat is one aphid/tiller at crop stage 59 (ear completely emerged), four at crop stage 69 (flowering completed) and seven at crop stage 75 (medium milk development) (Larsson, 2005). In the past, George and Gair (1979) demonstrated that one treatment with pirimicarb applied at the beginning of flowering when there were five or more *S. avenae* aphids per ear gave an increase in grain yield of 12.5%.

Aphids who are feeding from cereal ears cause direct damage and thus also yield losses. Large populations can remove nutrients from the plant, leading to reductions of dry mass and seed weight (Niehoff & Stablein, 1998), reductions in the number of spikelets per head when infestation occurs during boot stage (Voss *et al.*, 1997) and reductions of average seed weight during the later stages of plant growth (Havlickova, 1997, Voss *et al.*, 1997). Aphids occurring in ears can not only reduce grain yields but can possibly also diminish baking quality of grains due to changes in the protein composition (Basky & Fonagy, 2003, Basky *et al.*, 2006, Basky & Fonagy, 2007). Indirect damage includes the transmission of viruses like barley yellow dwarf virus, within and between different grain species (Blackman & Eastop, 2007).

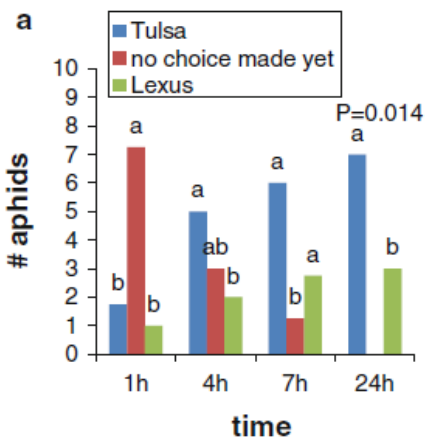
Starting the research: the initial preference and vectoring hypothesis

In a first attempt to elucidate the vectoring capacities of the *S. avenae* grain aphids the question arose whether or not the aphids occur on *Fusarium*–infected ears and if they consider these ears as suitable hosts? The initial hypothesis implied that if aphids are inhabiting infected ears or are even attracked by those ears, they have a greater chance to

act as a vector of the fungus. In order to find a suitable host, aphids depend on visual stimulation (*e.g.* colors) or olfactory cues ("smell of the plant") among others. They detect biogenic volatile organic compounds (BVOC) emitted by plants with their antennae. These BVOCs give the aphids a perception of the host's quality. The blend of volatiles emitted by a plant depends on plant species, age, welfare, etc. Plants under attack by pathogens or insects will emit different blends of volatiles compared to healthy plants. Based on these cues aphids choose a suitable plant. After landing on the plant, the aphids have several probings ("tastings"). *S. avenae* are well known piercing-sucking insects that suck up nutrients from the plant's phloem sap with their long flexible mouthparts, their stylets. After several probings, they will accept or reject a plant as host. This mode of feeding causes minimal damage to the plant in order to inhabit the ear and feed from its phloem sap for a long period of time. Several studies corroborate a role of plant volatiles in the attraction of aphids to plants. For example, Quiroz and Niemeyer (1998) noticed an elicited attraction of the cereal aphid *Rhopalosiphum padi* L. to volatiles produced by wheat and oat seedlings. Although plants produce volatiles during their physiological development, volatile biosynthesis is especially triggered when fungal or bacterial plant pathogens infect plants (Cardoza *et al.*, 2002, Obara *et al.*, 2002, Huang *et al.*, 2003, Vuorinen *et al.*, 2007, Toome *et al.*, 2010).

To delve into the preferential link between aphids and FHB-infected plants we conducted binary choice assays to elucidate the effect of *F. graminearum* on *S. avenae* aphids. In a first experiment, we investigated whether *S. avenae* preferred certain varieties of wheat and whether these varieties were also more susceptible to *Fusarium* infection (Fig. 2). However, no real parallelisms were uncovered (De Zutter *et al.*, 2012).

In a second approach, we investigated whether there was preference of aphids for FHB infected plants. Choice experiments in fields and in the laboratory revealed no preference for wheat ears inoculated with *F. graminearum* or treated with DON compared to healthy ears (Fig. 3) (De Zutter, unpublished data).



b

	Sahara (- - -)	Tulsa (- - +)	Homeros (- + +)	Lexus (+ + +)
Sahara (- - -)	0.777	0.397	0.397	0.397
Tulsa (- - +)	0.777	0.062	**0.014**	
Homeros (- + +)	0.777	0.260		
Lexus (+ + +)	0.777			

Fig. 2 Response in binary choice experiment at seedling level with different winter wheat varieties (Lexus, Homeros, Tulsa, Sahara) for *S. avenae*. Choice behavior at seedling level over time **(a)**. Different letters above the bars indicate a significant difference in preference between the wheat varieties using multinomial logistic regression. Table with *P* values over all variety combinations; (-) and (+) indicate tolerance and susceptibility for FHB, respectively **(b)** (De Zutter *et al.*, 2012).

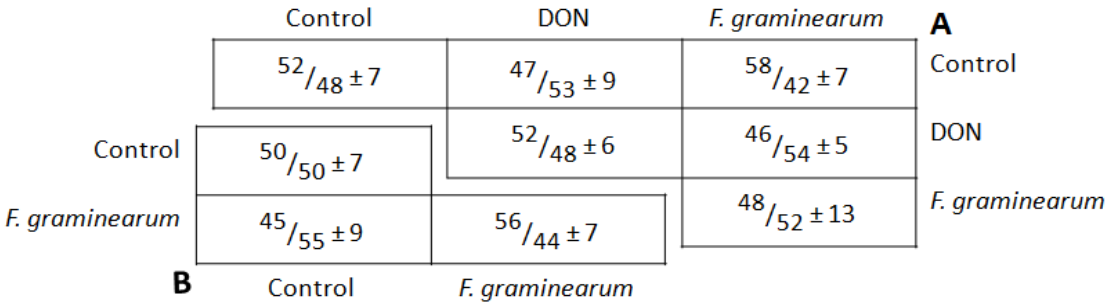


Fig. 3 The scheme represents aphid choice combinations in the laboratory **(A)** (N=6) and the field **(B)** (2014 as representative of two years, N=6). Percentages of aphids (± SE) on *F. graminearum*, deoxynivalenol (DON) or water treated ears 24 h after aphid introduction are shown. No significant differences were found among combinations of treatments (P > 0.05) using multinomial logistic regression.

Finally, in a third experiment, we verified whether aphids could serve as vectors to disperse conidia of *Fusarium* internally or externally. Aphids feeding on a parafilm sachet containing artificial diet and fungal spores (macroconidia) were put on petridishes with potato dextrose agar (whether or not crushed aphids to detect the fungus inside their body or in their honeydew). As we used a green fluorescent protein transformant of *F.*

graminearum, we could not detect any fluorescence on the plates. Macroconidia of *F. graminearum* are 41-60 x 4-5.5 µm (Samson *et al.*, 2004) while the stylet's food canal has a diameter of approximately 0.7 µm (Katis *et al.*, 2007). Internal uptake of fungal spores (conidia) by aphids is therefore impossible. Aphids were also externally examined, under the microscope after exposure to *F. graminearum* conidia in petridishes. As the majority of the aphids did not carry fungal particles, few of them were carrying spores on their legs or antennae. It seemed that their body was too smooth to carry fungal particles and even though some of them were carrying a single spore and theoretically this can cause ear disease, in reality this would not be enough to infect an ear as environmental conditions such as temperature and relative humidity must be optimal to ensure infection and one single spore cannot compete with the thousands or even millions of spores infecting a wheat field through natural processes. Moreover, even if *S. avenae* were vectors, which we think they are not, the conidia produced on infected tissue must be transferred to healthy ears within the crucial period of flowering, which is often short period of time and simultaneous with neighboring plants. Also Drakulic *et al.* (2015) demonstrated the failure of *S. avenae* aphids fed on symptomatic ears to produce disease in subsequent hosts.

In contrast, vectoring capacities of the orange wheat blossom midge (*Sitodiplosis mosellana* (Géhin)) are tentatively described. Mongrain *et al.* (2000) recovered *F. graminearum* from the spikes of wheat plants that had been exposed to artificially inoculated midges. Also on other plants, interactions between insects and fungal plant pathogens are already hypothesized. For example, pea aphids *Acyrthosiphon pisum* are a vector of *Verticillium albo-atrum* (Reinke & Berthold) on alfalfa (*Medicago sativa* L.) (Huang *et al.*, 1981). Adult shore flies, fungus gnats and moth flies are vectors of *F. avenaceum* that causes Fusarium crown and stem rot on lisianthus (*Eustoma grandiflorum*) (El-Hamalawi & Stanghellini, 2005).

Because the aphids were no vectors of *F. graminearum*, we had to delve into other interactions that are possible between fungi and insects inhabiting the same plant.

Thesis outline and research objectives

The doctoral thesis starts with **chapter 1** that gives a definition of tripartite interactions plant-pathogen-insect and screens all direct and indirect interactions playing between herbivores and fungal phytopathogens in plants. Influencing factors concerning the mediating role of the plant and the associated release of volatiles are highlighted. This is followed by a discussion of the human interferences on tripartite interactions in cereals.

In **chapter 2**, we investigated the influence of an earlier aphid infestation on the wheat expression profile of specific molecular markers associated with a *F. graminearum* infection. Using quantitative real time polymerase chain reaction (RT-qPCR) analysis, the expression of wheat key defense genes after *F. graminearum* infection and *S. avenae* infestation was tested at several time points. Aphids induced defense genes that are typically induced upon a *F. graminearum* infection. Moreover, we also assessed disease symptoms, fungal biomass, mycotoxin production and number of aphids at several time points during disease progress. Wheat ears infected with *F. graminearum* showed more disease symptoms and higher DON levels when ears were pre-exposed to aphids compared to a sole inoculation with *F. graminearum*.

DON, which is in Europe the most profound mycotoxin (Desjardins *et al.*, 2004), is one of the few mycotoxins of which the function is partially unraveled (Kazan *et al.*, 2012, Audenaert *et al.*, 2014) and is known to contribute to the virulence of the pathogen (Proctor *et al.*, 1995, Desjardins *et al.*, 1996, Bai *et al.*, 2002, Mesterhazy, 2002). TRI5-knock-out mutants of *F. graminearum* that possess an inactive *TRI5* gene are not able to produce DON. These mutants were less virulent and less able to colonize the rachis of ears, implying that DON is crucial for ear colonization (Desjardins *et al.*, 1996, Langevin *et al.*, 2004, Jansen *et al.*, 2005, Maier *et al.*, 2006). We hypothesized that, even though the aphids induce similar defense responses as against *F. graminearum*, the ears are still faster colonized by the fungus because of its higher DON production.

In chapter 2 we noticed that the grain aphids could survive on ears containing high concentrations of the toxin DON and its acetylated forms. Therefore, in **chapter 3** we investigated the (sub)lethal effects of DON on *S. avenae* aphids. These grain aphids are

under natural conditions exposed to several kinds of natural enemies like ladybugs, green lacewings and parasitoids. In this thesis we will thus also focus on the parasitic wasp *Aphidius ervi* Haliday (Hymenoptera: Braconidae), one of the most important parasitic wasps of *S. avenae* aphids in European cereal fields (Al Dobai *et al.*, 1999, Tomanovic *et al.*, 2008, Barczak *et al.*, 2014). *A. ervi* is included in the thesis because in a well functioning ecosystem they are indispensable and can also be affected by the tripartite interactions in which their host (*S. avenae*) plays a major role: we also tested the parasitism rate of the DON-contaminated aphids in presence of their endoparasitoid *A. ervi*. Here we wanted to learn more about food chain contamination with DON to higher trophic levels.

In a final research part of this doctoral thesis (**chapter 4**) we wanted to investigate whether *S. avenae* aphids can tolerate DON because of their exposure to the toxin during infestation of the wheat ear. Therefore we initiated a comparative study on the toxicity of DON for *S. avenae* and *A. pisum*. *S. avenae* inhabits cereals (monocots), whereas for example the pea aphids (*A. pisum*) has several species of legumes (dicots) as host. We demonstrated that *S. avenae* was more tolerant to DON than *A. pisum*. To elucidate the aphid's tolerance mechanisms for DON, several experiments were set up including amino acid sequence analysis of the DON target molecule 60S ribosomal protein L3 (RPL3) and experiments using targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) and non-targeted high resolution mass spectrometry (HR-MS^E) to detect DON and elucidate possible DON-derivatives in both aphid species. Using these approaches we detected several glucosylated forms of DON inside the aphids: DON-3-glucoside and DON-diglucosides. These data are indicatives of an adaptation by *S. avenae*, having stimulated DON detoxification processes whereas these detoxification mechanisms are not as efficient in other aphid species such as *A. pisum*.

Finally, in **Chapter 5**, the results throughout the chapters are linked together and the future perspectives of the research are given.

Chapter 1:

The tripartite interactions between herbivorous insects and fungal phytopathogens sharing the same host plant

De Zutter N, Audenaert K, Haesaert G and Smagghe G.

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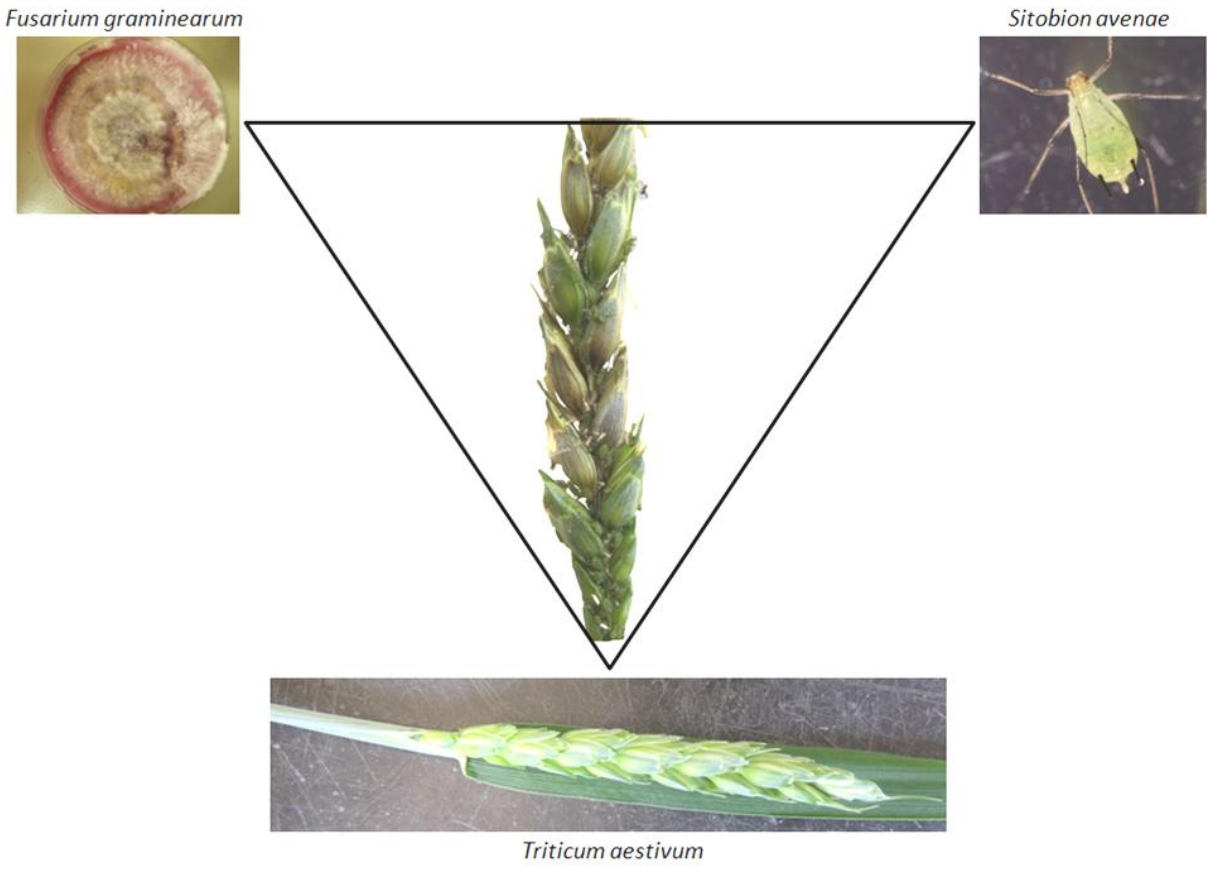

Abstract

In the past, research tended to focus on the plant-pathogen or plant-herbivore interaction. It is only recently that the tripartite interaction plant-pathogen-insect receives its needed attention. This chapter focuses on pests and fungal plant pathogens influencing each other directly and indirectly while inhabiting the same plant. Moreover, the mediating role of the plant and the associated release of volatiles are highlighted. Finally, a discussion on the direct and indirect anthropogenic impact on tripartite interactions through crop protection measures is given.

Keywords

Anthropogenic impact · Crop protection · Herbivorous insects · Fungal phytopathogen · Tripartite interactions

1.1 Introduction

Plants in nature and in agricultural fields are prone to many enemies, including herbivores (*i.e.* insects feeding on plants) and pathogens (*i.e.* organisms that can provoke disease). Both attackers can elicit phenotypical as well as molecular changes in plants. While in the past, research tended to focus on the plant-pathogen or plant-herbivore interaction, the interactions between pathogens and herbivores receives relative little attention. But in fact plants are often simultaneously attacked by pathogens and herbivores.

This chapter is a literature review that will focus on insect herbivores and fungal phytopathogens (*i.e.* fungi that are parasitic on a host plant) invading the plant on the same time. An attack of a plant by plant pathogens and plant pests is often a multi-phase event comprising first line attackers and secondary invaders which at a given time point co-occur on the plant and consequently impact each other directly or indirectly. More specifically, this PhD delves into the interactions present between grain aphids *Sitobion avenae* and the pathogen *F. graminearum*, who both inhabit wheat ears at the moment of anthesis.

Interactions between herbivores and pathogens can be direct, indirect (plant-mediated) or a combination of both. Direct interactions cover insect vectoring (*i.e.* dispersing fungal particles), fungivory or pathogens utilizing herbivorous feeding wounds to enter the plant. Indirect interplay implies the effect of the plant's defense towards one insect or fungal invader with respect to a second invader being a fungal or insect invader respectively. Plant changes and phytotoxic chemicals produced by pathogens and insects can influence the other parties with an altered performance as a result (Moran, 1998, Johnson *et al.*, 2003, Rostas *et al.*, 2003b, Stout *et al.*, 2006, Rohlfs & Churchill, 2011, Ponzio *et al.*, 2013, Tack & Dicke, 2013a, Lazebnik *et al.*, 2014). Both direct and indirect interactions (plant response, volatile emission and human interference) are schematically represented in Fig. 1.1.

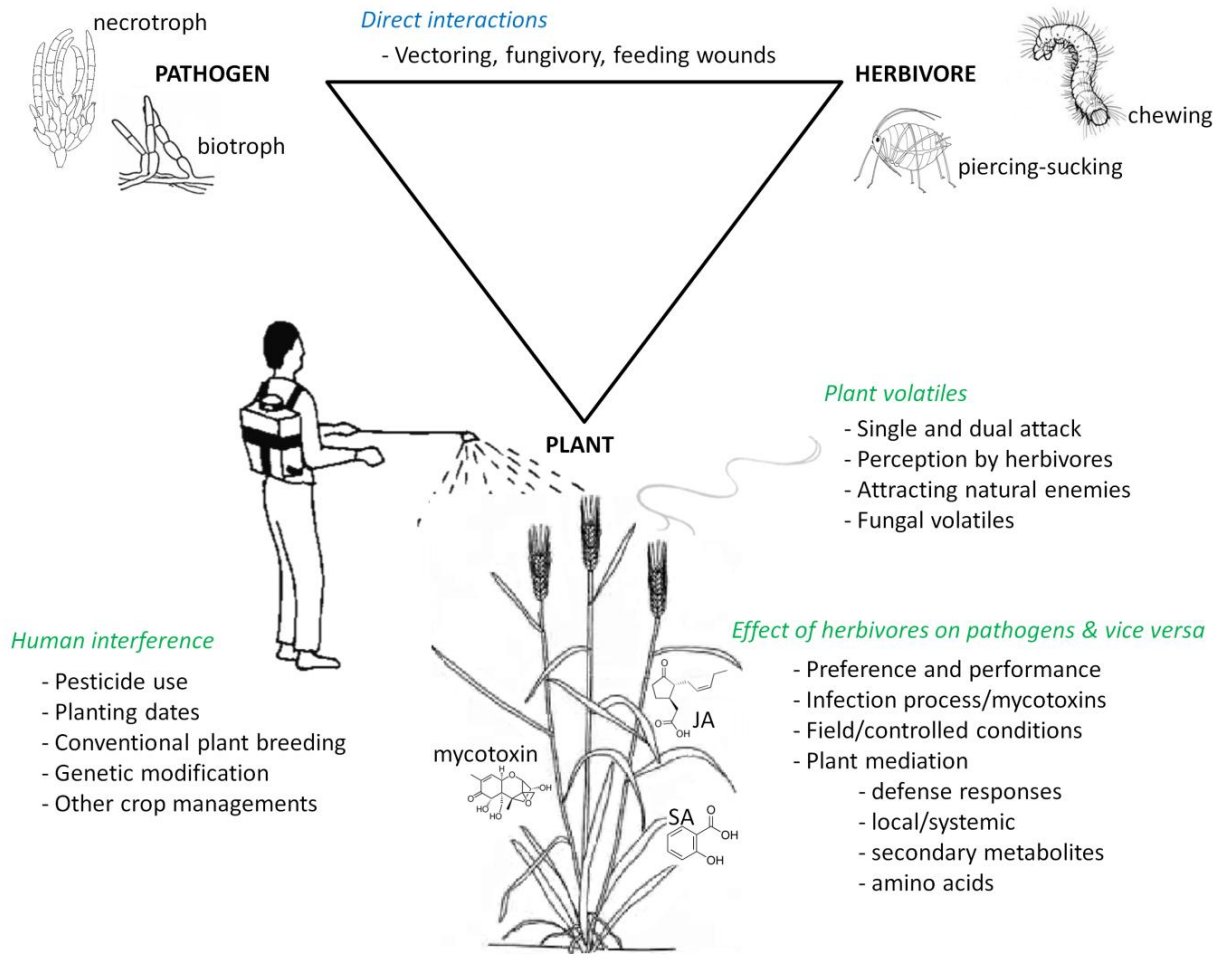

Fig. 1.1 Triangular scheme of three major organism classes (pathogen – herbivore – plant) occurring in plants to highlight the potential direct (blue) and indirect (green) interaction mechanisms.

1.2 Harmful organisms

1.2.1 Mode of feeding

Phytopathogens and herbivores are divided in groups according to their mode of feeding. Microbial pathogens are classified as biotrophs, necrotrophs and hemibiotrophs. Necrotrophs provoke cell death in order to obtain nutrients from dead tissue. In contrast, biotrophs can only maintain themselves on living plant tissue. Some fungi switch during development from a biotrophic to a necrothrophic mode of nutrition (*e.g.* the hemibiotroph *F. graminearum* causing Fusarium head blight (FHB) disease).

Herbivorous arthropods are divided into piercing-sucking and biting-chewing insects. Both groups are dependent in plant tissue throughout their life. This very close relationship with the plants makes them vulnerable to metabolic and physiological changes in their host plant. Piercing-sucking insects (*e.g.* aphids) use their stylet-formed mouth part to explore the host, penetrate a suitable plant and suck up the nutrients by ingesting large amounts of phloem sap (Will *et al.*, 2013). They inflict slight physical damage. Many piercing-sucking insects form intimate and long-lasting associations with their hosts, whereas chewing arthropods are usually more mobile. The latter removes large pieces of plant tissue. The feeding style of an attacker affects how the host will recognize the attack and respond to it.

1.2.2 Recognition of an attack by fungal phytopathogen or piercing sucking insects

A response against an attacker can only be effective when the enemy is recognized rapidly by the plant and when the switched-on defense responses are suitable to fight the intruder. Elicitors play a crucial role in recognition. An elicitor is any molecule that can activate plant defense reactions. Elicitors can be associated with the attacker itself or with components from the plant that are modified by the attacker (Ferreira *et al.*, 2006, Howe & Jander, 2008).

When a plant is attacked by a phytopathogen, it is important for the plant to rapidly recognize the attacker. In the past, literature reported on the production of elicitors (coded by avirulence genes (Avr) of the pathogen) and the subsequent recognition of these elicitors by the plants receptors (products of the resistance (R) genes). This is called the gene-for-gene complex (Avr-R). This interaction is expanded with the zigzag model of Jones and Dangl (2006) (Fig. 1.2): plants detect the PAMPS (pathogen-associated molecular patterns, red crystals) of the pathogen and trigger PTI (PAMP-triggered immunity). This is followed by ETS (effector-triggered susceptibility): a successful pathogen delivers effectors that interfere with PTI, enabling it to disperse in and feed from the plant. In a third step, the red effector is recognized by the plant, activating ETI (effector-triggered immunity). This immunity passes the threshold for hypersensitive cell death response (HR). In a fourth step, pathogen isolates

are selected that have lost the red effector and perhaps gained new effectors through horizontal gene flow (blue dots). These can suppress ETI and the zigzag model continues.

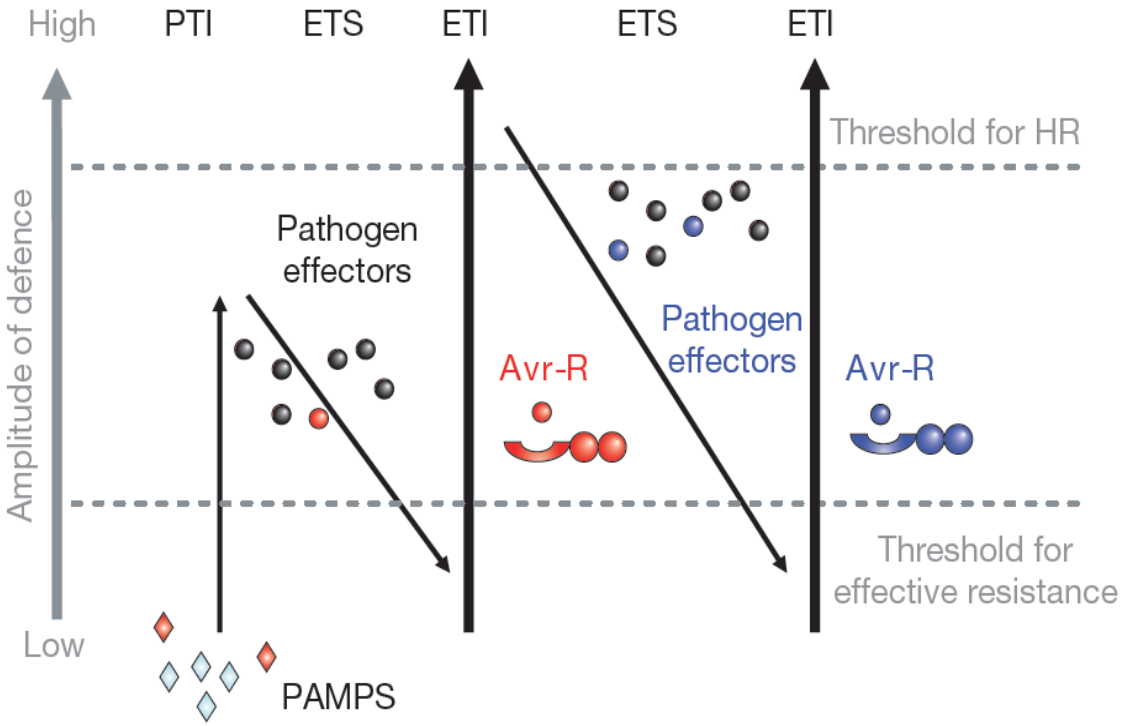

Fig. 1.2 Zigzag model illustrates plant-pathogen recognition (Jones & Dangl, 2006).

One of the best studied piercing-sucking insects are aphids. Aphids are especially feared because of their fast reproduction and population build-up under favorable circumstances, leading to yield reduction of grain crops. At higher temperatures, the development and reproduction of the aphids are faster. For example, the bird cherry-oat aphid *Rhopalosiphum padi* (a typical grain aphid in Europe) had high fecundity rates between 16 °C and 24 °C, with up to 4 nymphs produced per female per day. At even higher temperatures (24 °C - 28 °C) the time interval between each generation became shorter (Auad *et al.*, 2009).

The host selection behavior of aphids is dependent on a series of stages. First, aphids use visual stimuli like color and shape as well as olfactory stimuli like aphid pheromones and plant volatiles to find a suitable plant. After landing on that plant and assessing surface cues (*e.g.* are there trichomes present?) (second stage) aphids start probing the epidermis and underlying mesophyll- and parenchyma tissues and eventually phloem sieve elements (Fig. 1.3) to ingest small quantities of plant sap for gustatory discrimination. This is the third stage

in selecting a host. After accepting a plant as their host, aphids start feeding with their piercing-sucking mouthparts from the phloem sap of the plant during a long time. This mouthpart is called a stylet. These needle-like stylets can penetrate between plant cells and puncture individual cells to suck up nutrients. This stylet contains a salivary and a food canal. With the salivary canal aphids secrete sheath/gelling saliva to form canals in the plant's apoplast that can remain after stylet retraction (Tjallingii & Esch, 1993).

After penetrating the cell, plants react with some early events like callose depositions and protein plugging, both mechanisms are activated by a sudden Ca^{2+}-influx in order to prevent cell contents of leaking (Will *et al.*, 2013). Aphids must avoid or sabotage plant defenses and keep the phloem sieve tubes alive if they want to keep the phloem sap available for feeding (Giordanengo *et al.*, 2010). In this regard, plant defenses are counteracted by the aphids by excreting gelling and watery saliva, prior to ingestion, using the food canal in the stylet. The stylet's food canal of *Myzus persicae* for example had a diameter of approximately 0.7 µm (Katis et al., 2007). The saliva contains a mixture of enzymes such as pectinases, peroxidases and polyphenol oxidases (Baumann & Baumann, 1995, Urbanska *et al.*, 1998, Will *et al.*, 2009). Such enzymes like *e.g.* pectinases and phenol oxidases in the saliva of *S. avenae* can be recognized and activate defense mechanisms in wheat (Liu *et al.*, 2009, Ma *et al.*, 2010). Effector-proteins in the aphid's saliva can modulate plant-insect interactions (Hogenhout & Bos, 2011). Another early event is the production of reactive oxygen species (ROS), rapidly induced upon injury, that are toxic for insects (Kehr, 2006) but counteracted by salivary secretions containing *e.g.* NADH-dehydrogenases (Harmel *et al.*, 2008). These are all examples of defense mechanisms by plants to counteract herbivore attack and subsequently, defense mechanisms of aphids who on their turn must offset these plant defenses in order to maintain interactions with their host during several hours or even days.

While passively feeding from the phloem sap by means of high pressure within the sieve elements, aphids are provided with high concentrations of sugars and unbalanced, low concentrations of amino acids (Guerrieri & Digilio, 2008). The phloem structure transports a wide range of compounds like water, minerals, amino acids, organic acids, sugars and sugar alcohols (Kehr, 2006). The excess amount of sugars is secreted in the form of honeydew, consisting of an aqueous mixture of sugars (90-95%) and amino acids (Auclair, 1984). The sugar composition of honeydew reflects the composition of phloem sap; however, a number

of other mono-, di-, and oligo-saccharides are also synthesized by the sap feeder (through the action of gut enzymes on plant derived sucrose) (Hendrix *et al.*, 1992, Wackers, 2000, Sabri *et al.*, 2013). High quantities of honeydew on leaf surfaces can lead to a black layer of saprophytic fungi that block stomata and impair photosynthesis (Morkunas *et al.*, 2011).

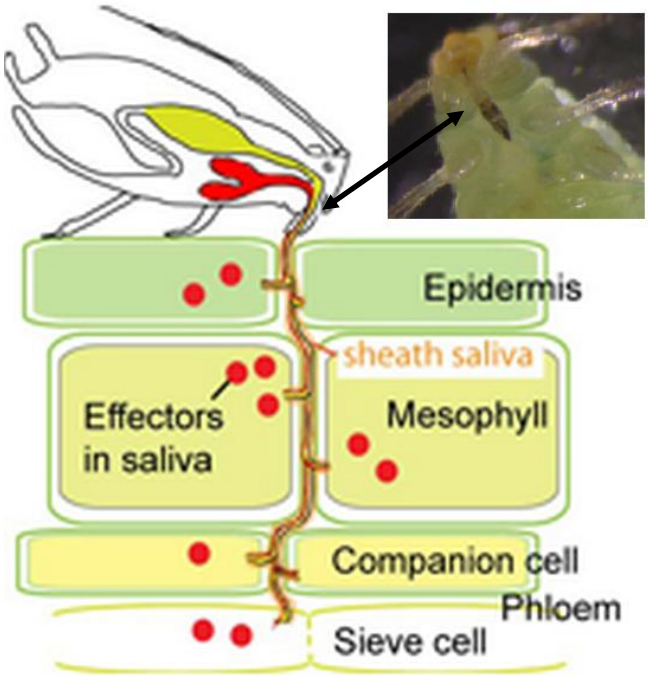

Fig. 1.3 Aphids penetrate the plant with their stylet (red: salivary canal, green: food canal) and puncture phloem sieve tubes (adjusted from Hogenhout (consulted 05-10-2015)).

Plants have developed different mechanisms to reduce aphid attack. It has been suggested that two different processes are involved in the elicitation of plant defense (Smith & Boyko, 2007). Whether a plant is susceptible or resistant to aphids, depends on how fast and efficient the plant can recognize an attack (Fig. 1.4). During incompatible interactions between aphids and plants, the plant with a resistance (R) gene rapidly recognizes aphid attack and infestation is counteracted (Kaloshian & Walling, 2005). This implies a gene-for-gene recognition of aphid-derived elicitors, followed by activation of aphid-specific resistance and defense responses (Smith & Boyko, 2007). It is also possible that the plant recognizes tissue damage without knowing that this damage is caused by aphids. This leads to a more general stress response that can, but not always, lead to the resistance of the plant against the aphids.

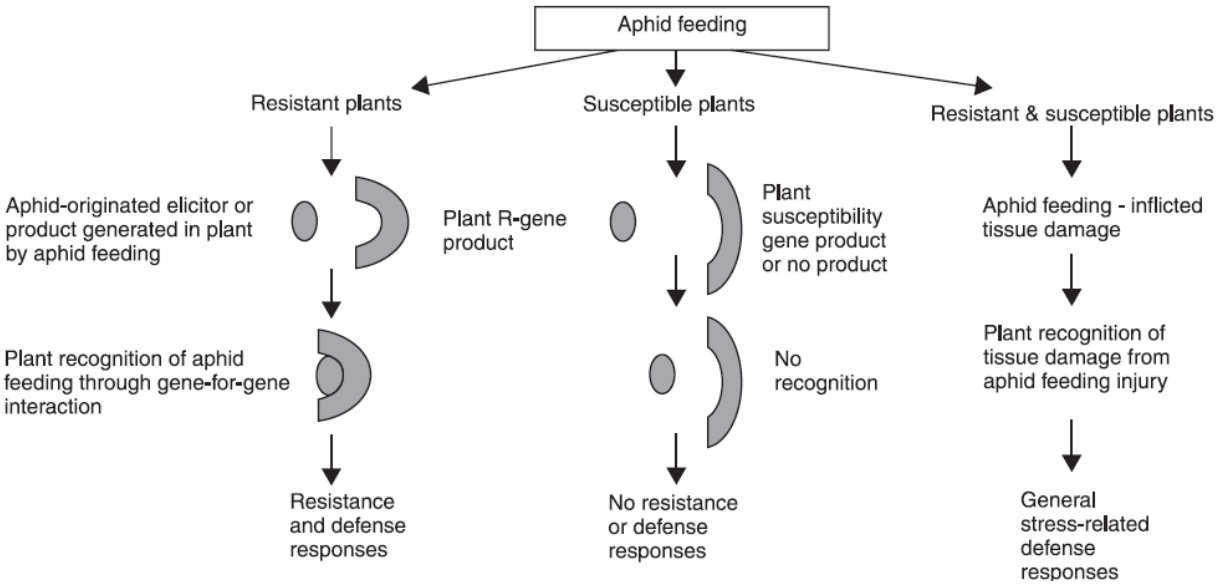

Fig. 1.4 Model of recognition of aphid feeding (Smith & Boyko, 2007).

1.2.3 Plant response induced after recognition: different attackers lead to different defense responses

Plants have evolved multiple layers of passive (constitutive) and active (induced) defense mechanisms to combat attackers in order to maintain their growth. Constitutive defenses, which are constantly activated, exists of physical barriers like cuticula and trichomes, preformed secondary metabolites and proteins that have antixenotic and antimicrobial effects. Induced defenses are changes in resistance-related traits that occur following attack. Both herbivore and pathogen attack can lead to such induced changes in the metabolism of the host plant. On plant cellular level, ion fluxes are induced and ROS are produced. Induced defense against pathogens involves the hypersensitive response. This is a programmed cell death at the site of infection in order to restrict the pathogen. This defense is only effective against biotrophs who cannot utilize dead tissue (Govrin & Levine, 2000, Heath, 2000, Wang *et al.*, 2013, Rojas *et al.*, 2014, Zhou *et al.*, 2014, Choi & Hwang, 2015). When plants survive an initial attack, this often renders them more resistant to a second attacker by responding more rapidly (the "priming" effect). This is called systemic acquired resistance (SAR) and is effective against both bio- and necrotrophs and some aphid species (Walling, 2000, Conrath *et al.*, 2002, Durrant & Dong, 2004, Pusztahelyi *et al.*, 2015). Also other defense related genes and metabolites are induced upon herbivore or pathogen attack (*e.g.* feeding

deterrents and pathogenesis-related (PR) proteins) (Dangl & Jones, 2001, Gatehouse, 2002). Also the fortification of cell walls is an example of direct induced defense (Kang & Buchenauer, 2000). Moreover, other changes can occur upon attack that are more indirect. Such a change is the production of volatile compounds (see 1.4.2) that can attract natural enemies of the attacker (Kessler & Baldwin, 2001, Ponzio *et al.*, 2013).

These direct and indirect induced responses are regulated by a signal transduction network in which jasmonic acid (JA) and salicylic acid (SA) play the most important role (Pieterse *et al.*, 2012). Although many studies have explored the antagonistic relationship between JA and SA pathways, exceptions have been noted depending in how particular enemies are perceived (Beckers & Spoel, 2006, Smith *et al.*, 2009, Pieterse *et al.*, 2012). According to the attacker (insect or pathogen) encountered, different pathways are activated. In general, SA is predominantly associated with resistance against biotrophic pathogens and JA with necrotrophs, though cross talk between SA and JA depends on the pathogen's nature (Rojo *et al.*, 2003, Glazebrook, 2005, Beckers & Spoel, 2006, Smith *et al.*, 2009, Morkunas *et al.*, 2011). For induced defenses against herbivores it is assumed that the JA-dependent pathway is effective but phloem feeding insects are also associated with SA-responsive genes (Gatehouse, 2002, Kessler & Baldwin, 2002, Howe & Jander, 2008). The timing of defense reaction activation and the strength of the defense response is crucial for resistance.

1.2.4 Attackers can hijack the plant defense responses to their benefit

Pathogens are able to activate a phytohormone signaling pathway that promotes disease by suppressing another phytohormone pathway that confers resistance. To enable such a successful colonization pathogens secrete effectors in an effective and timely way. Both effector-mediated manipulation of SA and JA pathways are known (Kazan & Lyons, 2014, Asai & Shirasu, 2015). Pathogens (especially biotrophic ones) need to attenuate SA signals to promote their fitness. Therefore, many pathogen effectors target the SA biosynthesis. For example, *Phytophthora sojae* and *Verticillium dahliae* secrete isochorismatase effectors that disrupt the plant SA pathway by suppressing its precursor (Liu, TL *et al.*, 2014). There are also effectors known that target components of JA signaling. The JA pathway provides resistance to various necrotrophic fungal pathogens, some of which have evolved abilities to suppress

this pathway. For example, *Sclerotinia sclerotiorum* produced a protein effector (SSITL) that played a significant role in the suppression of JA/ethylene(ET) signal pathway mediated resistance at early infection stages (Zhu *et al.*, 2013). Moreover, some effectors also alter the antagonistic relationship between SA and JA. The oomycete downy mildew pathogen of Arabidopsis, *Hyaloperonospora arabidopsidis*, was able to shift the balance of defense transcription from SA-responsive defense to JA/ET-signaling, and enhancing susceptibility to biotrophs by attenuating SA-dependent gene expression (Caillaud *et al.*, 2013). Effector-mediated manipulation of the gibberellic acid, auxin, abscisic acid, ET, cytokinin, and brassinosteroid pathways is also possible (Kazan & Lyons, 2014).

Also insects can deceive plants in order to trigger non-effective defense strategies. Evidence arises from the following examples: the silver leaf whitefly *Bemisia tabaci* can manipulate plant signaling (*Arabidopsis thaliana*) to suppress effective defenses. Under normal circumstances, the SA-based defense is upregulated upon whitefly feeding, while the JA-based defense is unchanged. Experiments show that the JA-regulated defenses are important to deter whitefly development. But when using a plant mutant, with impaired SA-regulated defenses and uncoupled SA-JA cross talk, that is treated with methyl jasmonate, the whitefly development is severely delayed. This demonstrates that JA controls defenses that actively impede the insects' development. Whiteflies are able to trigger a non-effective defense based on SA in order to suppress the effective JA defenses (Kempema *et al.*, 2007, Zarate *et al.*, 2007).

Similarly, sorghum (*Sorghum bicolor*) seedling plants that were attacked by *Schizaphis graminum* aphids displayed SA-dependent PR genes and only a weak induction of methyl-JA-regulated defense genes. However, infestation tests confirmed that the JA-regulated pathways were effective in plant defense against the aphids (Zhu-Salzman *et al.*, 2004). The authors of this study declare that these results indicate that aphids are able to avoid triggering of potentially effective plant defense machinery (probably through their particular mode of feeding). Phloem feeding insects can be perceived as pathogens (Walling, 2000) due to similarities in the manner of penetration of plant tissues (stylet vs. fungal hyphae) (Fidantsef *et al.*, 1999). In our experiment, genes that are typically upregulated upon *Fusarium* attack were also shortly upregulated during aphid infestation (deception of the plant) (see chapter 2) (De Zutter *et al.*, 2016a).

1.3 Direct interactions between herbivores and fungal pathogens

1.3.1 Herbivores can vector fungal particles

When an insect can transport another organism (*e.g.* fungal particles) within or between plants, it is called a vector. Insects can carry particles internally and/or externally and help spreading disease resulting in higher yield losses and disease pressure. Often several insects are vectors of a certain fungus on a certain plant species. These findings arise from several examples on different plant species.

The first example is *F. avenaceum* that causes Fusarium crown and stem rot on lisianthus (*Eustoma grandiflorum*). Adult shore flies (*Scatella* spp.), fungus gnats (*Bradysia* spp.) and moth flies (*Psychoda* spp.) serve as vectors of the aboveground life stage of this soilborne plant pathogen, namely macroconidia produced on stem lesions. They acquire and transport these fungal particles through the air to healthy plants which on their turn developed disease symptoms. Using microscopy, it was found that all three insect species had macroconidia externally on the body but only *Scatella* spp. deposited macroconidia in their frass (El-Hamalawi & Stanghellini, 2005). *Scatella* spp. can also transmit *Verticillium dahlia*, *Fusarium oxysporum* f.sp. *basilici* and *Thielaviopsis basicola* (El-Hamalawi, 2008).

On alfalfa (*Medicago sativa*) several insects serve as vectors for the effective transmission of *Verticillium albo-atrum* causing Verticillium wilt. One example are the pea aphids (*Acyrthosiphon pisum*). Their host-seeking and feeding behaviors help contribute to the spread of the pathogen. Piercing-sucking insects acquire spores of *V. albo-atrum* from diseased plants, transport them, and release inoculum onto the probing and feeding wounds, which are ideal sites for infection and development of the pathogen. In contrast to these insects, chewing insects (*e.g.* alfalfa weevil (*Hypera postica*) and grasshoppers (*Melanoplus sanguinipes* and *M. bivittatus*)), that feed on infected leaf tissue, acquire the pathogen internally and deposit fungal particles in their feces. The pathogen is able to survive in the gut system. In case of planthoppers, feces become free of contamination when the diet changed from diseased to healthy leaf tissue. This means that the pathogen survived in a non-persistent manner (Huang *et al.*, 1981, Huang & Harper, 1985, Huang, 2003).

In case of cereal insects, authors can only hypothesize the capacities of the picnic beetle *Glischrochilus quadrisignatus*, the western corn rootworm *Diabrotica virgifera*, the European corn borer *Ostrinia nubilalis,* the orange wheat blossom midge (OWBM) *Sitodiplosis mosellana* and the western flower trips *Frankliniella occidentalis* to vector several *Fusarium* spp. (Windels *et al.*, 1976, Gilbertson *et al.*, 1986, Farrar & Davis, 1991, Sobek & Munkvold, 1999, Mongrain *et al.*, 2000). Mongrain *et al.* (1997) could not directly link the correlation between OWBM and *Fusarium* spp. to transmission of the pathogen by the insect. It could also be that host disease increased because of larval feeding damage (see 1.3.2), recruitment of the insects to infected hosts (preference because several members of the Cecidomyiidae family feed from fungal material, see 1.3.3), the adult may lay their eggs preferentially on *Fusarium*-infected ears or the involvement of plant response (see 1.4.1). In many studies, the acquisition of fungal inoculum was not satisfactorily demonstrated and there was no careful distinction between direct and indirect (plant-mediated) effects.

Another example of *Fusarium* transmission in cereals arises from the study of Kemp *et al.* (1996). Mites *Siteroptes avenae* that were fed with *F. poae* growing on agar plates were placed in open petridishes between rows of wheat plants at ear emergence. These ears became symptomatic because of the transmitting capacities of the mites. Similarly, in this PhD we tried to reveal the ways of vectoring of *Fusarium* by insects, more in particular *F. graminearum* dissemination by grain aphids *S. avenae*. As mentioned in the research objectives of this thesis, the aphids were not able to get internally contaminated with the fungus (their stylet is too narrow) but also not externally contaminated due to their smooth body. The latter was examined by putting aphids in petridishes containing potato dextrose agar medium with the sporulating fungus and examining the aphids under the microcope. These kinds of experiments (artificially inoculating insects) are unrealistic approaches. In reality, the inoculum source is an infected host (*e.g.* infected leaves or crop debris) rather than a fungal colony growing in a petridish. This fungal colony is probably a much denser source of inoculum/spores (worst-case scenario) compared to inoculum sources in fields. This problem was solves in a correct manner by Drakulic *et al.* (2015) who demonstrate that *S. avenae* aphids fed on symptomatic ears could not produce disease in subsequent hosts. *F. graminearum* could be promoted by aphid infestation without acting as a vector for the pathogen (Bagga, 2008, Drakulic *et al.*, 2015, De Zutter *et al.*, 2016a).

Several insects (with different feeding modes) can contribute to the dissemination of a fungus but it is dependent on the population density of the transmitting insects, the quantity of infected plants and the inoculum density present for acquisition. Dispersal of fungal particles by insects also implies that there should be an alignment between the life cycle of the arthropod and the infection period of the pathogen. This alignment is crucial for the uptake of fungal particles. In addition, dispersal of these particles should also be done in a (often short) time period when other (healthy) host plants are still receptive for disease. In order to help control fungal diseases, it is advisable to also control the insects that are possible vectors of fungi (or contribute to the disease pressure in any other way). This can be done by using insecticides and insect resistant plant cultivars (see 1.4.3). Unfortunately, many studies do not conclusively demonstrate the direct interactions between herbivores and pathogens because it can be easily be entangled with plant-mediating effects.

1.3.2 Herbivorous feeding wounds as entrance sites for pathogens

Feeding wounds made by herbivorous insects are not only deleterious for the plant tissue but also make an important contribution to pathogen infection and disease severity. The wounds can facilitate penetration and colonization of the plant by fungi. On macro scale this can lead to more disease pressure in the field. These findings are very much depending on the feeding nature of the insects. Parsons and Munkvold (2010) found strong associations between thrips and Fusarium ear rot symptoms (caused by *F. verticillioides*) in maize. These correlations were not as strongly present for corn earworms *Helicoverpa zea*. It could be partly accounted by the nature of their feeding damage. Unlike thrips which do not completely consume maize kernels but damage them by weakening the pericarp tissue, corn earworms destroy the individual kernel completely but leave adjacent kernels mostly intact, rendering thrips damaged kernels more prone to infection. A similar phenomenon was demonstrated for the European corn borer *Ostrinia nubilalis* larval feeding damage that poorly correlated with *Aspergillus flavus* infection of corn kernels (Mencarelli et al., 2013), a pathogen that occurs in the field at a later time point, during crop ripening. Larvae only occurred on the tip of the ears, whereas the fungus infected the whole ear, explaining the poor correlation.

Moreover, studies indicate a positive correlation between presence of insects on plants and mycotoxin production by the fungus. For example, the production of moniliformin and deoxynivalenol (DON) by *Fusarium* spp. (*F. proliferatum* and *F. graminearum*) are closely linked with insect injury caused by *O. nubilalis* larvae (Folcher *et al.*, 2012, Scarpino *et al.*, 2015). Higher mycotoxin occurrence (*e.g.* fumonisins, moniliformins, etc.) was associated with *O. nubilalis* feeding activity and it was suggested that reducing feeding damage by insect control measurements could be an effective solution to minimize mycotoxins (Mazzoni *et al.*, 2011, Blandino *et al.*, 2015) (also see 1.4.3).

1.3.3 Fungivory

Like plants, fungi are immobile organisms unable to escape from predator attack. The most obvious direct interaction between an insect and a fungus is fungivory (*i.e.* feeding from a fungus). As mentioned before several members of the Cecidomyiidae family are known to feed from fungal particles. Similar to plant adaptations to herbivore attack, fungal secondary metabolites are increasingly recognized to mediating resistance against fungivore grazing (Rohlfs, 2015). This is the case for the filamentous fungus *Aspergillus nidulans* that is being eaten by the soil arthropod, *Folsomia candida*. As a response, *A. nidulans* produced higher amounts of toxic secondary metabolites and invested more in sexual reproduction relative to unchallenged fungi (Doll *et al.*, 2013). In contrast to inducible defense strategies that are well known in plants attacked by herbivorous insects, induced resistance of fungi against fungivorous animals remain largely unknown.

1.4 Indirect interactions between herbivores and fungal pathogens

1.4.1 Role of the host plant as intermediary agent

In ecological systems, indirect interactions between plant pathogens and phytophagous arthropods can arise when infestation by a first attacker alters the common host plant so that although a second attacker could be spatially or temporally separated from the first one, the former could be affected. The induction of plant defense reactions leading to the

production of secondary metabolites is thought to have an important role since it involves antagonistic and/or synergistic cross-talks that may determine the outcome of such interactions (Mouttet *et al.*, 2011). Plant-mediated indirect effects of pathogens on herbivores and vice versa is variable (related to different tissues of the same plant (systemic) or related to the same plant part (local)) and also dependent on the feeding mode of the attackers (biotrophic or necrotrophic; chewers or piercing-sucking insects). Since results obtained in laboratory experiments (under controlled conditions) not always reflect field conditions, we differentiate between field and controlled conditions: all examples are controlled unless otherwise stated.

- ***Plant-mediated effect of herbivore infestation on fungal pathogens***

Chewing insects vs. biotrophic infections. The tripartite interaction between willow hybrid *Salix cuspidata*, the biotrophic rust *Melampsora allii-fragilis* and the willow leaf beetle *Plagiodera versicolora* was studied in laboratory assays and greenhouse experiments. The rust infection was not affected by herbivore feeding in a local scale (feeding on the same leaf). However, the susceptibility of the plants for rust was increased by herbivore feeding because more rust sori were found on the leaves adjacent from feeding-damages leaves (this is a systemical plant effect) (Simon & Hilker, 2003).

Grazing by the beetle *Gastrophysa viridula* (chewing) on *Rumex obtusifolius* led to a decrease in lesion density *Venturia rumicis* (hemibiotrophic) and *Uromyces rumicis* (biotrophic) but not *Ramularia rubella* (necrotrophic) in field experiments during autumn. For *V. rumicis* and *U. rumicis* significant reductions in lesion density occurred on the undamaged leaves of damaged plants, compared with similar leaves on undamaged plants, suggesting a systemic induced resistance (Hatcher & Paul, 2000).

Chewing insects vs. nectrophic infection. Herbivory by the leaf beetle *Phaedon cochleariae* did not influence fungal growth of *Alternaria brassicae* on Chinese cabbage neither locally (the same leaves) nor systemically (adjacent leaves). (Rostas & Hilker, 2002).

Populus hybrids previously exposed to the cottonwood beetle *Chrysomela scripta* (chewers) affected (positively/negatively depending in the hybrid clones) the subsequent susceptibility of the plants to the necrotrophic *Septoria musiva* (Klepzig *et al.*, 1997).

Piercing-sucking insects vs. biotrophic infection. The fungal pathogen *Magnaporthe grisea* (biotrophic) was less likely to cause symptoms of leaf blast on rice plants that had previously been infested with the white-backed planthopper *Sogatella furcifera* compared to uninfested plants. Resistance to *M. grisea* was induced in rice plants by planthopper infestation. The observed phenomenon could not be explained by the feeding behavior of the plant hoppers (stylet insertion, piercing-sucking) because no significant difference in leaf blast incidence was observed between damaged plants treated with needling and untreated control plants. In the insect infested plants, the expression of two genes regarding beta-1,3-glucanase (which indicates a strong antimicrobial activity), *Gns4* and *Gns5*, was confirmed by real time polymerase chain reaction analysis. These results indicated that infestation with plant hoppers apparently induced physiological changes including gene expression that were related to *M. grisea* resistance in rice plants (Kanno et al., 2005). The effect was also observed when plant hopper infestation (restricted to the stems) and blast infection were spatially separated (different plant parts) which indicated an induced systemic resistance (Kanno & Fujita, 2003). This evidence suggests that piercing-sucking insects will upregulate SA which can also lead to the inhibition of biotrophic pathogens (Lazebnik *et al.*, 2014).

Piercing-sucking insects vs. necrotrophic infection. One of the oldest evidences of insect infestation influencing the pathogen is the example of Leath and Byers (1977). Significantly more root rot caused by *F. roseum* (synonym *F. graminearum*) (hemibiotrophic) developed in alfalfa, red and white clover when the plants were subjected to *A. pisum* aphid feeding. It was not known whether this was due to an increased susceptibly of the plants or to increased pathogenecity of the fungus or to insect feeding injury itself.

Similar and more recent examples of herbivores causing increased pathogen infection arises from the work of Drakulic *et al.* (2015) but also out own work (chapter 2). Drakulic *et al.* (2015) demonstrated that wheat ears exposed to both *S. avenae* cereal aphids and *F. graminearum* showed accelerated disease progression, an increase in disease severity and mycotoxin accumulation compared to plants treated only with *F. graminearum*. The authors suggested that honeydew deposits on the plant could promote fungal colonization and on top of that, molecular mechanisms induced by coincidental aphid stress must also play a significant role in the increase of host susceptibility to FHB disease. The role of wheat defense in the increased *Fusarium* disease symptoms and mycotoxin accumulation was

confirmed in this PhD thesis (see chapter 2) (De Zutter *et al.*, 2016a). Expressions in ears containing both *F. graminearum* and aphids were observed earlier, similar and/or enhanced compared to ears containing only *F. graminearum*. We cannot directly link these enhanced expressions to the aphids but it seemed that if plant genes were already upregulated previously because of the aphids presence, they were able to react faster/better to a subsequent attack of the fungus. This enhanced response was circumvented by the fungus itself by producing higher levels of its virulence factor DON which could explained the accelerated disease progression and increase in disease severity (De Zutter *et al.*, 2016a). In these experiments, both the aphids and the phytopathogen inhabited the same plant part (wheat ears). It is thus tempting to assume that the plant responses involved were locally induced.

On the other hand, piercing-sucking insects can impact the infection of necrotrophic pathogens negatively. Mouttet *et al.* (2011) found a negative interaction between the necrotrophic fungus *Botrytis cinerea* and the aphid *Rhodobium porosum*, which is conveyed by reduced fungal lesion area.

- ***Plant-mediated effect of a fungal pathogen infection on herbivores***

Host plants infected by pathogenic fungi represent a complex feeding niche for herbivores. The fungus induces changes in the plant metabolites but also produces secondary metabolites itself (*e.g.* fungal toxins). For the influence of fungal mycotoxins on herbivores, see chapter 4. Performance of herbivores feeding from fungi-infected plant tissue can be positively or negatively influenced and this often linked intrinsically with plant-mediated reactions.

Biotrophic infection vs. chewing insects. Larvae of the stem-boring weevil *Apion onopordi* (chewing) developing in creeping thistle (*Cirsium arvense*) infected with *Puccinia punctiformis* rust showed higher survival, laid more eggs and were larger than weevils developing in healthy thistles (Bacher *et al.*, 2002).

In contrast, larvae of the butterfly *Melitaea cinxia* (chewing) developed more slowly and weighed less at diapause when feeding on leaves of narrowleaf plantain *Plantago lanceolata* infected with the biotrophic powdery mildew *Podosphaera plantaginis* compared

to healthy leaves. In a behavioral experiment larval groups tended to leave the original host plant when it was infected by *P. plantaginis*. The latter was confirmed under laboratory conditions as well as under common garden conditions. Although the exact mechanisms of the observed negative effects remained unclear, it seems likely that they were mediated by the host plant (Laine, 2004).

Biotrophic infection vs. piercing-sucking insects. In field studies, there were no interactions found between the population densities of *Aphis gossypii* and *Anasa tristis* aphids and the presence of powdery mildew *Erysiphe cichoracearum* (biotroph) on leaves of quash plants (*Cucurbita pepo x texana*) (Moran & Schultz, 1998).

Euceraphis betulae aphid performed better (weighed more, displayed enhanced embryo development) on silver birch (*Betula pendula*) infected with *Marssonina betulae* (biotroph) causing lesions on leaves compared to aphids reared on asymptomatic leaves (Johnson *et al.*, 2003). Plant-mediated interactions can be the underlying reason for both examples: it is known necrosis after pathogen infection represents accelerated senescence, and on healthy plants, several aphid species perform better on senescing leaves. Indeed, enhanced aphid performance can be the result of improvement in leaf nutritional quality. Leaves inoculated with the fungus in the manipulative field experiment of Johnson *et al.* (2003) contained higher concentrations of free amino acids. Free amino acids from mesophyll cell degradation are translocated out of infected leaves through the phloem as a result of the plant's response to the fungal attack. These changes are similar to leaf senescing and are proposed as the positive interaction between fungus and aphid (Johnson *et al.*, 2003).

Rust (*Uromyces viciae-fabae*) infection of *Vicia faba* plants enhanced the performance of *Aphis fabae* aphids. The aphid's response to rust infection was attributed to an increase in leaf total nitrogen concentration (Al-Naemi & Hatcher, 2013).

Necrotrophic infection vs. chewing insects. Evidence of a positive impact arises from the study of Carruthers *et al.* (1986). *O. nubilalis* larvae (chewing) developed faster on maize tissues showing symptoms of stalk rot caused by *Colletotrichum graminicola* (hemibiotrophic) than on non-inoculated tissue (Carruthers *et al.*, 1986). The authors suggested that the accelerated development of the larvae was attributed to improvement of

the nutritional value of tissues via maceration of tissues and breakdown of complex carbohydrates by fungal enzymes.

Necrotrophs can also impact chewers in a negative way. The phytophagous leaf beetle *Cassida rubiginosa* (chewing) consumed significantly more leaf tissue from healthy creeping thistle plants than from thistle plants infected with the necrotrophic fungus *Phoma destructiva*. Development time from freshly hatched larvae until pupation was significantly longer for larvae fed on infected leaves. Also the weight of last-instar larvae and pupae was lower, and larval and pupal mortality was higher when larvae had been fed with infected leaves (Kruess, 2002). Although this study could not easily attribute the negative effects to a specific mechanism it could be explained by the plant-mediated production of pathogenesis-related enzymes (*e.g.* peroxidases) or to the production of toxins by the fungus.

In laboratory studies, larvae of the above-ground diamondback moth larvae *Plutella xylostella* feeding (chewing) on leaves of cabbage plants that are inoculated with the soil-borne endophytic fungi *Acremonium alternatum* suffered from increased mortality and other negative effects. Since the experiments were conducted before the endophyte reached the green plant parts, *P. xylostella* came not in direct contact with the endophyte and thus the negative effects on the insect must result from a systemic plant response effect (Raps & Vidal, 1998). This indicates that the systemic changes to the host's biochemistry that are induced upon fungal attack can influence performance of insects feeding from the same plant but not necessarily the same plant part (*e.g.* above and below ground).

Rostas and Hilker (2002) found that when the chewing leaf beetle *Phaedon cochleariae* fed on Chinese cabbage leaves infected with the necrotroph *Alternaria brassicae* had a prolonged larval development and reduced pupal weight. Adult beetles avoided feeding and egg deposition on fungus-infected leaves. In contrast to these local effects, no systemic effect of phytopathogenic infection on the herbivore was detected. The mechanism behind this local effect (on the same leaf) was not fully elucidated but could be attributed to noxious compounds released by the fungus in the infected tissue (*e.g.* destruxins) or the fungus induced metabolic changes in the infected tissue that negatively influenced the beetles.

Necrotrophic infection vs. piercing-sucking insects. Infection of cucumber plants (*Cucumis sativus*) with necrotrophic pathogens *Cladosporium cucumerinum* (cucurbit scab fungus) and

Colletotrichum orbiculare led to an enhanced reproduction of melon aphid *Aphis gossypii* (Moran, 1998).

Mouttet *et al.* (2011) found a negative interaction between *Botrytis cinerea* (necrotroph) and *Rhodobium porosum* aphids, expressed by decreased aphid growth rate.

The necrotrophic fungus *Botrytis cinerea* had an inhibitory effect on development, survival and fecundity of individual *Aphis fabae* aphids. Infection of *Vicia faba* plants with this necrotroph led to reduction in leaf nitrogen concentration and this could possibly explain the negative effects on the aphid (Al-Naemi & Hatcher, 2013).

- ***Defense responses and amino acid profiles influencing interactions between fungal pathogens and herbivores***

Few studies have investigated tripartite interactions. In almost all studies mentioned above, the phytohormones were not tested. Lazebnik *et al.* (2014) did an effort to include phytohormones in this context in a hypothetical manner (Fig 1.5) but came across some counterintuitive phenomenons. For example, in experiments with young rose plants under controlled conditions, Mouttet *et al.* (2011) found a negative interaction between the necrotrophic fungus *Botrytis cinerea* and the aphid *Rhodobium porosum*, which is conveyed by decreased aphid growth rate and reduced fungal lesion area. These results are counterintuitive because a necrotroph is expected to stimulate JA dependent defense pathways, at the cost of SA expression, which would in turn benefit the aphid. Similarly, piercing-sucking insects induce SA dependent pathways, which should be positive for necrotrophs (due to a cross talk between JA and SA). These contrasting results could be explained by the possible role of other plant defense mechanisms such as a change in phenolic compounds and free amino acids that occurs downstream from phytohormonal signaling (Johnson *et al.*, 2003, Lazebnik *et al.*, 2014).

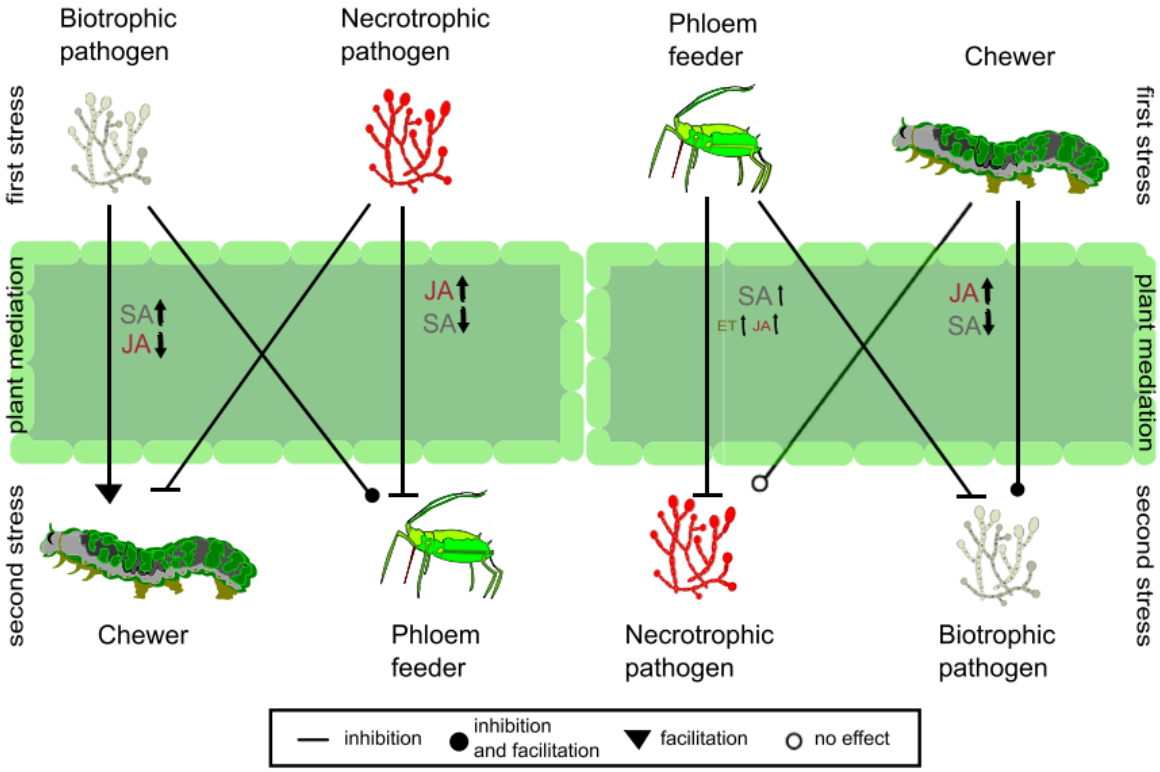

Fig. 1.5 Overview of plant-mediated effects of pathogens on insects and vice versa with different feeding modes, including hypothetical phytohormone-mediated mechanisms. This scheme was made after reviewing literature about sequential tripartite interactions among plants, pathogenic microbes and herbivorous insects (Lazebnik *et al.*, 2014).

The amino acid (AA) composition of infected leaves can be altered by fungal infection. Given that aphids are sensitive to changes in composition of phloem AAs it would be instructive to determine the effect of plant pathogens on AA profile of host plants.

Nitrogen mobilization in the plant can impact the available plant's AA. The glutamate metabolism in the plant has a pivotal role in AA metabolization and plays a key role in the plant's defense against pathogens (Seifi et al., 2013). Winter wheat grains infected with *F. graminearum* showed increasing levels of alanine, lysine and tyrosine and decreasing glutamic acid contents with a simultaneous increase in percentage of *Fusarium* damaged kernels or DON contents (Beyer & Aumann, 2008). Wheat ears treated with mycotoxin DON showed elevated levels of aromatic phenylalanine, tyrosine and tryptophan (Warth *et al.*, 2015b). Note that piercing-sucking insects like aphids ingest large amounts of phloem sap to obtain enough nutrients like AA that are indispensible compounds for the aphid survival. To

make up for the lack of essential AA (*e.g.* lysine, phenylalanine and tryptophan) in the phloem, aphids possess symbiotic intracellular bacteria *Buchnera* spp., that provide their host with certain essential AA (Douglas & Prosser, 1992). This indicates that host diet quality changes like altered AA concentrations induced by a preceding pathogen attack influences the insect performance. Changes to free AA composition of plants has already been suggested as a mechanism behind the altered fitness of *Euceraphis betulae* aphids on birch leaves infected with *Marssonina betulae* (Johnson *et al.*, 2003) and *Aphis fabae* when feeding on bean plants infected with *Botrytis fabae* causing chocolate spot disease (Zebitz & Kehlenbeck, 1991). In support of this concept, it was previously suggested that *R. padi* aphid rejection of his primary host and subsequent migration to secondary hosts was stimulated by the decline in levels of free AA within primary host leaves caused by long-term *R. padi* feeding (Sytykiewicz *et al.*, 2011). Moreover, Hale *et al.* (2003) indicated a correlation between aphid performance and essential AA availability.

In contrast (the other way around), it is also conceivable that the competition between herbivores and fungi for nitrogen compounds in the plant tissue can also impact the pathogen itself. The ability of aphids to alter their host's plant phloem has been suggested in several studies (Telang *et al.*, 1999, Sandstrom *et al.*, 2000, Petersen & Sandstrom, 2001, Wilson *et al.*, 2011). Stylet exudates were analyzed (stylectomy) from *Diuraphis noxia* aphids feeding from wheat. Comparison of samples from undamaged and damaged susceptible wheat revealed changes in AA composition and an increase in levels of essential AAs, indicating a nutritionally enhanced ingesta (Telang *et al.*, 1999). Such nutritional enhancement of host plants by aphids is dependent on the aphid species (Sandstrom *et al.*, 2000, Petersen & Sandstrom, 2001). Changes in the AA profile can have an influence on pathogens. For example, it is assumed that the pathogen *F. graminearum* may perceive polyamines and related AAs as cues for the production of toxins (sometimes virulence factors) during the infection process (Gardiner *et al.*, 2009, Gardiner, DM *et al.*, 2010).

To impede an attack plants are able to induce multilayered defense responses. It is possible that some PR genes are essential for defense against one organism and play little or no role in defense against another. But some set of PR proteins display broad responses to both pathogens and insects. In this respect, Wu *et al.* (2014) studied the expression profile of PR

genes during *Triticum* species defense against *Fusarium*, aphid-transmitted Yellow Dwarf Virus and Hessian fly *Mayetiola destructor* (Diptera: Cecidomyiidae). They showed that PR12 and PR14, encoding low molecular membrane acting protein, defensin and lipid transfer protein respectively, show broad responses to the pathogens and insects in their study. Similarly, microarray analysis of barley gene expression after *R. padi* aphid infestation of susceptible and partially resistant genotypes indicated that most induced genes belonged to the PR classes of proteins (Delp *et al.*, 2009). These genes (such as PR1 and PR5, chitinases and β-1,3-glucanase) were also known to be induced in barley leaves encountering powdery mildew *B. graminis* (Erysiphales: Erysiphaceae) (Gregersen *et al.*, 1997).

Botha *et al.* (2005) reviewed the cereal host interactions with Russian wheat aphid *Diuraphis noxia* and suggested that *D. noxia* feeding displayed both SA- and JA/ET-dependent signaling pathways by mimicking aspects of both pathogen and herbivorous insect attacks.

- ***Conclusion of plant-mediated interactions***

Interactions between fungal phytopathogens and herbivores inhabiting the same plant as host are very complex (positive, negative of no effect at all). The outcome is dependent on the ability of the host to induce resistance, the biology and mode of feeding of the attackers, the way the experiments are performed (way of infecting the plant tissue, field versus laboratory conditions) but also the spatial scale (the same or different plant part) and timing of the interactions. In regard to the latter, Drakulic *et al.* (2015) demonstrated that *S. avenae* grain aphids could accelerate *F. graminearum* disease progression depending on the period of aphid colonization before wheat ear inoculation.

To date little is known about how plants can convert their induced signals against multiple attackers (herbivores and phytopathogens) into a response that can increase plant fitness. Knowledge about plant mediated interactions against pathogens and herbivores are of interest in order to learn more about population dynamics of arthropods and pathogens in managed and natural ecosystems but also because they shed light on plant defenses against multiple attackers (Stout *et al.*, 2006). A lot of research is necessary to better understand the role of plant hormones in mediating interactions between pathogens and herbivores, that are to date still poorly understood. Moreover, a lot of examples mention

the possible role of toxin produces by the pathogens that can have a serious impact on the arthropods feeding from the fungus-infected tissue. Performance of herbivores coping with secondary metabolites from fungi is explained in chapter 4.

1.4.2 Role of plant and fungal volatiles

Since plants are sessile, it is impossible for them to evade environmental challenges. Chemical plant defense as consequence of elevated defense gene expression leading to the production of secondary metabolites having a negative influence on attacking enemies is not the only plant defense tool. Inventive dodging of attackers implies the production of biogenic volatile organic compounds (BVOCs). The chemical composition of plant-emitted volatile blends and their intensity can carry information about the plants' physiological status and the stresses they have been subjected to (Dudareva *et al.*, 2006). Insects can perceive these volatiles and adjust their behavior accordingly. BVOCs can act repelling for insects (Birkett *et al.*, 2000, Aharoni *et al.*, 2003), can be attractive for natural enemies of herbivores (Birkett *et al.*, 2000, Kessler & Baldwin, 2001, Shiojiri *et al.*, 2006) and even possess antifungal properties (Hammer *et al.*, 2003, Shiojiri *et al.*, 2006, Terzi *et al.*, 2007) among other functions. These secondary metabolites comprise terpenoids, phenyl-propanoids/benzenoids, fatty acid derivatives and amino acid derivatives (Dudareva *et al.*, 2004). Insects use their highly sensitive olfactory system located in the antennae contain receptor neurons in the sensillae to detect volatile compounds and differentiate between hosts and non-hosts causing attractance and repellence respectively (Bruce & Pickett, 2011). They are able to perceive the total array of plant BVOCs. Remarkably, the whole is more than the sum of parts as Bruce and Pickett (2011) formulated.

- ***The volatile blend upon single enemy attack***

The plant volatile blend becomes more complex upon attack by either insects, pathogens, or both. When a plant is attacked by a herbivore, the emission of a specific blend of volatile compounds is induced, known as herbivore-induced plant volatiles (Ponzio *et al.*, 2013). Joo *et al.* (2010) investigated the volatile spectrum of *Fagus sylvatica* trees under natural field conditions and in a growth chamber and saw that the emission spectrum shifted from

monoterpenes to linalool, α-farnesene, *(E)*-β-ocimene and *(E)*-4,8-dimethyl-1,3,7-nonatriene due to infection with *Phyllaphis fagi* aphids.

Plant pathogens are also capable of inducing plant volatiles, but this has been far less studied than the induction by herbivores. The ecological function of pathogen-induced plant volatiles is not very clear yet but it is thought that they have an antimicrobial function (*e.g.* (*Z*)-3-hexenol and (*E*)-2-hexenal) (Croft *et al.*, 1993, Ponzio *et al.*, 2013). Piesik *et al.* (2011b) found that vegetative tissues of wheat artificially infected in the greenhouse with one of the three *Fusarium* spp. (*F. graminearum*, *F. avenaceum* or *F. culmorum*) had a significant increase in BVOC production compared to undamaged plants. Green leaf volatiles (GLVs) like (*Z*)-3-hexenal, (*E*)-2-hexenal, (*E*)-2-hexenol, (*Z*)-3-hexenyl acetate and 1-hexenyl acetate, and terpenes like β-linalool and β-caryophyllene were released in greater amounts after infection by all fungal pathogens.

- ***Herbivore response to altered volatile blend***

When the volatile blend of a plant changes after a pathogen attack, it will impact the behavior of the herbivores. Preference of cereal leaf beetles *Oulema cyanella* for cereal plants was influenced by the volatile blend after *Fusarium* attack. GLVs like (*Z*)-3-hexenal and (*Z*)-3-hexenyl acetate attracted the beetles in a Y-tube olfactometer (Piesik *et al.*, 2011a) at doses comparable with herbivore-injured plant emission levels (Piesik *et al.*, 2010) while at high doses beetles were repelled by these two GLVs and by terpenes (*Z*)-β-ocimene and linalool (Piesik *et al.*, 2013) indicating that concentrations of individual BVOCs are important.

In light of the VOC blend after pathogen infestation, volatile emissions from plants infected with pathogens can provide the pathogen with their own benefit: attraction of insect vectors which can carry the pathogen to new hosts. This is described for bacterial pathogens (Mayer *et al.*, 2008), viruses (Eigenbrode *et al.*, 2002) and also for fungal pathogens (McLeod *et al.*, 2005). The latter performed laboratory and field studies using volatiles from American elm wood and suggested that the fungus *Ophiostoma novo-ulmi* manipulates host trees to enhance their appearance to foraging beetles *Hylurgopinus rufipes*, a strategy that increases the probability of transportation of the pathogen to new hosts (vectoring) (McLeod *et al.*, 2005).

Behavior responses of herbivores are thus influenced by pathogen-infected versus healthy plants. Leaf beetles *Cassida rubiginosa* preferred feeding on healthy tissue compared to thistles plants infected with *Phoma destructiva* (Kruess, 2002), in the olfactometer larvae of the herbivorous moth *Lobesia botrana* were attacked to volatiles emitted from *Botrytis cinerea*-infected fruits of grapevine *Vitis vinifera* (Mondy et al., 1998). Preference of insects towards (or away from) infected plants could explain higher insect densities found on the plants (or more specifically on certain plant parts).

- ***The volatile blend upon simultaneous attack***

When two plant enemies occupy the plant simultaneously, both the volatile blends and their proportional concentrations will alter. Maize seedlings in a climate chamber subjected to a concomitant attack of fungus *Setosphaeria turcica* and *Spodoptera littoralis* larvae emitted lower concentrations but qualitatively similar volatiles as when seedlings were only damaged by the herbivore (Rostas *et al.*, 2006). The change in volatile blend can here possibly be explained by the underlying plant defenses. While *S. turcica* is a hemibiotroph (Chung *et al.*, 2010) inducing SA- and ET-dependent pathways during early infection stages (Erb *et al.*, 2009), *S. littoralis* is a leaf-chewer (typical induction of JA-dependent defenses). The reduced volatile emissions from the dual-infestation could be explained by a cross-talk between SA and JA pathways (Ponzio *et al.*, 2013).

Nevertheless, knowledge about BVOC induction under multiple attack is still scarce to draw solid conclusions on how plants determine their volatile blend emissions and is dependent on many factors like attacker identity, severity of attack, sequence and timing of attack, abiotic conditions and phytohormones (Ponzio *et al.*, 2013).

- ***Fungal volatiles and subsequent insect behavior***

Analysis of the volatile blend can be a useful tool for early detection of fungal infection. Not only the plant can produce volatiles, also fungi can. Trichodiene is a volatile intermediate in the production of trichothecenes and can be used as a useful marker in the detection of toxigenic *Fusarium* and trichothecenes formation (Jelen *et al.*, 1997a, Jelen *et al.*, 1997b, Perkowski *et al.*, 2008, Girotti *et al.*, 2012, Becker *et al.*, 2014). This was also shown in a previous study of Jelen *et al.* (1995), which investigated the production of volatile

sesquiterpenes by *F. sambucinum* strains, having different abilities to synthesize trichothecenes. Strains that did produce toxins released high amounts of sesquiterpenes like β-farnesene, β-chamigrene, β-bisabolene, α-farnesene, trichodiene, etc. compared to strains that did not produce trichothecenes. These non-toxic strains showed besides a lower sesquiterpene production, also less chemical diversity.

Fungal volatiles are also affect the insect's behavior. *Tenebrio molitor* beetles larvae, a pest of stored products, were either repelled or attracted by grains according to their infection with different *Fusarium* spp. (probably due to fungal VOC cues) and could lead to increased mortality (Guo *et al.*, 2014). Attraction to infected grains could potentially benefit the fungus in terms of dispersal or feeding damage by the beetle facilitating fungal infection. Females of the yellow peach moth *Conogethes punctiferalis* showed clear preferences for oviposition substrates baited with mouldy codling, mouldy rice cake or fungi-inoculated agar media. Since the insects were not allowed to contact the fungi, it was suggested that the moth's response was induced by olfactory stimuli from volatile compounds associated with fungi like *Penicillium* spp., *Aspergillus* spp., *Mucor* spp., etc. (Honda *et al.*, 1988). It was suggested that fungal volatiles could provide a useful tool for oviposition monitoring or can be used as a mass-trapping agent.

- ***Altered volatile blend and natural enemies***

The plant-mediated response results in the release of BVOCs can affect the herbivore but can also mediate the behavior of natural enemies. Evidence of fungus-infected plants affecting the parasitoids arises from the next example: preference of the parasitoid wasp *Cotesia marginiventris* was influenced by plant volatiles upon infection with beet armyworms (*Spodoptera exigua*). The wasps were even more responsive to these volatiles when plants were infected with both catterpillars and white mold fungus *Sclerotium rolfsii*. This means that parasitoid behaviour is also influenced by the effect of pathogen-induced biochemical changes in plants (Cardoza *et al.*, 2003). In this way, pathogens can also modify the interaction between herbivores and their natural enemies. Parasitism rate is likely to vary between pathogen-infected and non-infected host plants.

1.4.3 Human interference in tripartite interactions

- ***Pesticides and planting dates***

Since crops must cope with many pests damages, humans have created several strategies to limit yield losses. Chemical pesticides are frequently used and interfere with the tripartite interaction of insects and pathogens inhabiting the same plant. In 1991 Farrar and Davis associated reductions in thrips populations by using insecticides with reductions of Fusarium ear rot incidence caused by *F. verticillioides* (Farrar & Davis, 1991). This pathogen is besides yield reduction also feared because of fumonisin contamination in grains. Foliar spray insecticide treatment led to ear rot and fumonisin B1 reduction. This result was influenced by the planting date, since at later planting dates (often associated with hot and droughty conditions, more favorable for thrips) higher thrips infestations led to more ear rot symptoms and higher toxin levels (Parsons & Munkvold, 2010). It was unclear how thrips influenced Fusarium ear rot risk but it may be accounted for by feeding damage.

A similar phenomenon was recorded for *O. nubilalis* damaging maize. Insecticide treatments applied at the beginning of insect flight activity were most effective in controlling insect damage on ears resulting in lower fumonisin contamination (Blandino *et al.*, 2009). The optimal treatment window was between the beginning of consistent adult flight activity and the flight peak. If treatment is delayed (after the adult flight peak), larvae from the eggs deposited early in the laying period enter the plant and are not controlled effectively by the insecticide. This results in more ear damage and fumonisin contamination. Also, earlier sowing dates reduced *O. nubilalis* damage leading to diminished ear rot incidence (Blandino *et al.*, 2008). Folcher *et al.* (2009) did not notice *Fusarium* spp. reduction in maize trails when caterpillars of *O. nubilalis* and *Sesamia nonagrioides* were controlled with insecticides but did report on reduced mycotoxin (trichothecenes, fumonisins and zearalenone) levels.

Instead of insecticide application altering disease epidemiology also the opposite is possible namely, fungicide (chlorothalonil) application in tomato crops (*Lycopersicon esculentum*) showed an inverse relationship between arthropod pest numbers (potato aphid *Macrosiphum euophorbiae*, green peach aphid *Myzus persicae*, flower thrips *Thrips* spp., flea

beetles *Epitrix* spp.) and disease (*Alternaria solani*) severity and a causative relationship could not be confirmed, though it was suggested that fungicide application provided more nutritious and suitable habitats for the pests by suppressing the disease of the tomatoes (Yardim & Edwards, 1998).

- ***Plant modifications***

Besides pesticide treatment and altered planting dates, humans can affect the tripartite relationship between insects and pathogens in cereals by modifying the plant itself. An extensively studied example is corn. Transgenic corn hybrids inserted with a *cry* gene from bacterium *Bacillus thuringiensis* (Bt) have in some parts of the world been proposed as an alternative for insecticide application. Several Bt hybrids are developed with the *cry1Ab* gene and are designed to specifically target *O. nubilalis*. Transgenic Bt maize, highly resistant to *O. nubilalis* injury, is subjected to much lower levels of Fusarium ear rot and fumonisins, compared to conventional hybrids (Munkvold *et al.*, 1999). This was confirmed by Clements *et al.* (2003) who examined the impact of Bt corn hybrids on the Fusarium ear rot severity and fumonisin contamination in grain. In seasons favoring *O. nubilalis* Bt hybrids were able to reduce fumonisin concentrations. Fungal biomass and fumonisin B1 in Bt maize were lower compared to isogenic maize (Bakan *et al.*, 2002).

In field trials positive correlations were found between Fusarium ear rot or grain fumonisin levels and injury from *O. nubilalis*, *H. zea* and western bean cutworms *Striacosta albicosta* (Bowers *et al.*, 2013). Maize hybrids expressing two transgenic insect resistance proteins (*cry1Ab x vip3Aa*) were more likely to yield low fumonisin grain compared to *cry1Ab*-hybrids or hybrids expressing no insect resistance. Bt hybrids also showed lower DON levels (Schaafsma *et al.*, 2002). As Munkvold (2003) stated, transgenic insect control plays a major role in prevention of mycotoxins in maize. Interestingly, fungal species composition in the maize stalk rot complex (*Gibberella zeae*, *Colletotrichum graminicola*, *Stenocarpella maydis*, and several members of the *Fusarium* genus) can differ between Bt maize hybrids and non Bt hybrids (Gatch & Munkvold, 2002).

In contrast, plants protected from a particular pest can become more favorable for another. For example, von Burg *et al.* (2012) and Alvarez-Alfageme *et al.* (2011) tested the effects of powdery mildew *B. graminis* on cereal aphids. A genetically modified mildew-

resistant wheat line and its non-transgenic sister line used in the experiments differed only in the presence of the transgene and in powdery mildew resistance. Pm3b wheat plants were generated by biolistic transformation of the spring wheat cultivar bob white, which has no endogenous *pm3* gene and is susceptible to powdery mildew. Pm3b plants have shown an enhanced resistance against powdery mildew under protected glass house conditions and in the field (Zeller *et al.*, 2010, Brunner *et al.*, 2011, Brunner *et al.*, 2012). The abundance of cereal aphids was negatively correlated with powdery mildew with transgenic powdery mildew-resistant spring wheat plants hosting more aphids than their mildew-susceptible controls under glasshouse conditions. In the field, there was no difference in aphid density between transgenic and susceptible wheat, probably due to low mildew and aphid pressure. For the negative correlation, the authors hypothesized that the fungal pathogen could change the allocation of plant metabolites and induce plant defense mechanisms which might change the nutrition provided to aphids by its host plant. These findings challenge the common assumption of transgenic plants counteracting a second party.

- ***Chemical elicitors***

Another human interference in the tripartite interactions between plants, phytopathogens and insects is the use of chemical elicitors that induce resistance in plants against a broad spectrum of pathogens, insects and abiotic stresses (Small *et al.*, 2012, Gordy *et al.*, 2015). BTH (SA-mimic benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester) treatment alone resulted in significant systemic resistance of maize seedlings against the pathogen *Setosphaeria turcica* (Rostas & Turlings, 2008). Moreover, when BTH was applied prior to *Spodoptera littoralis* caterpillar-feeding the plants became far more attractive to the parasitoid *Microplitis rufiventris* than plants that were only damaged by the herbivore (Rostas & Turlings, 2008). Also maize plants and cotton plants treated with BTH were more attractive for several parasitic wasps (Sobhy *et al.*, 2012, Sobhy *et al.*, 2015). Their studies confirmed that elicitors of pathogen resistance are compatible with the biological control of insect pests and may even help to improve it.

- ***Take home message of human interference in tripartite interactions***

Together, the abovementioned findings highlight the complex impact of humans on tripartite cereal interactions due to agrochemical treatment, altered planting dates that impede or favor insect populations or pathogen infection and modification of plants (genetic modification or by use of plant fortifiers) for higher resistance against attackers. Crop protection targeting a particular plant enemy, whether that protection is based in genetic modification or conventional control methods, should always account for the possibility of an altered attack by other enemies.

1.5 General recap and perspective

This chapter gave an overview of herbivorous pests and pathogens occurring together on plants and how they interact with each other. Given the frequent co-occurrence of herbivores and pathogens on plants, insights in these tripartite interactions in crucial to learn more about epidemiology of both plant enemies. In light of this PhD thesis, focusing in cereal aphids *S. avenae* and the mycotoxin producing fungal phytopathogen *F. graminearum*, we demonstrate for this particular tripartite interaction that herbivores can impact pathogen epidemiology through plant-mediated defenses (De Zutter *et al.*, 2016a), but vice versa, pathogens can impact the performance (De Zutter *et al.*, 2016b) and preference of the herbivore. This chapter took into account both direct as indirect interactions. Direct interactions have not yet been satisfactorily demonstrated or distinguished from plant-mediated interactions. Indirect interactions involved the mediating role of the plant when insects encounter pathogens on the same plant and vice versa as well as the associated release of volatiles. Finally, a discussion on the anthropogenic impact on tripartite interactions through crop protection measures was given.

In general, much remains to be discovered about the exact role of plants in relation with different kind of attackers and their different feeding strategies. In context of this PhD, it must be mentioned that a lot of research has been done on mycotoxin production in grains. Further research is needed to provide new insights in insect tolerance or resistance to secondary metabolites provoked or produced by phytopathogens. It is not inconceivable

that during co-evolution of herbivores and pathogens sharing the same cereal plant, herbivores have evolved mechanisms to cope with such deleterious components (see chapter 4).

In nature, plants are faced with multiple attackers and they need to adapt to the ever-changing environment. The dynamic three-way interactions of insects, pathogens and plants can constantly change and can be subject to influences from the environment like humans (see 1.4.3), weather, climate change, etc. Crop protection measurements must be taken in order to prevent or reduce crop damage and yield loss resulting from pathogens and herbivorous pests. Finding a balance between pest reduction and pesticide application to an economically and ecologically acceptable level is the core idea of integrated pest management (Oerke, 2006). Further research on these unique complexities is necessary to provide more insights in the cereal's ecosystem. This will provide us with knowledge about the plant's ecology and tolerance mechanisms resulting from co-evolution of insect-pathogen-plant interactions.

Chapter 2:

The plant response induced in wheat ears by a combined attack of *Sitobion avenae* aphids and *Fusarium graminearum* boosts the fungal infection and its deoxynivalenol production

Redrafted from De Zutter N, Audenaert K, Ameye M, De Boevre M, De Saeger S, Haesaert G* and Smagghe G*. Accepted in *Molecular Plant Pathology*

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Abstract

The pathogen *Fusarium graminearum*, producer of the mycotoxin deoxynivalenol (DON), and *S. avenae* aphids both reside on wheat ears. We explored the influence of an earlier aphid infestation on the expression profile of specific molecular markers associated with *F. graminearum* infection. Using quantitative real time polymerase chain reaction (RT-qPCR) analysis, we followed the expression of wheat defense genes upon *S. avenae* infestation and explored the effect on a subsequent *F. graminearum* infection. This was done by assessing disease symptoms, fungal biomass, mycotoxin production and number of aphids at several time points during disease progress. Wheat ears infected with *F. graminearum* showed more disease symptoms and higher DON levels when ears were pre-exposed to aphids compared to a sole inoculation with *F. graminearum*. Aphids induced defense genes that are typically induced upon a *F. graminearum* infection. Other defense genes showed earlier and/or enhanced transcription after exposure to both aphids and *F. graminearum*. In the discussion, we link symptomatic and epidemiological parameters with the transcriptional induction pattern in the plant. Our study suggests that pre-exposal of wheat ears to aphids affect the plant response which plays a role in the subsequent attack of *F. graminearum*, enabling the fungus to colonize the ears faster.

Key words

Fusarium graminearum · Mycotoxin production · Plant defense · *Sitobion avenae* · *Triticum aestivum*

2.1 Introduction

Fusarium head blight (FHB) is a disease on wheat caused by a complex of toxigenic wheat pathogens all belonging to the *Fusarium* genus. *Fusarium* spp. (Hypocreales: Nectriaceae) infect ears and can cause yield losses up to 40% (Parry *et al.*, 1995). They also produce a plethora of mycotoxins with diverse chemical structures (Bottalico & Perrone, 2002, Goswami & Kistler, 2004). Within the FHB pathogen complex *Fusarium graminearum*, a hemi-biotrophic fungus, is a common causal agent of FHB in Europe (Xu *et al.*, 2005). According to the type of mycotoxins produced, this species is divided into two different chemotypes producing primarily either DON and acetylated forms of DON (3-acetyl DON (3-ADON) and 15-acetyl DON (15-ADON)) or primarily nivalenol and/or fusarenone-XS (Miller *et al.*, 1991). High concentrations of DON in wheat ears can lead to human and animal health issues. DON has been notorious because it provokes acute and chronic disease symptoms like nausea, vomiting and diarrhea (Bennett & Klich, 2003). In regard to the massive yield losses and serious health concerns, profound research in *Fusarium* epidemiology is important.

Upon pathogen attack, plants can activate different defense mechanisms (Fig. 2.1). The infection process of *F. graminearum* being a hemibiotrophic fungus is complicated. Successful defense implies a first line of defense mainly made up by salicylic acid (SA)-directed responses during the biotrophic phase (Ameye *et al.*, 2015). SA is related with the production of reactive oxygen species (ROS) which leads to programmed cell death (PCD). Because a biotrophic pathogen needs living plant tissue to be provided with nutrients, this PCD is effective to retain the fungus (Heath, 2000, Qi *et al.*, 2012). The second line of defense comprises JA-directed defense mechanisms during the necrotrophic phase (Ameye *et al.*, 2015). The PCD response is favorable for necrotrophic pathogens, which can live on dead plant material. This necrotrophic phase is accompanied by the production of its virulence factor DON.

To date different layers of complexity in the interaction between *Fusarium*/DON and wheat have been uncovered. A first layer is situated at the pathogen exploiting the plants generic host stress response of polyamine and putrescine synthesis. It is assumed that the pathogen may perceive polyamines and related amino acids as cues for the production of toxins during the infection process (Gardiner *et al.* (2009), Gardiner, DM *et al.* (2010)). A

second layer of complexity is situated at the level of DON accumulation in infected tissue. As all other plant species when attacked by a pathogen, the wheat plant produces ROS to limit pathogen spread and induce antimicrobial plant response (O'Brien *et al.*, 2012). However, ROS stimulate PCD is not efficient against *Fusarium* having a necrotrophic phase at later time points in the infection. The third layer is situated at the interphase of ROS interfering with the toxigenic metabolome of *Fusarium*. Pursuing an *in vitro* approach, it was demonstrated that exogenously administered hydrogen peroxide (H_2O_2) to liquid *F. graminearum* cultures at time of spore germination resulted in higher DON and its acetylated form levels (Ponts *et al.*, 2006). Also Audenaert *et al.* (2010) suggested that after a sublethal application of fungicide, H_2O_2 can trigger DON accumulation. All the above mentioned examples show the role of ROS and polyamines in the interaction between *Fusarium* and wheat. A fourth and final layer of complexity involves plant detoxification mechanisms to weaken the detrimental effects of DON. This implies the binding of hydrophilic molecules like glucose (G) and glutathione (GSH) to DON and subsequently the transportation of the conjugated DON to vacuoles or apoplastic space (Coleman *et al.*, 1997, Bowles *et al.*, 2006).

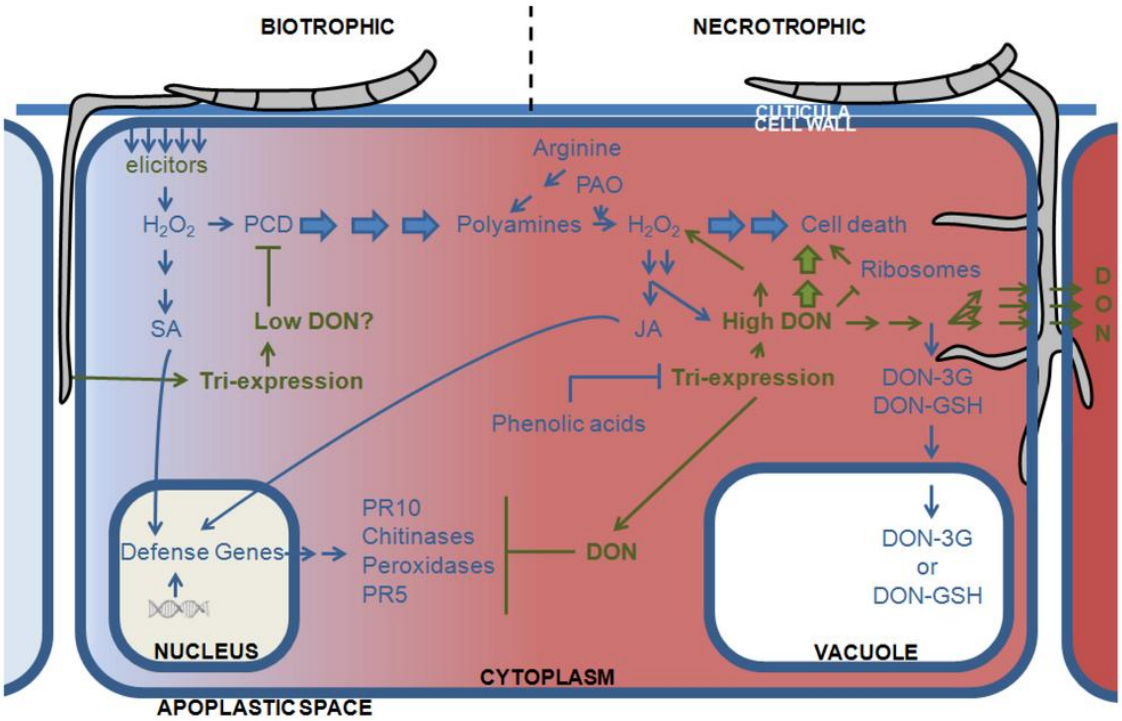


Fig. 2.1 Hypothetical model of the effect of deoxynivalenol (DON) during *F. graminearum* infection, based on defense-related responses in wheat (Green: pathways of the fungus, blue: pathways of the plant) (Audenaert *et al.*, 2014).

To date, DON is one of the few mycotoxins of which the function is partially unraveled (Kazan *et al.*, 2012, Audenaert *et al.*, 2014) and is known to contribute to the virulence of the pathogen in wheat (Proctor *et al.*, 1995, Desjardins *et al.*, 1996, Bai *et al.*, 2002, Mesterhazy, 2002). Nevertheless, DON plays a role in many other processes. It has been shown that the presence of other fungi influences DON production by *F. graminearum* (Von der Ohe & Miedaner, 2011, Muller *et al.*, 2012, Muller *et al.*, 2015), also the relationship with insects inhabiting the same plant tissue can possibly lead to differential colonization of plant tissue and therefore needs our attention.

Upon aphid attack, plants activate defense responses. Early events (recognition of elicitors, ROS, calcium influx etc.) lead to activation of multiple phytohormone-dependent pathways by the plant (Fig. 2.2). Phloem feeding insects like *S. avenae* can activate both the jasmonic acid (JA) and SA-dependent pathways (Zhao et al., 2009) and may act antagonistically (Koornneef & Pieterse, 2008, Vlot et al., 2009). SA promotes development of systemic acquired resistance, a broad range resistance against pathogens and some aphid species, and is crucial for localized hypersensitive response (Alvarez, 2000, Vlot *et al.*, 2009). Moreover, SA stimulates expression of defense response genes like pathogenesis related (PR) proteins (Smith & Boyko, 2007). PR-gene RNAs, proteins and protein activities are elevated after hosts are attacked by phloem-feeders (Walling, 2000). The octadecanoid pathway can lead to JA. JA induces the accumulation of hydrogen peroxide in response to wounding in different plant species, but can also act as a plant defense against both herbivores and pathogens (Orozco-Cardenas & Ryan, 1999). ROS are elicitors of defense signaling pathways with known involvement in the elicitation of plant response to aphid feeding (Divol *et al.*, 2005, Boyko *et al.*, 2006) but may also be, as said before, toxic to the aphids and have direct adverse effects on arthropod midgut tissue. Also ethylene (ET) (Argandona *et al.*, 2001), abscisic acid (ABA), giberellic acid (GA) (Boyko *et al.*, 2006, Park *et al.*, 2006) and nitric oxide (NO) (Moloi *et al.*, 2015) play a role (Fig. 2.2).

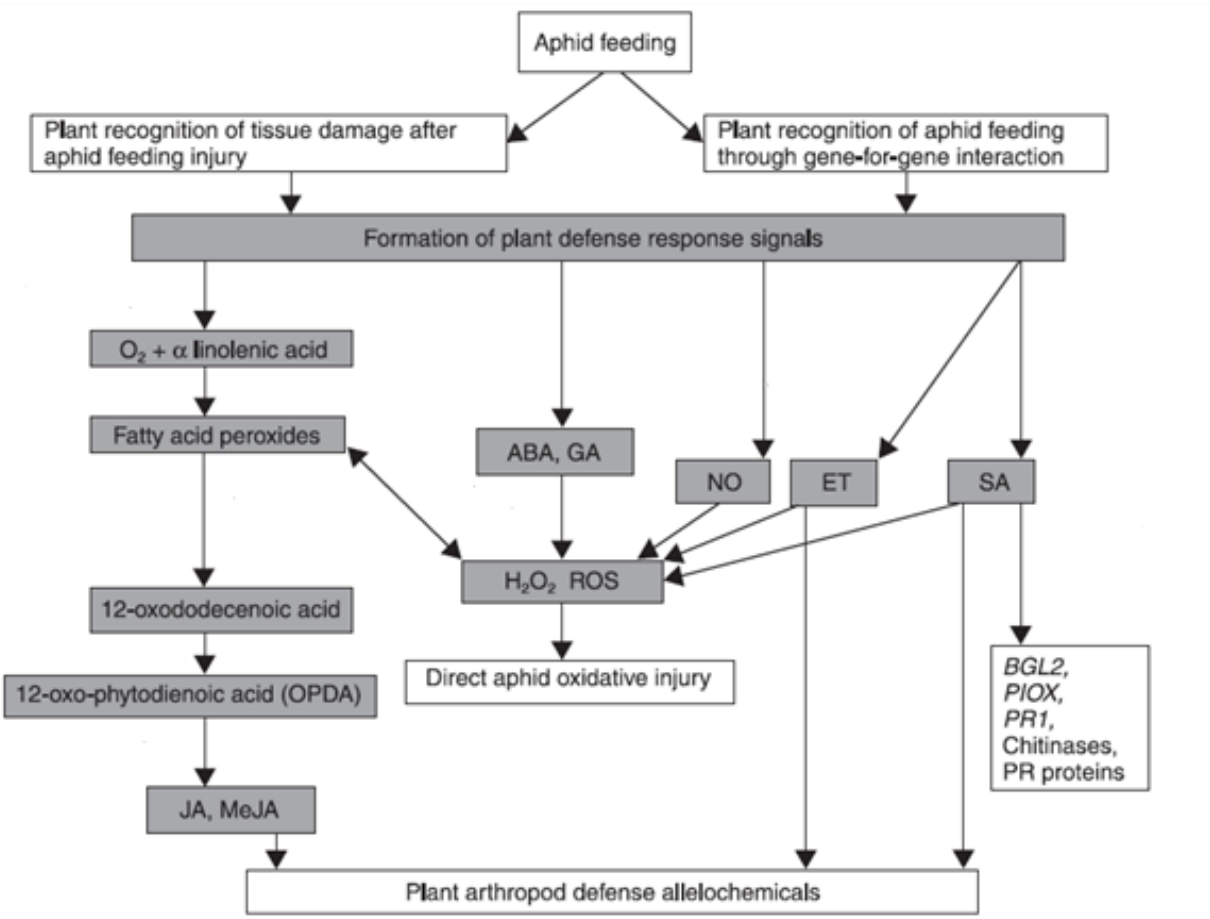


Fig. 2.2 Representative plant signaling pathways involved in aphid resistance and aphid defense response signaling. Arrows indicate pathway activation (adjusted from Smith and Boyko (2007)).

The interaction between fungi and insects has been studied by several research groups and involves several layers of complexity. Interactions can be direct, indirect or both. Direct interactions can include insects dispersing (vectoring) or feeding on fungal particles, or fungi utilizing feeding wounds made by the herbivore as entry points into the plant (Windels *et al.*, 1976, Martin, 1979, Mondy & Corio-Costet, 2004). Insects and fungi can also influence each other in an indirect manner which is often plant-mediated. Either party can bring about changes in plant quality, chemical composition or result in allelochemical production, thereby influencing the second party (Moran, 1998, Rostas *et al.*, 2003a, Stout *et al.*, 2006, Ponzio *et al.*, 2013, Lazebnik *et al.*, 2014). In addition, this can modulate the insect performance and affect parameters of reproduction, population size and survival rate (Kruess, 2002, Johnson *et al.*, 2003, Tack & Dicke, 2013b).

Although several studies investigated tripartite insect-plant-pathogen interactions (Leath & Byers, 1977, Moran, 1998, Kruess, 2002, Johnson *et al.*, 2003, Mondy & Corio-Costet, 2004, Al-Naemi & Hatcher, 2013), aphid-cereal-*Fusarium* interactions remained elusive for a long time. Bagga (2008) demonstrated that FHB severity in wheat was significantly reduced by more than 30% when aphids were controlled by insecticide application. Also in maize, another host that is susceptible to *Fusarium*, a clear link between *Fusarium* or its toxins and insect control was demonstrated (Degraeve *et al.*, 2016).

Recently, Drakulic *et al.* (2015) explored the interaction between aphids, wheat and *F. graminearum* with focus on the aphid and the disease caused by *F. graminearum*. Pursuing this approach, they were able to discover synergistic effects both on the level of disease symptoms and DON production. They demonstrated that *S. avenae* grain aphids could accelerate disease progression and DON accumulation depending on the period of aphid colonization before *F. graminearum* ear inoculation. Drakulic *et al.* (2015) also showed that volatile production by FHB infected plants negatively impacts on the preference and performance of aphids rendering the host inhospitable. However, the role of the plant's defense system in this tripartite interaction remained unexplored.

Therefore, our study explored the interaction between *S. avenae* and the fungal pathogen *F. graminearum* but at the level of plant defense as this provides valuable information to interpret the outcome of the interaction. We hypothesize that plant defense signaling plays a major role in the enhanced *F. graminearum* disease progression and mycotoxin production when both the pathogen and the aphids inhabit ears of wheat. We monitored the expression of wheat defense genes after *S. avenae* infestation and *F. graminearum* infection during several days. We measured disease symptoms, fungal biomass and mycotoxin production at several time points during the disease progress and coupled it with a time course analysis of the transcriptional grid of well-known defense genes. Because of the hemibiotrophic lifestyle of *F. graminearum*, we selected biosynthesis genes for both the SA and JA pathways. Additionally, we chose plant defense genes encoding PR proteins which are known to play a role in the defense against *F. graminearum* (Bertini *et al.*, 2009, Makandar *et al.*, 2012, Gao *et al.*, 2013) and genes involved in the redox state of plant cells because *F. graminearum* can interfere with this mechanism through the action of the mycotoxin DON (Desmond *et al.*, 2008). We also selected genes encoding for lignin biosynthesis because cell wall reinforcement plays a role in plant defense against fungal

pathogens (Bi *et al.*, 2011). To our knowledge, this is the first in depth transcriptional analysis in wheat plants of a tripartite plant-insect-fungal model system.

2.2 Experimental procedures

2.2.1 Insect, plant and fungal material

The laboratory stock culture of *S. avenae* aphids was maintained on wheat seedlings (cv. Passat) at a constant temperature of 22 °C and a photoperiod of 16 h:8 h, light:dark, stimulating parthenogenesis.

Spring wheat cv. Passat was sown in universal potting soil (3 seeds per pot). The plants were kept in the glasshouse and each plant received one application of 0.1 g 27% NH_4NO_3 during stem elongation.

The strain of *F. graminearum* used in ear experiments was a constitutively green fluorescence protein-expressing *Fusarium* strain 8/1 (kindly provided by K. Heinz-Kogel, Justus Liebig University, Giessen, Germany) (Jansen *et al.*, 2005). The fungus was cultivated on potato dextrose agar under a light regime of UV/darkness (12 h 365 nm 10W/12 h) to promote sporulation. The macroconidia were harvested by adding a suspension of sterile water amended with 0.01% Tween 80 and rubbing the mycelium with a sterile spatula.

2.2.2 Experimental setup

Spring wheat plants (cv. Passat) (N = 92) were used to examine the influence of aphid infestation on the ear colonizing of *F. graminearum*. Ears at anthesis were infested with 100 aphids of different developmental stages (4 dbi, days before infection). The ears (still attached to the plant) were placed in a plastic cup with netting on top to prevent the aphids from escaping. After 4 days, this was followed by *F. graminearum* spray inoculation (dai 0, days after infection) with 10 sprays of 100 µl 5 x 10^5 spores mL^{-1} per ear. Control-ears were sprayed with sterile water. All ears were kept at 100% relative humidity for 24 h to ensure spore germination. Overall the experimental setup contained ears infected with *F. graminearum* and aphids (Fg+aphids, N = 16), ears infected with *F. graminearum* (Fg, N =

16), ears with aphids (Aphids, N = 28) and control-ears (Control, N = 32) (sprayed with sterile water). Ears with different treatments were chosen at random. At several time points in the experiment (see Fig. 2.3) ears were cut off, flash frozen in liquid nitrogen, and stored at -80 °C until further analysis. This analysis included counting the number of aphids on the ears, sampling three spikelets haphazardly in the middle of the ear from each ear for RNA extraction to investigate plant defense, and crushing the remaining spikelets of the ear with liquid nitrogen to use for fungal DNA extraction and DON analysis. The experiment was repeated twice and this chapter represents the results of one representative experiment.

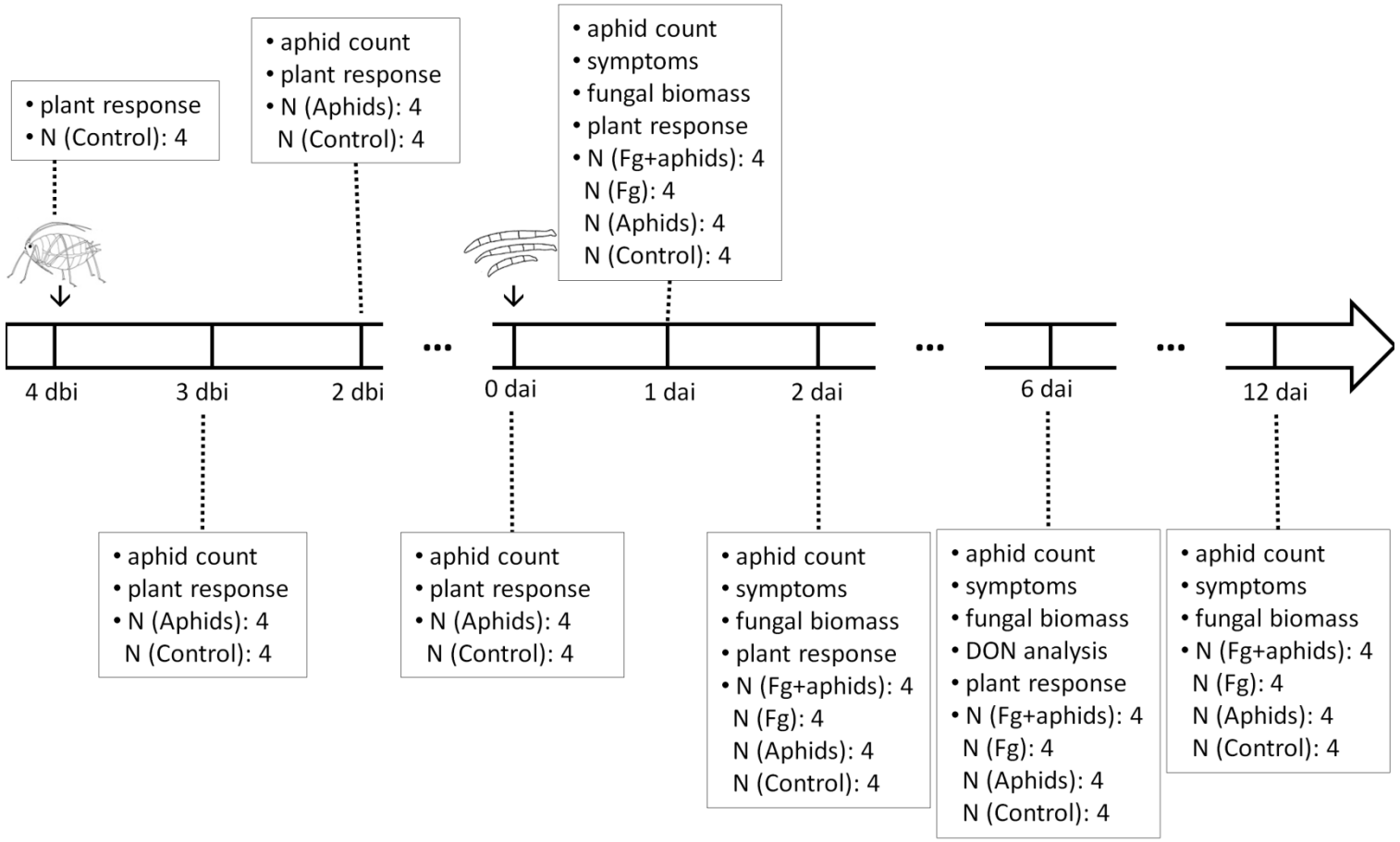


Fig. 2.3 Time line indicating important time points in the experiment elucidating the effect of *S. avenae* aphids on wheat response and *F. graminearum* infection. Each time point contains a summary of performed analyses and number of ears sampled. In total a batch of 92 wheat ears was used.

2.2.3 F. graminearum infection parameters: symptom assessment, analysis of fungal biomass and measurement of DON and acetylated forms

Diseased ears were evaluated by scoring the percentage of diseased spikelets per ear with visible *F. graminearum* blight symptoms (Isebaert *et al.*, 2009).

DNA was extracted with an Invisorb Spin Plant mini kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. To calculate the fungal and plant biomass RT-qPCR assays were performed using primers based on the elongation factor 1 alpha gene and the thermal profile described in Nicolaisen *et al.* (2009). The reaction mixture consisted of 6.25 µl GoTaq qPCR Master Mix (Promega), 0.208 µl CRX reference dye (Promega), 250 nM of each primer and 2.5 µl DNA. Analysis was performed with an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). *F. graminearum* DNA from the ear samples was quantified using five DNA standards in ten-fold dilutions. Linear regression was used to calculate the quantity of *F. graminearum* DNA. *F. graminearum* DNA was normalized to the amount of plant DNA.

The wheat ears were analyzed according to a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) procedure for DON, 3-ADON and 15-ADON (Monbaliu et al., 2010, Monbaliu, 2011). A Waters Acquity ultra high performance liquid chromatography system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) was used to analyze the samples, equipped with MassLynxTM version 4.1 and QuanLynx(r) version 4.1 software (Waters, Manchester, UK) for data acquisition and processing. Sample preparation was performed using an extraction with acetonitrile/H_2O/acetic acid (79/21/1, v/v/v). The identity of the analytes was controlled according to Commission Decision 2002/657/EC (European Commission, 2002). In case that the obtained results were out of the range of the calibration curve, the sample was re-analyzed in order to fit in the range of a new constructed calibration plot. Every analytical run consisted of a standard control mix, calibrants, a blank sample, a maximum of 20 samples and a control sample (a re-injection of a spike of the calibration curve).

2.2.4 *Analysis of the plant response against F. graminearum after infestation with S. avenae aphids: RNA extraction and RT-qPCR analysis of plant response genes*

This protocol is adapted from Ameye et al. (2015). RNA from ear spikelets was extracted using TRI reagent (Sigma-Aldrich, Saint-Louis, MO, USA) according to the instructions of the manufacturer. The extracted RNA was quantified using a Quantus Fluorometer (Promega, Madison, WI, USA). With a GoScript Reverse Transcription System (Promega) first-strand cDNA was synthesized and used to perform RT-qPCR assays elucidating plant response against *F. graminearum* and aphids. These assays were conducted with an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with following thermal settings: 50 °C for 2 min; 95 °C for 10 min; 35 cycles of 95 °C for 15 s and 60 °C for 1 min; dissociation curve analysis was performed using a temperature profile of 95 °C for 15 s, cooling to 60 °C for 20 s and subsequently heating to 95 °C for 15 s. Primers for all genes used can be found in Table 2.1. The reaction mix consisted of 6.25 µl GoTaq qPCR Master Mix (Promega), 0.208 µl CRX reference dye (Promega), 250 nM of each primer and 2 µl cDNA. Each sample was repeated two-fold or more if Ct-values differed more than one cycle. Normalization of wheat defense genes was carried out using ADP-ribosylation factor (Ta2291) and GABARAP (GABA-receptor-associated protein) (Ta54963) as reference genes (Paolacci et al., 2009). Selection of reference genes (Ta2291 and Ta54963) was based on a GeNorm analysis performed using qBase+ software (Biogazelle NV, Zwijnaarde, Belgium) which was also used for all other calculations of the RT-qPCR data (Hellemans et al., 2007).

2.2.5 *Statistical analyses*

Data were analyzed using IBM SPSS software (Statistical Package for Social Sciences) version 22.0 for Windows. All tests were conducted with a significance level of $\alpha = 0.05$. For comparing the symptoms, fungal biomass, content of DON and acetylated forms between two groups ('Fg' and 'Fg+aphids') t-tests was used when data were normally distributed according to a Shapiro-Wilk test or Mann-Whitney U tests (non-parametric) when data were not normally distributed. For the expression data t-tests (3 dbi, 2 dbi and 0 dai, comparing two treatments) and one-way Anova's post-hoc LSD (least significant difference) (1, 2 and 6 dai, comparing four treatments with each other) were used for statistical analysis of the fold

increase over control. Boxplots of symptoms were generated in R Software version 3.1.0 (R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Table 2.1 List of primers used to analyze wheat defense gene expression against *F. graminearum* and/or cereal aphids *S. avenae*. Normalization of wheat defense genes was carried out using *Ta2291* and *Ta54963* as reference genes.

Primer	Sequence (5'-3')	Reference
LOX	F: AACAAGTTCGCCGTCACCTT; R: TTGTCGAGGGTGATGGTCTT	Beccari *et al.* (2011)
AOS	F: TCTCATAGCAGCCGTCAATC; R: AAAACACGCACACACATACA	Zhao *et al.* (2009)
PAL	F: TTGATGAAGCCGAAGCAGGACC; R: ATGGGGGTGCCTTGGAAGTTGC	Ding *et al.* (2011)
ICS	F: AGAAATGAGGACGACGAGTTTGAC; R: CCAAGTAGTGCTGATCTAATCCCAA	Ding *et al.* (2011)
PEROX	F: GAGATTCCACAGATGCAAACGAG; R: GGAGGCCCTTGTTTCTGAATG	Desmond *et al.* (2005)
NADPHOX	F: ATGCTCCAGTCCCTCAACCAT; R: TTCTCCTTGTGGAACTCGAATTT	Ding *et al.* (2011)
CCR3	F: CTGTCGGCTAGTTAATTCTATG; R: ATATGATCGCCAACCAACC	Bi *et al.* (2011)
CAD1	F: AGATACCGCTTCGTCATCG; R: GAATCGCACGCACCAACC	Bi *et al.* (2011)
FPS	F: TCAAGACGGCTTCAGGG; R: TCGCCAAAGTTATCCAAAT	Zhao *et al.* (2009)
PR1	F: CGTCTTCATCACCTGCAACTA; R: CAAACATAAACACACGCACGTA	Gao *et al.* (2013)
PR2	F: CCGCACAAGACACCTCAAGATA; R: CGATGCCCTTGGTTTGGTAGA	Gao *et al.* (2013)
PR3	F: CAGGAAAATCAACAGTGGCGA; R: GCGTCGATCAAGAATCTAGCAA	Gao *et al.* (2013)
PR4	F: ACACCGTCTTCACCAAGATCGACA; R: AGCATGGATCAGTCTCAGTGCTCA	Qi *et al.* (2012)
PR5	F: ACAGCTACGCCAAGGACGAC; R: CGCGTCCTAATCTAAGGGCAG	Gao *et al.* (2013)
Ta2291	F: GCTCTCCAACAACATTGCCAAC; R: GCTTCTGCCTGTCACATACGC	Paolacci *et al.* (2009)
Ta54963	F: AGGAGAACAAGGACGAGGAC; R: AGGAGGCATTCAGAGCGATTG	Paolacci *et al.* (2009)

2.3 Results

To evaluate the impact of *S. avenae* aphids on the plant response in wheat ears and subsequent *F. graminearum* infection process, we infested wheat ears with aphids four days before fungus inoculation. We investigated all agents of the tripartite interaction (wheat, aphids and *F. graminearum*) at several time points during the disease progress (Fig. 2.3) and coupled it with a time course analysis of the transcriptional grid of well-known defense genes. Different infection parameters for *F. graminearum* (symptoms, fungal biomass and production of DON and its acetylated forms), infestation of aphids (number of aphids at different time points) and gene response patterns using RT-qPCR assays were analyzed.

2.3.1 Infection and infestation parameters of F. graminearum and aphids

Diseased ears were evaluated by scoring the percentage of spikelets per ear with visible *F. graminearum* symptoms (Isebaert *et al.*, 2009). At 6 days after infection with *F. graminearum* (dai) we observed 2.5 times more bleaching symptoms on ears containing both aphids and fungus compared to ears containing solely *F. graminearum* (mean of 73% vs. 29% respectively, P = 0.014). This difference was no longer observed at 12 dai (mean of 94% vs. 81% respectively, P = 0.121) (Fig. 2.4). No bleaching symptoms were observed at 1 and 2 dai, nor for ears containing solely aphids and the control-ears. For each time point (1, 2, 6 and 12 dai) there were no significant differences found for the fungal biomass between *F. graminearum* infected ears with aphids and ears infected with *F. graminearum* alone. There was no fungal biomass retrieved in the ears with only aphids and the control-ears (Table 2.2).

Concentrations of DON and acetylated forms (3-ADON and 15-ADON) were measured in ears sampled 6 dai using LC-MS/MS. Significantly higher DON, 3-ADON and 15-ADON concentrations were found (P = 0.031, 0.023 and 0.019 respectively) in ears containing both *F. graminearum* and aphids compared to ears containing solely *F. graminearum* (Table 2.3). There was no DON or acetylated forms of DON found in ears containing solely aphids and control-ears.

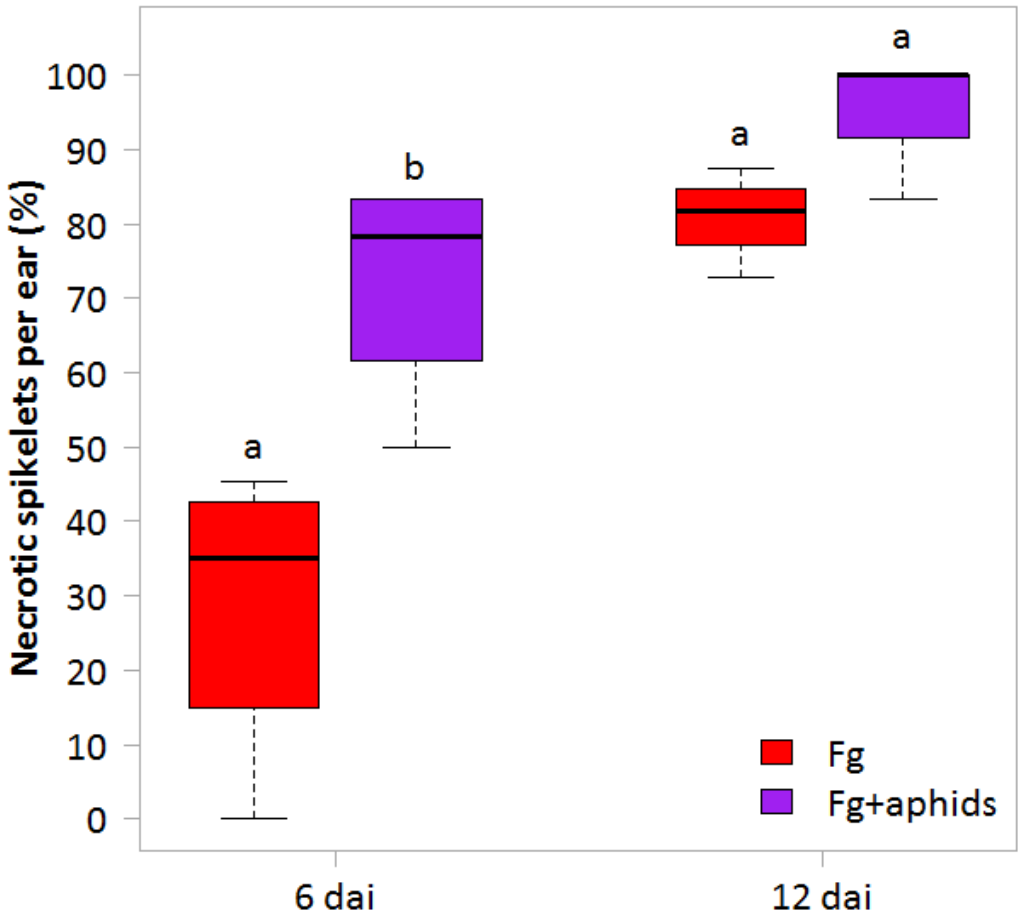


Fig. 2.4 Disease symptoms expressed as percentage of symptomatic spikelets per ear (number of bleached spikelets / number of total spikelets *100) of *F. graminearum* 6 and 12 dai for ears containing the following treatments: *F. graminearum* and *S. avenae* aphids (Fg+aphids) or solely *F. graminearum* (Fg). Data represent four ears for each treatment with different letters indicating significant differences between both treatments (6 dai: P = 0.014, two-sided t-test; 12 dai: P = 0.121, Mann-Whitney U tests). No bleaching symptoms were observed at 1 and 2 dai, nor for ears containing solely aphids and the control-ears.

Table 2.2 Fungal biomass (mean ± SE) found in *F. graminearum* infected ears with aphids (Fg+aphids) and without aphids (Fg) at different time points. No significant differences were found between both treatments using t-tests. There was no fungal biomass retrieved in the ears with only aphids and the control-ears.

pg *Fg* DNA per ng plant DNA	**Fg**	**Fg+aphids**	**P-value**
1 dai	0.597 ± 0.200	1.085 ±0.420	0.334
2 dai	5.328 ±3.329	3.381 ±1.025	0.596
6 dai	296.3 ±127.4	303.0 ±147.3	0.974
12 dai	432.6 ±93.11	731.8 ±245.1	0.317

Table 2.3 Concentrations of deoxynivalenol (DON) and acetylated forms (3-ADON and 15-ADON) (mean ± SE) found in *F. graminearum* infected ears with aphids (Fg+aphids) and without aphids (Fg) 6 dai. Different letters indicate significant differences between both treatments using one-sided Mann-Whitney U tests. There was no DON or acetylated forms of DON found in ears containing solely aphids and control-ears.

mg kg^{-1}	**Fg**	**Fg+aphids**	**P-value**
DON	32.77 ± 12.73 a	65.83 ± 20.15 b	0.031
3-ADON	0.53 ± 0.20 a	2.05 ± 0.83 b	0.023
15-ADON	2.39 ± 0.96 a	9.60 ± 3.88 b	0.019

At 4 days before infection with *F. graminearum* (4 dbi), 100 *S. avenae* aphids were put in a plastic cup containing one wheat ear. Number of aphids on ears containing only aphids ranged from a mean of 56 to 112 aphids per ear (from 3 dbi till 12 dai respectively). Not all 100 aphids found the ear during the first 24 h after they were introduced (3 dbi) and thus died of starvation. Over time, aphids reproduced and populations increased. Aphid population showed a little decline at 1 dai probably because of the spray inoculation and the related high humidity. Populations on ears containing both aphids and *F. graminearum* varied from a mean of 94 to 2 aphids per ear (from 1 dai till 12 dai, respectively). When fungal symptoms started to develop, aphids moved to the remaining green parts of the ear. At 6 dai aphid populations on ears containing *F. graminearum* (symptoms) were lower than on ears without fungus (a mean of 85 vs. 111 aphids per ear respectively, P = 0.048). At 12 dai (fully diseased ears) this difference became even more pronounced (a mean of 2 vs. 112 aphids alive per ear respectively, P = 0.004) (Table 2.4).

Table 2.4 Number of aphids (mean ± SD) on ears during all time points in the experiment for ears containing only aphids (Aphids) and ears containing both aphids and *F. graminearum* (Fg+aphids). Different letters between both treatments indicate significant differences using t-tests.

	4 dbi	**3 dbi**	**0 dai**	**1 dai**	**2 dai**	**6 dai**	**12 dai**
Aphids	56 ± 11	90 ± 14	113 ± 39	88 ± 39 a	136 ± 29 a	111 ± 15 a	112 ± 37 a
Fg+aphids				94 ± 23 a	122 ± 21 a	85 ± 15 b	2 ± 1 b
P-value				0.809	0.456	0.048	0.004

2.3.2 *Wheat response after exposing ears to aphids and followed by F. graminearum infection*

To elucidate the effect of aphids on the defense response in wheat and the infection process of *F. graminearum*, we exposed ears to 100 *S. avenae* aphids and examined the wheat defense response against both the aphids and the fungus over a period of 10 days (Fig. 2.5). We tested several defense related genes. We selected lipoxygenase (*LOX*) and allene oxide synthase (*AOS*) as markers genes for the JA biosynthesis pathway (Zhao *et al.*, 2009, Feng *et al.*, 2010, Beccari *et al.*, 2011), and phenylalanine ammonia lyase (*PAL*) and isochorismate synthase (*ICS*) as marker genes for the SA biosynthesis/signaling pathway (Ding *et al.*, 2011). Peroxidase (*PEROX*) and NADPH oxidase (*NADPHOX*) play a role in the plant's redox state (Desmond *et al.*, 2005, Desmond *et al.*, 2008, Ding *et al.*, 2011), while cinnamoyl CoA reductase 3 (*CCR3*) and cinnamyl alcohol dehydrogenase 1 (*CAD1*) indicate cell wall reinforcement (lignin biosynthesis) (Bi *et al.*, 2011). We also tested farnesyl pyrophosphate synthase (*FPS*) which plays a role in isoprene biosynthesis (Zhao *et al.*, 2009). *PR* genes were used to indicate a more downstream plant response: basic *PR1* proteins (Makandar *et al.*, 2012, Gao *et al.*, 2013), *PR2* (β-1,3-glucanase) (Gao *et al.*, 2013), *PR3* (class-VII acidic chitinases) (Gao *et al.*, 2013), *PR4* (antifungal properties against *Fusarium*) (Bertini *et al.*, 2009, Qi *et al.*, 2012) and *PR5* (thaumatin-like protein) (Gao *et al.*, 2013).

At 4 dbi (ears without aphids), none of the tested genes were upregulated. Other genes such as *PEROX*, *ICS*, *LOX*, *AOS* and *FPS* did not show any significant induction at the tested time points (Fig. 2.6). These comprise mainly genes known to be involved in early steps of defense gene activation. However, *PAL*, a marker gene for the SA biosynthesis and signaling pathway, was significantly different for ears containing solely *F. graminearum* at 2 dai compared to the other treatments.

PR1 gene expression (indicating SA mediated defense response) was significantly higher for ears exposed to *S. avenae* and ears exposed to both *F. graminearum* and *S. avenae* than control ears starting from 2 dbi till 2 dai. To explore the link between *PR1* gene expression and aphid numbers, a correlation analysis was conducted. The Pearson correlation coefficient between aphid numbers and *PR1* expression in ears was 0.608 (P =

0.002) for ears exposed to aphids and 0.849 (P = 0.002) for ears exposed to both *F. graminearum* and aphids (Fig. 2.7). This tight correlation might explain the lower induction of *PR1* in ears exposed to aphids at 1 dai and in ears exposed to both aphids and *F. graminearum* at 6 dai. Both time points are characterized by a clear decline in aphid numbers. The slope of the linear trend line for *PR1* expression in ears containing both aphids and *F. graminearum* was 2.45 times higher than for ears containing solely aphids (Fig. 2.7).

PR2 (β-1,3-glucanase) gene expression in ears exposed to *F. graminearum* was initiated at 2 dai and became significantly different from control ears at 6 dai (P = 0.036). None of the other treatments showed a significant induction of *PR2* gene expression. These results show that presence of aphids before a *F. graminearum* infection suppresses the *PR2* gene expression initiated by *F. graminearum*.

Expression patterns of *PR3* (class-VII acidic chitinases) and *PR4* (antifungal properties against *Fusarium*) showed many parallels. The first induction of *PR3* and *PR4* appeared in ears exposed to aphids at 0 dai (P = 0.021 and 0.046 respectively), demonstrating that aphids induced *PR3* and *PR4*. From 1 dai till 2 dai, the expression of *PR3* and *PR4* steadily increased and was significantly different from control ears in both ears exposed to aphids and ears exposed to both *F. graminearum* and aphids (*PR3* at 2 dai: P = 0.002 and < 0.001 respectively, *PR4* at 2 dai: P = 0.015 and < 0.001 respectively). In addition, a remarkable inductive effect between ears containing aphids and ears with both *F. graminearum* and aphids was observed for *PR4* gene expression (P = 0.001) and to a lesser extend of *PR3* gene expression at 1 dai and 2 dai. This induction disappeared at time point 6 dai.

The time lapse experiment on *PR5* (Thaumatin-like protein) gene expression showed a clear induction at 2 dai in all treatments containing aphids (Aphids: P = 0.008, Fg+aphids: P = 0.004). Moreover, at 6 dai aphids were observed to have a predisposing effect on *PR5* gene expression upon *F. graminearum* infection as *PR5* gene expression in ears exposed to both *F. graminearum* and aphids showed higher induction than both ears exposed to aphids or *F. graminearum* alone (P = 0.001 and 0.029 respectively).

NADPHOX gene expression (membrane bound precursor of H_2O_2) was clearly induced by aphids 2 dbi and 0 dai pointing to a limited oxidative burst induced by aphids in these ears. Remarkably, this early induction of *NADPHOX* gene expression clearly prompted wheat ears to activate *NADPHOX* gene expression faster and to a higher extend upon *F. graminearum* infection. Indeed, the *NADPHOX* gene was significantly induced at 1 dai in ears

exposed to both aphids and *F. graminearum* where this induction only became apparent at 6 dai in ears solely infected with *F. graminearum* (P = 0.024).

For *CAD1* and *CCR3,* two genes involved in lignin biosynthesis, several similarities were observed. Both genes were induced in ears containing aphids and ears with both aphids and *F. graminearum* (*CAD1* at 2 dai: P = 0.004 and 0.001 respectively, *CCR3* at 2 dai: P = 0.024 and 0.02 respectively). In addition, combined exposure of wheat ears to *F. graminearum* and aphids resulted in a higher expression of *CAD1* and *CCR3* at 6 dai compared to sole exposure to *F. graminearum* (P = 0.015 and < 0.001 respectively). In addition, the clear induction of *CAD1* and *CCR3* gene expression in ears colonized by aphids disappeared at 6 dai.

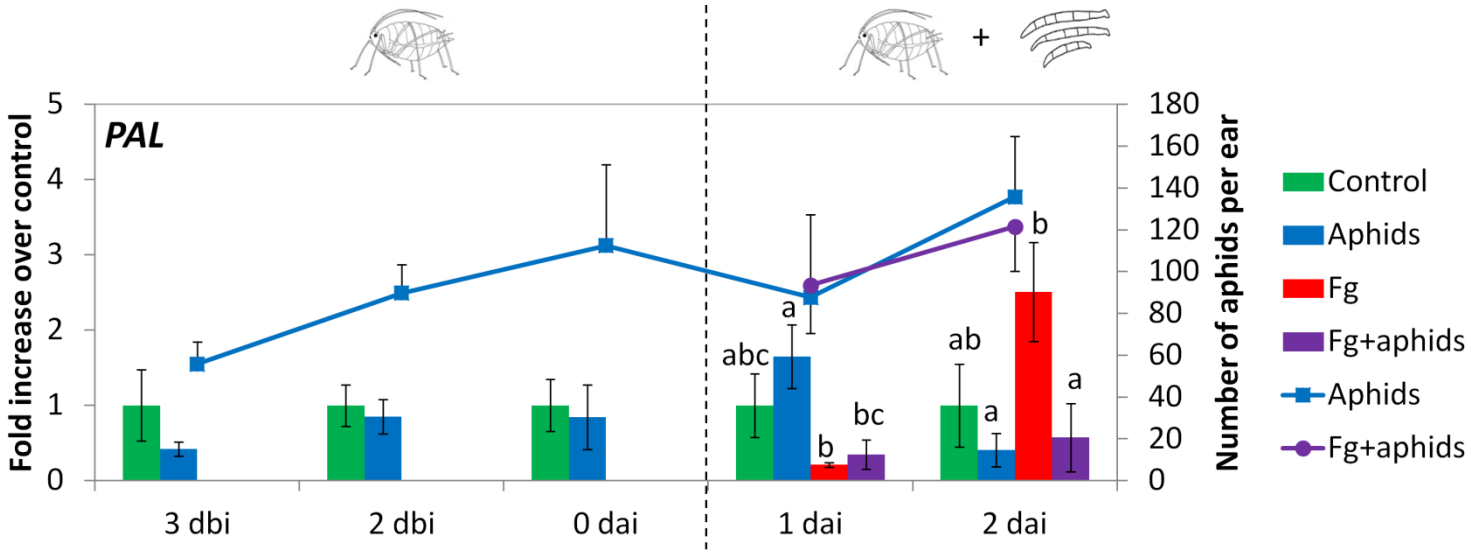


Fig. 2.5 Wheat response against *F. graminearum* after pre-exposing ears with *S. avenae* aphids.

Expression profile of *PAL*, *PR1* to *5*, *NADPHOX*, *CAD1* and *CCR3* in wheat ears infested with *S. avenae* aphids and/or infected with *F. graminearum*. Each bar represents the mean fold increase over control (± SE) of four ears or less, each consisting of three pooled spikelets. Data were normalized for *Ta2291*, *Ta54963* and control treatments ('Control') with 'Aphids': ears with aphids, 'Fg': ears containing *F. graminearum* and 'Fg+aphids': ears containing *F. graminearum* while being infested by aphids. Two-sided t-tests (3 dbi, 2 dbi and 0 dai) and one-way Anova's (post-hoc LSD) (1, 2 and 6 dai) were used for statistical analysis with different letters indicating significant differences between treatments ($P < 0.05$) for each time point. Bars without letters are not significantly different ($P > 0.05$). The line chart above the bars indicate the mean number of aphids (± SD) at the different time points. ND: not detected.

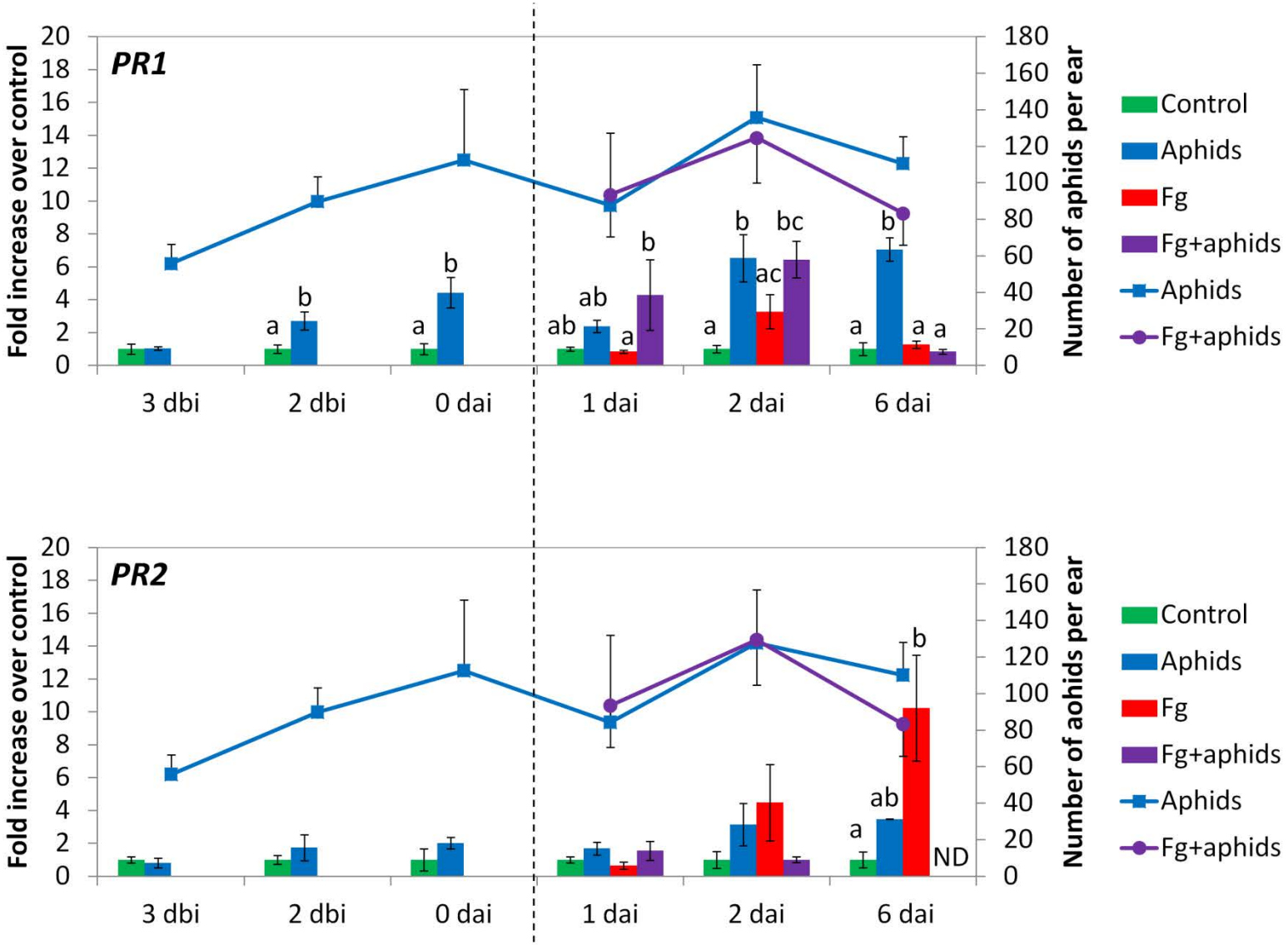


Fig. 2.5 continued

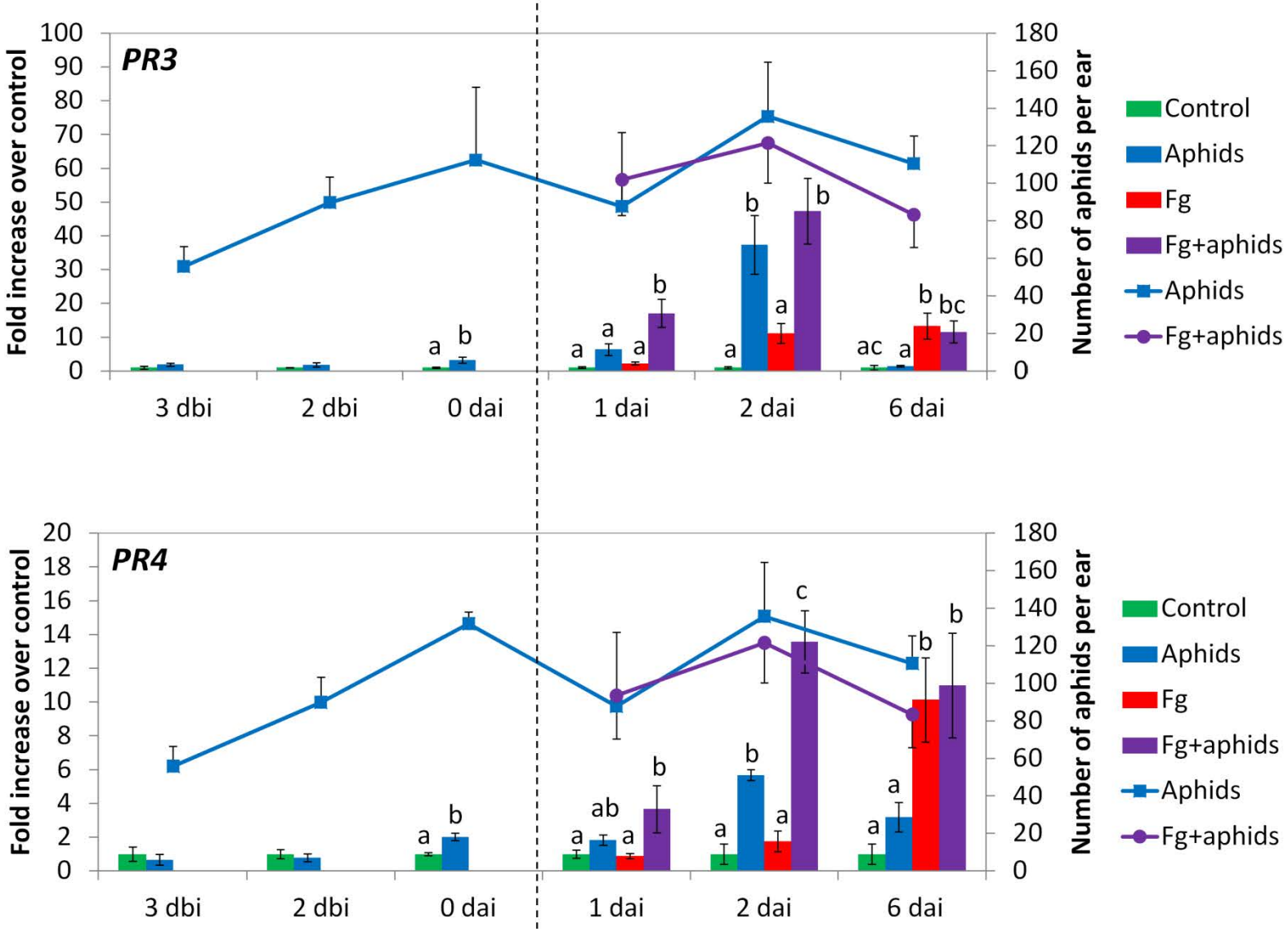


Fig. 2.5 continued

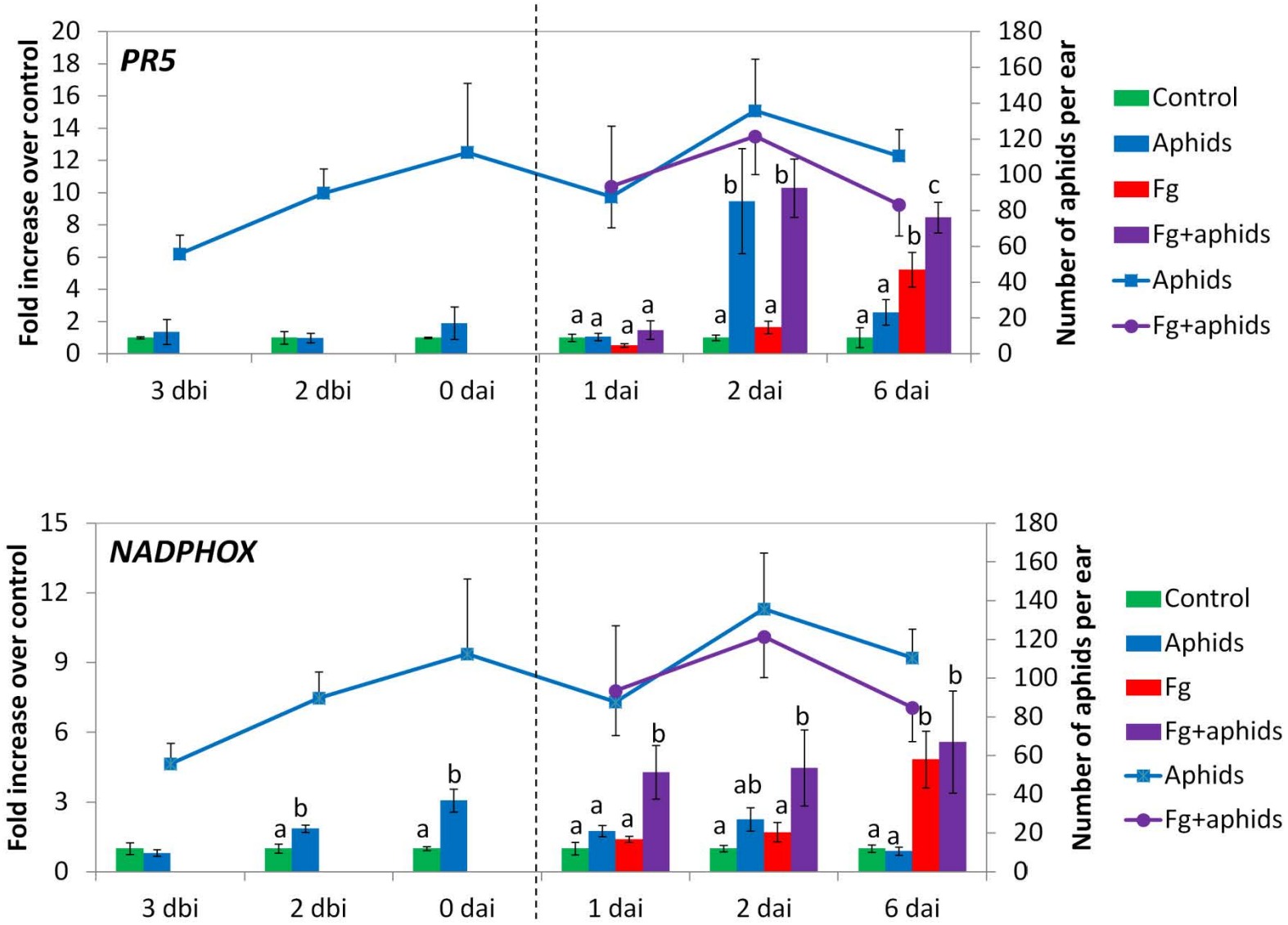
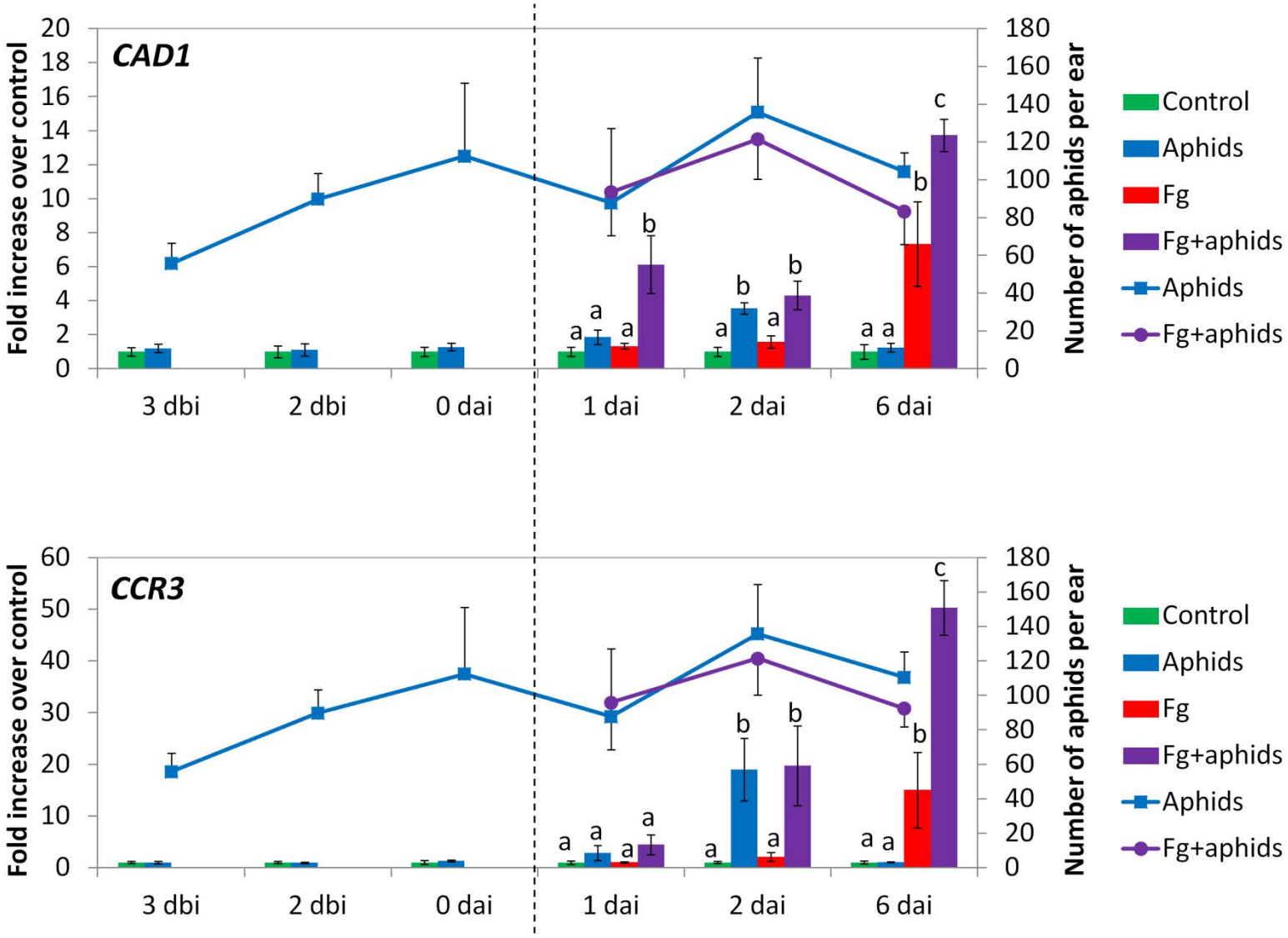


Fig. 2.5 **continued**



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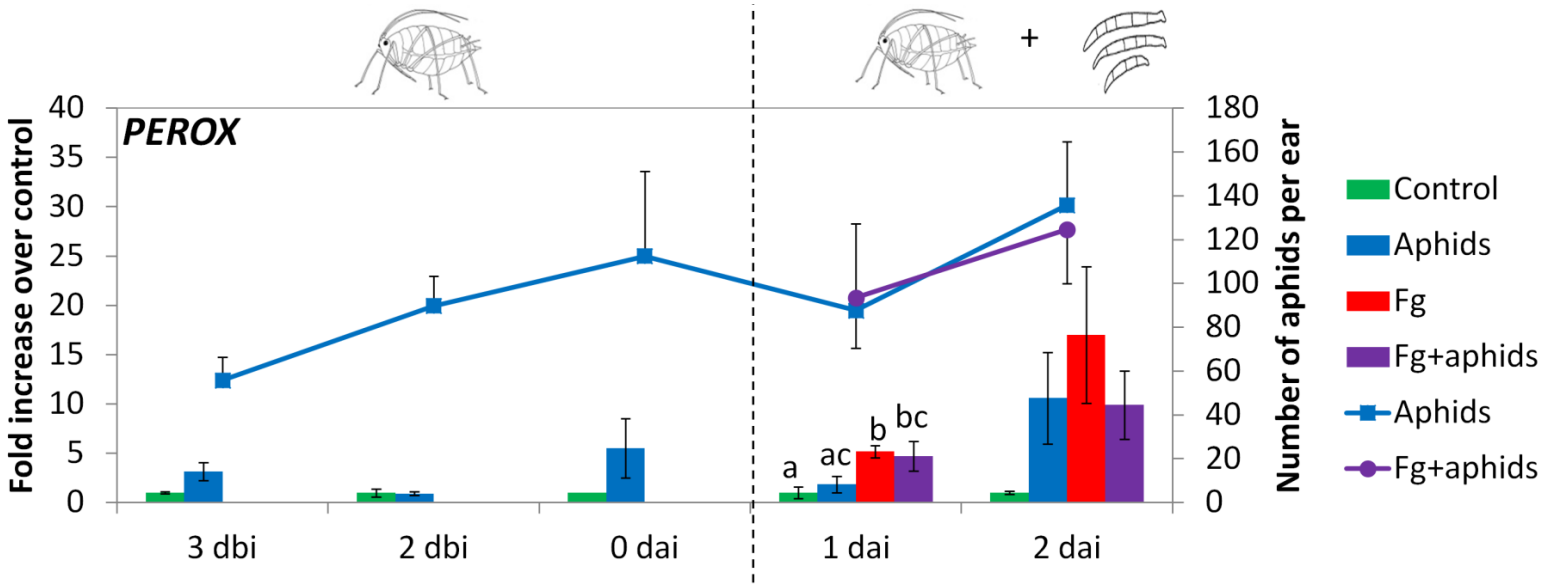


Fig. 2.6 Wheat response against *F. graminearum* after pre-exposing ears with *S. avenae* aphids.

Expression profile of other genes tested in wheat ears infested with *S. avenae* aphids and/or infected with *F. graminearum*. Each bar represents the mean fold increase over control (± SE) of four ears or less, each consisting of three pooled spikelets. Data were normalized for *Ta2291*, *Ta54963* and control treatments ('Control') with 'Aphids': ears with aphids, 'Fg': ears containing *F. graminearum* and 'Fg+aphids': ears containing *F. graminearum* while being infested by aphids. Two-sided t-test (3, 2 and 0 dai) and one-way Anova's (post-hoc LSD) (1, 2 and 6 dai) were used for statistical analysis with different letters indicating significant differences between treatments ($P < 0.05$) for each time point. Bars without letters are not significantly different ($P > 0.05$). The line chart above the bars indicate the mean number of aphids (± SD) at the different time points.

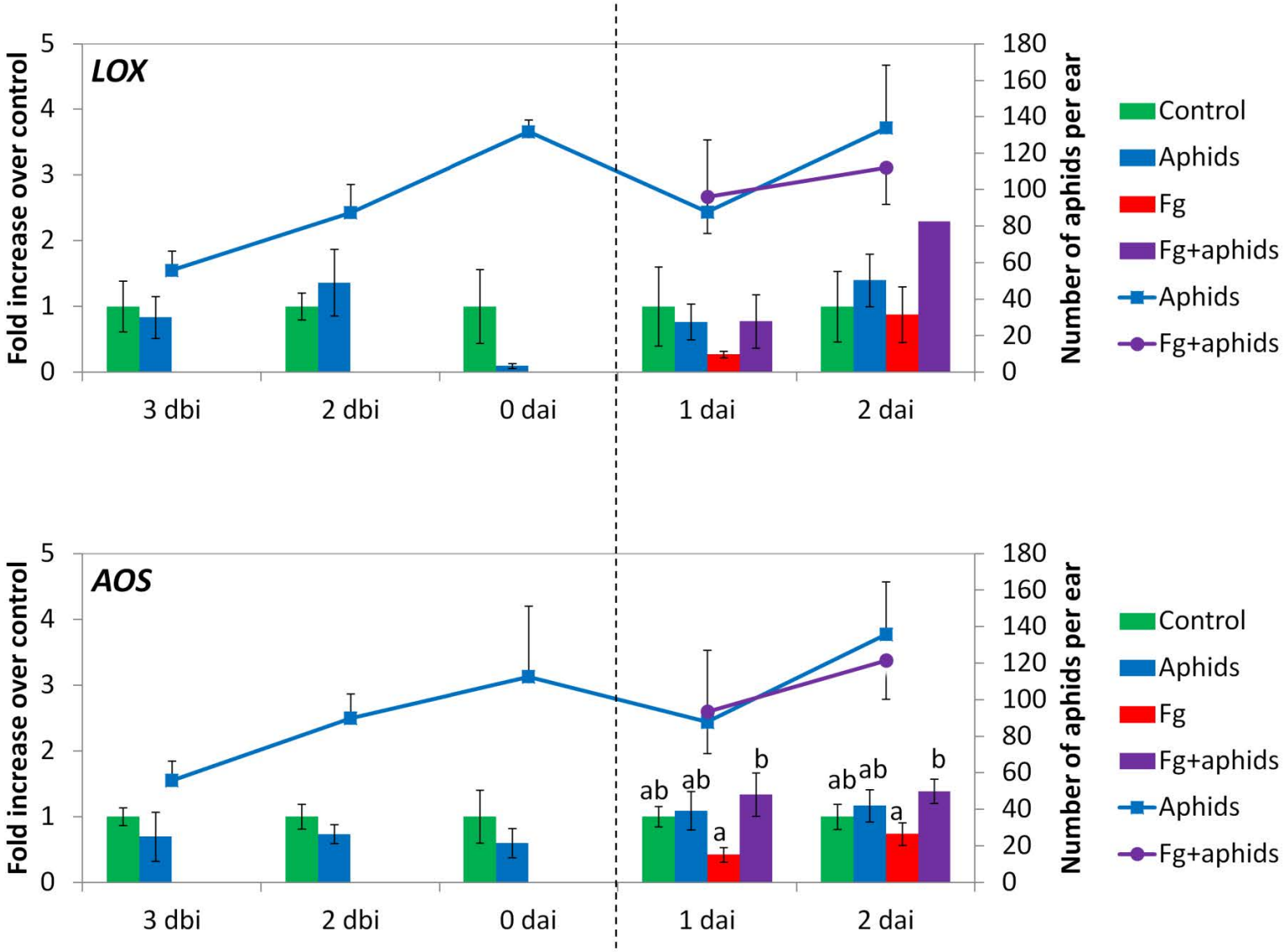
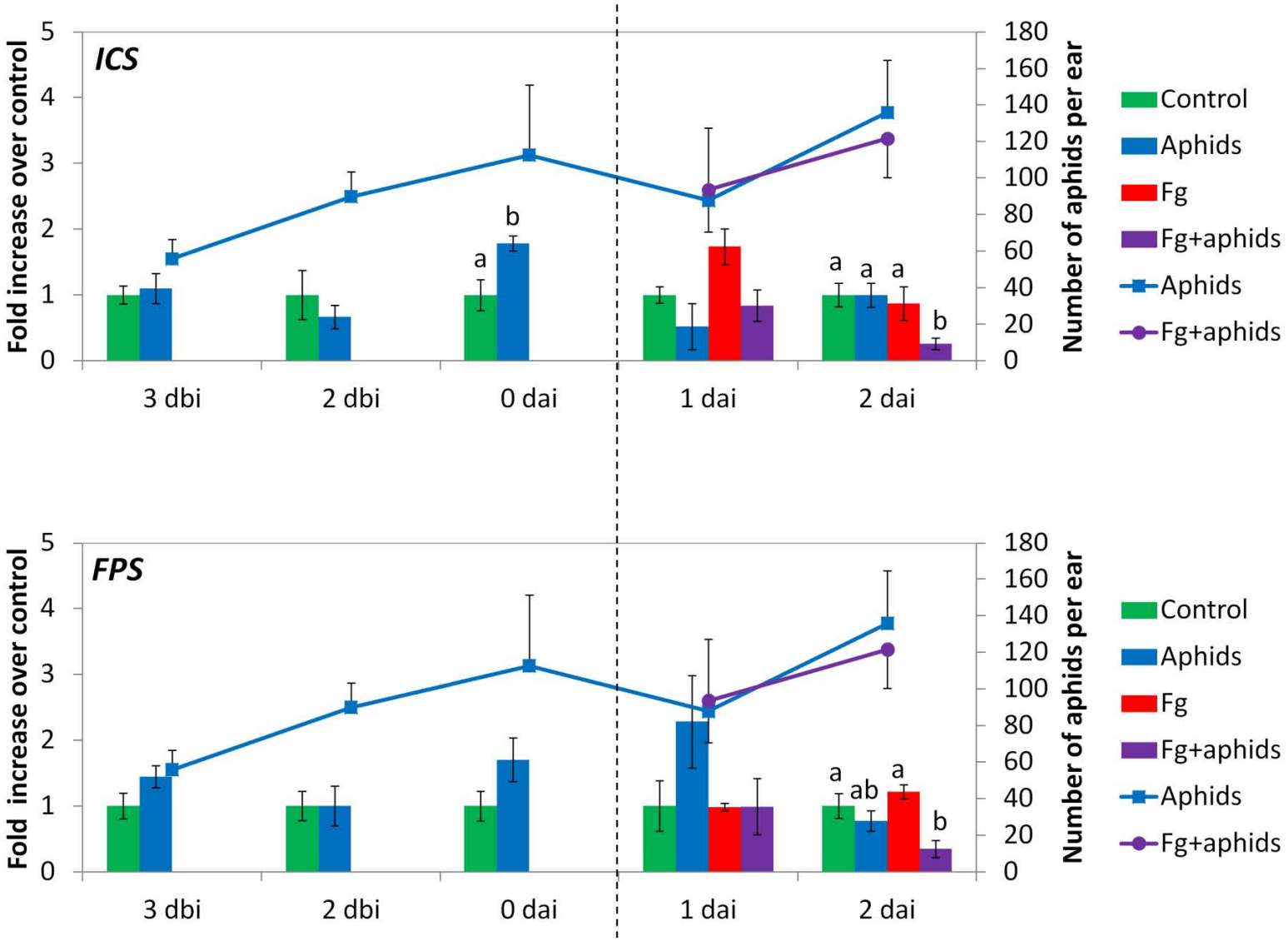


Fig. 2.6 **continued**



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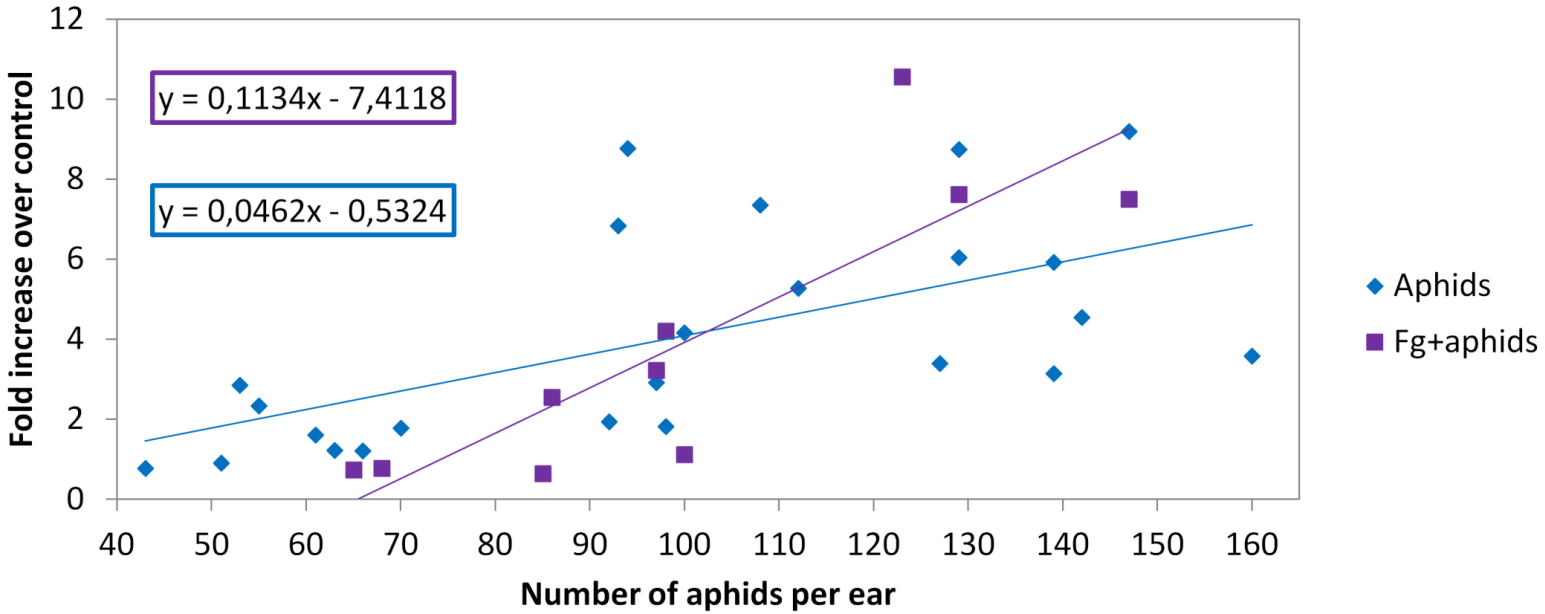

Fig. 2.7 Correlation between aphid population and gene expression of *PR1* on ears infested with *S. avenae* aphids (blue dots, Pearson coefficient = 0.608, P = 0.002, N = 24) and ears with both aphids and *F. graminearum* infection (red dots, Pearson coefficient = 0.849, P = 0.002, N = 10). Equations correspond with the linear trend lines in the same color. A significant linear regression for both treatments was demonstrated ($P < 0.01$).

2.4 Discussion

2.4.1 Infection and infestation parameters of F. graminearum and aphids

Ears infected with both *F. graminearum* and aphids showed more disease symptoms and higher DON and acetylated DON concentrations at 6 dai compared to ears with a sole *F. graminearum* infection (Fig. 2.4 and Table 2.3). The fungal biomass was not significantly different between ears treated with *F. graminearum* and ears treated with both *F. graminearum* and aphids at any of the time points. We hypothesize that the higher gene expressions in the treatment with both aphids and *F. graminearum* compared to solely *F. graminearum* are triggered by the activation of the fungal metabolism rather than more fungal biomass triggering more plant cells to defend themselves.

In our experiment ears were infested with 100 *S. avenae* aphids. By the time the whole ear became symptomatic aphid populations dropped rapidly. Their death could be explained by the lack of phloem sap and by accumulating DON concentrations. In the field (where aphids are not trapped in plastic cups), aphids would move away from the diseases ears to find healthier ears to feed on. Drakulic *et al.* (2015) also found a greater mortality rate and a depressed reproductive rate after exposure of *S. avenae* to diseased ears, compared to healthy ears.

2.4.2 Wheat response after exposing ears to aphids and followed by F. graminearum infection

Although studies of defense gene expression against aphids in wheat ears remain scarce, there have been several studies investigating cereal defense responses against grain aphids at wheat seedling or leaf stage. Both JA- and SA-mediated signaling pathways play a role in the attack of wheat by *S. avenae* (Zhao *et al.*, 2009, Cao *et al.*, 2014). According to infestation tests of Zhu-Salzman *et al.* (2004) JA-regulated pathways were effective in sorghum defense against greenbugs (*Schizaphis graminum*). Liu *et al.* (2011) suggested that *PR1* expression (indicating SA defense response) is of limited importance in

wheat defense responses against Russian wheat aphids *Diuraphis noxia*. In our study, we showed an upregulation of *PR1* in wheat ears with *S. avenae* starting from 2 dbi onwards (Fig. 2.5).

It is suggested that aphids are able to suppress or avoid activation of potentially effective plant defensive machinery, possibly through their particular mode of feeding (Zhu-Salzman *et al.*, 2004, Walling, 2008, Elzinga *et al.*, 2014). Phloem feeding insects probe plant tissue intercellulary to establish feeding sites in the phloem sieve elements. This mode of feeding minimizes wounding and limits local induction of defense responses to a minimal number of cells (Tjallingii, 2006). Indeed, no induction of upstream biosynthesis genes (*LOX* and *AOS*, or *PAL* and *ICS* as marker genes for the JA and SA biosynthesis pathway, respectively) was observed in our experiment.

Besides *PR1*, a diverse spectrum of defense related genes in wheat ears was upregulated upon aphid attack. Our experiments showed upregulation of defense genes *NADPHOX* (starting from 2 dbi), *PR3* and *PR4* (starting from 0 dai and peaking at 2 dai) and *PR5*, *CAD1* and *CCR3* (single peak at 2 dai) (Fig. 2.5). After 2 dai, these genes (except PR1) become similar in expression as the control-treatment. Several of these genes were previously reported to be induced upon grain aphid feeding. Moloi and van der Westhuizen (2006) noticed an early accumulation of H_2O_2 and an early increase of NADPH oxidase activity in wheat seedlings infected with *D. noxia* aphids (Moloi & van der Westhuizen, 2006). Moreover, these data suggested a possible signaling role for H_2O_2 by activation of downstream defense enzymes like peroxidase and β-1,3-glucanase (Moloi & van der Westhuizen, 2006). Chitinase and glucanase activities were also highly induced in wheat subject to *D. noxia* feeding (van der Westhuizen *et al.*, 1998b, van der Westhuizen *et al.*, 1998a). In our experiment we demonstrated an early induction of *NADPHOX* and induction of *PR* genes at later time points.

Once the plant response upon aphid feeding was clarified, we wanted to explore the response against infection with *F. graminearum*. As *F. graminearum* is a hemibiotrophic fungus its infection process is complicated. Successful defense employs a first line of defense mainly made up by SA-directed responses during the biotrophic phase followed by a second line of defense comprising JA-directed defense mechanisms during the necrotrophic phase (Ameye *et al.*, 2015). Although Ameye *et al.* (2015)

noticed a SA-dependent response after 24 h and a JA-dependent response after 48 h, our experiment indicated an SA-dependent response after 48 h (upregulation of *PAL* at 2 dai). Possibly this difference can be explained by the different physiological stage between seedlings and ears. *PR1*, also an indicator of SA, was no longer upregulated at 6 dai (Fig. 2.5).

Other studies conducted experiments with wheat spikelets being infected with *F. graminearum* during 48 h. Gene expression of *CCR3* and *CAD1* in those ears increased more than 9-fold and 7-fold respectively compared to mock-inoculated controls (Bi *et al.*, 2011). Ears also showed an increase in expression of *PR1* and *PR4* (Qi *et al.*, 2012) and according to Pritsch *et al.* (2000) *PR1*, *2*, *3*, *4* and *5* expression peaked at 36 to 48 h after *F. graminearum* inoculation. Our data show similar results but at later time points.

Once the response of wheat ears upon sole infection with *F. graminearum* or sole infestation with *S. avenae* was uncovered, the mutual interaction was explored. In general, early induction of defense genes *NADPHOX*, *CAD1*, *CCR3*, *PR3*, *PR4* and *PR5* by aphids is proliferated upon *F. graminearum* infection compared to a sole *F. graminearum* infection. Expressions in ears containing both *F. graminearum* and aphids were earlier, similar and/or enhanced compared to ears containing only *F. graminearum* even though the fungal biomass was not significantly different.

NADPHOX, a membrane bound precursor of H_2O_2, was significantly upregulated in ears containing both *F. graminearum* and aphids. We hypothesize that this increased induction of *NADPHOX* is a consequence of the aphids inducing *NADPHOX* at early time points. H_2O_2 is known to be an inducer of DON production once the fungus becomes necrotrophic (Ponts *et al.*, 2006, Audenaert *et al.*, 2010, Audenaert *et al.*, 2014). DON is a virulence factor of *F. graminearum* and appears to be crucial for ear colonization (Bai *et al.*, 2002, Langevin *et al.*, 2004, Jansen *et al.*, 2005). In our experiment, ears containing both *F. graminearum* and aphids had a higher DON content compared to ears with solely *F. graminearum*. This statement corroborates with the recent findings by Drakulic *et al.* (2015).

DON-nonproducing *F. graminearum* strains are unable to prevent thickening of cell walls and thus impede colonization of the ear by the fungus (Bai *et al.*, 2002, Jansen *et al.*, 2005, Maier *et al.*, 2006). Thus, lignin accumulation in host cell walls neighboring necrotic cells may play an important role in restricting the spread of the pathogen in host tissues

(Kang & Buchenauer, 2000). Our experiment demonstrated earlier and higher expressions of genes (*CAD1* and *CCR3*) indicated lignin biosynthesis (cell wall reinforcement) in ears containing both *F. graminearum* and aphids. Even though, one would suspect more lignin biosynthesis, and thus decreased spread of *F. graminearum*, ears containing both *F. graminearum* and *S. avenae* showed more symptoms. We hypothesize that the cell wall degrading properties of DON were able to render upregulation of *CAD1* and *CCR3* insufficient to impede ear colonization. It is also possible that these genes were more upregulated in an attempt of the plant to overcome degrading cell walls.

It is likely to assume that if aphids induce similar defense responses as against pathogens, pre-exposure to aphids would help protect plants from a subsequent fungal infection. However, this is not the case in this tripartite interaction because *F. graminearum* has an additional infection mechanism against plant defense, namely its production of the mycotoxin DON which acts as a virulence factor. It is possible that pathogens without specialized virulence factors cannot overcome the enhanced plant response due to pre-treatment with aphids. In latter case, the aphids indirectly service the plant to avoid colonization by non-virulence-factor producing pathogens. These pathogens will not be able to overcome the enhanced plant response and disease in the field could be less severe compared to when aphids were absent.

In contrast to the other genes (*PR3-4-5*, *NADPHOX*, *CAD1* and *CCR3*), *PR1* and *PR2* expression were exceptions. *PR1* was never induced in ears containing solely *F. graminearum*, but slopes of trend lines in Fig. 2.7 indicate a boost in *PR1* expression when both fungus and aphids were present compared to the expression in ears with solely aphids. *PR2* (β-1,3-glucanase, breakdown of the fungal cell wall) expression was not upregulated in ears containing only aphids but showed a high increase in ears infected with *F. graminearum*. At 6 dai aphids inhibited *PR2* expression in ears infected with *F. graminearum*. This indicates that *F. graminearum* would not be restricted by this defense strategy. Indeed, after 6 dai we observed more symptoms on ears with aphids.

In general, the plant response to a dual infestation of ears with *S. avenae* and *F. graminearum* gives a rather complex interaction: in order to establish a long term interaction with the plant, aphids must limit or avoid the plant defense. Indeed, we saw no induction of upstream biosynthesis genes. However at later time points, we saw induction of

several PR genes, but only for a short period of time. These genes show clear overlap with genes typically induced during a successful infection of ears with *F. graminearum*. It is known that insect deceive plants in order to trigger non-effective defense strategies. Evidence arises from the following example: the silver leaf whitefly *Bemisia tabaci* can manipulate plant signaling (*Arabidopsis thaliana*) to suppress effective defenses. Under normal circumstances, the SA-based defense is upregulated upon whitefly feeding, while the JA-based defense is unchanged. Experiments show that the JA-regulated defenses are important to deter whitefly development. But when using a plant mutant, with impaired SA-regulated defenses and uncoupled SA-JA cross talk, that is treated with methyl jasmonate, the whitefly development is severely delayed. This demonstrates that JA controls defenses that actively impede the insects development. Whiteflies are able to trigger a non-effective defense based on SA in order to suppress the effective JA defenses (Kempema *et al.*, 2007, Zarate *et al.*, 2007). In our experiment, genes that are typically upregulated upon *Fusarium* attack were also shortly upregulated during aphid infestation (deception of the plant). Expressions in ears containing both *F. graminearum* and aphids were observed earlier, similar and/or enhanced compared to ears containing only *F. graminearum*. We cannot directly link these enhanced expressions to the aphids but it seemed that if plant genes were already upregulated previously (because of the aphids), they were able to react faster/better to a subsequent attack with *F. graminearum*.

Recently, work by Drakulic *et al.* (2015) demonstrated that wheat ears exposed to both aphids and *F. graminearum* showed accelerated disease progression, an increase in disease severity and mycotoxin accumulation compared to plants treated only with *F. graminearum*. Similarly to these results, we demonstrated that ears pre-infested with *S. avenae* could exacerbate *F. graminearum* infection because we found more disease symptoms and DON production compared to ears without aphids. We were able to clarify these physiological results by investigating the transcriptional induction pattern in the wheat ears. Aphids induced some defense genes that are also typically induced upon a *F. graminearum* infection. Other defense genes showed earlier and/or enhanced transcription after exposure to both aphids and *F. graminearum*.

Ameye *et al.* (2015) demonstrated that *F. graminearum* produces more DON in an attempt to circumvent enhanced defense. In their study they showed that wheat seedlings

primed with the volatile Z-3-hexenyl acetate and infected with *F. graminearum* produced 48 h after inoculation lower fungal biomass, but a stronger upregulation of defense genes coinciding with a massive increase in DON. This is in agreement with our data, ears containing both *F. graminearum* and aphids show in general an earlier and higher response and higher DON, 3-ADON and 15-ADON contents.

Overall, this chapter provides valuable information on the impact of cereal aphids on the proliferation of *F. graminearum* and concomitant DON load in wheat ears. We demonstrated that infestation with *S. avenae* aphids provoked earlier, similar and/or enhanced typical sensitive plant responses against *F. graminearum*. This led to more symptoms and higher DON contents in the wheat ears containing aphids compared to ears without aphids.

Contributions:

Measurements of DON and acetylated forms were done by the Department of Bioanalysis, Laboratory of Food Analysis

Chapter 3:

Effect of mycotoxin deoxynivalenol on grain aphid *Sitobion avenae* and its parasitic wasp *Aphidius ervi* through food chain contamination

Redrafted from De Zutter N, Audenaert K, Ameye M, Haesaert G* and Smagghe G*.

Arthropod-Plant Interactions, 10(4), 323-329

**: equal last author*

Abstract

We investigated the influence of the *Fusarium* mycotoxin deoxynivalenol (DON) on the English grain aphid *Sitobion avenae* (Hemiptera: Aphididae) and its parasitic wasp A*phidius ervi* (Hymenoptera: Braconidae) using in-vitro laboratory experiments. In this tritrophic interaction, DON caused lethal (declined survival) and sublethal (prolonged nymphal development and reduced reproduction) effects on *S. avenae* aphids and consequentially led to a decreased production of parasitoid offspring resulting from parasitized DON-contaminated aphids. This chapter highlights that the presence of mycotoxins should be considered in environmental risk assessment tests because they may alter the efficiency of biological control agents such as parasitoids through food chain contamination.

Keywords

Cereal aphids · Deoxynivalenol · Food chain contamination · Parasitic wasps · Tritrophic interactions

3.1 Introduction

The trichothecene DON is a mycotoxin produced by fungal species of the genus *Fusarium* that causes Fusarium head blight (FHB) disease on cereals (Bottalico & Perrone, 2002, Goswami & Kistler, 2004). DON can be translocated inside the plant through xylem vessels and phloem sieve tubes (Kang & Buchenauer, 1999). Concentrations of DON found in wheat samples from fields all over Flanders (Belgium) fluctuate around 0.1-10 mg kg^{-1} although concentrations in individual spikelets are probably higher (Audenaert *et al.*, 2009, Isebaert *et al.*, 2009, Landschoot *et al.*, 2013). DON has been notorious because it provokes acute and chronic disease symptoms in humans and animals that consume contaminated grains (Bennett & Klich, 2003). Its toxic effects range from diarrhea, vomiting, gastro-intestinal inflammation, necrosis of the intestinal tract, the bone marrow and the lymphoid tissues. It causes inhibition of mitochondrial function and has effects on cell division and membrane integrity and induces apoptosis (Pestka, 2010). Finally, it also inhibits protein, DNA and RNA synthesis (Rocha *et al.*, 2005).

To date, little research has been done regarding the influence of trichothecenes on insects, even though it is an interesting topic to explore because trichothecenes can pose a threat for insects feeding on contaminated plant tissue and alter insect-plant interactions. It was found that trichodermin and other 12,13-epoxytrichothecenes have larvicidal activity against the mosquito *Aedes aegypti* (Grove & Hosken, 1975). DON and especially nivalenol have toxic effects on lepidopteran *Spodoptera frugiperda* cells (Fornelli *et al.*, 2004). Diacetoxyscirpenol and neosolaniol are potent antifeedants against larvae of *Galleria mellonella* (Mule *et al.*, 1992) and a novel isoquinoline type pigment from *F. moniliforme* showed larvicidal activity against *A. aegypti* and *Anopheles stephensi* (Pradeep *et al.*, 2015).

Also the impact of mycotoxins on the survival of insect natural enemies, developing in herbivorous insects that feed on mycotoxin-contaminated plants, is not well explored. Cereal aphids in Europe are prone to different natural enemies like predators and parasites. Aphid-specific predators consist of three groups: the coccinellids (Coccinellidae) like *e.g. Harmonia axyridis* and *Coccinella septempunctata* (Jansen, 2000, Vandereycken *et al.*, 2013), the syrphids (Syrphidae) like *Episyrphus balteatus* and *Syrphus vitripiennis* (Chambers & Adams, 1986, Jansen, 2000, Vandereycken *et al.*, 2013) and the chrysopids (Chrysopidae) *e.g. Chrysoperla carnea* (Jansen, 2000). Aphids are also subject to polyphagous predators like

carabids (Carabidae) *e.g. Pterostichus melanarius* (Harwood *et al.*, 2009) and spiders (Linyphiidae) *e.g. Erigone atra* (Harwood *et al.*, 2009). Two important groups of parasites are the parasitoids (Aphididae) e.g *Aaphidius ervi* and *Aphidius rhopalosiphi* (Olmez & Ulusoy, 2003, Stary & Havelka, 2008) and the entomophthoralean pathogens like *Pandora neoaphidis* and *Entomophthora planchoniana* (Barta & Cagan, 2006).

In this chapter we will focus on the Hymenopterous endoparasitoids which are key biological control agents contributing to biological control of economically important pests in both agricultural and natural ecosystems. Endoparasitoids spend a significant proportion of their life inside the host organism and kill their host (Fig. 3.1). As they are developing inside the host, they can come into contact with eventual toxic compounds *e.g.* mycotoxins originating from a plant infected by a toxigenic fungus which can be detrimental for the developing parasitoid.

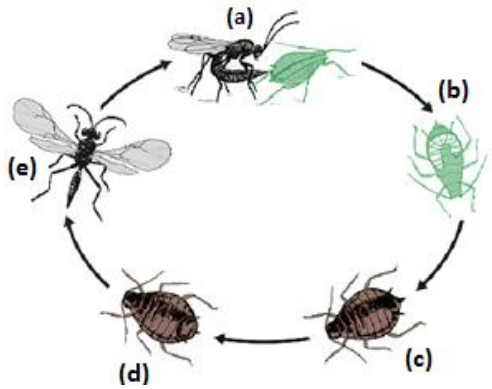

Fig. 3.1 Life cycle of an aphid endoparasitoid (adjusted from Knutson (2011, consulted 28-09-2015)): oviposition **(a)**, growth of parasite larva (aphid alive) **(b)**, parasite pupates (aphid dies and becomes mummy) **(c)**, adult parasite emerges **(d)**, adult parasite **(e).**

On a higher trophic level, natural enemies such as parasitic wasps developing in contaminated herbivores, can be directly exposed to these secondary metabolites or indirectly be affected by the reduced growth of the host (Bukovinszky *et al.*, 2012, Gols, 2014). Endoparasitoids can be especially vulnerable to mycotoxins because they develop for a period of time inside the mycotoxin-contaminated host. It is already known that plant secondary metabolites can pose a threat to natural enemies (Campbell & Duffey, 1981, Barbosa *et al.*, 1991, Roth *et al.*, 1997, Ode *et al.*, 2004, Harvey *et al.*, 2007). The effect of fungus-infected plants on adult female parasitoid behavior and development has been

investigated (Cardoza *et al.*, 2003, Harri *et al.*, 2008, van Nouhuys & Laine, 2008, Bultman *et al.*, 2009). Still, to our knowledge, research remains scarce on the impact of mycotoxins on parasitoids which develop inside herbivorous insects feeding directly from those mycotoxins.

To fill this knowledge gap, we performed a case study investigating the influence of the mycotoxin DON on the phloem-feeding cereal aphids *S. avenae*. Both the mycotoxin-producing *Fusarium* spp. and the aphid *S. avenae* reside on wheat ears during anthesis, the critical fungal infection period. This can increase the chances that the cereal aphids come into contact with DON when feeding from the DON- contaminated phloem. Moreover, we examined the effect of DON-contaminated *S. avenae* on aphid parasitism by the parasitic wasp *A. ervi*, one of the most important parasitic wasps of *S. avenae* aphids in European cereal fields (Al Dobai *et al.*, 1999, Tomanovic *et al.*, 2008, Barczak *et al.*, 2014).

3.2 Material and Methods

3.2.1 Insects and deoxynivalenol

A laboratory stock culture of *S. avenae* aphids was maintained on wheat seedlings at constant temperature of 22 °C, 60% relative humidity and a photoperiod of 16 h light, stimulating parthenogenesis (De Zutter *et al.*, 2012).

S. avenae aphid mummies containing *A. ervi* were acquired from Biobest (Westerlo, Belgium). Newly emerged parasitoids were sexed and pairs consisting of one female and one male were put individually in Petri dishes to allow mating overnight in order to obtain naive females (*i.e.* females that have never laid an egg before) (Joseph *et al.*, 2011, Pan & Liu, 2014). All experiments were conducted in a climate chamber at 22 ± 2 °C, 60% relative humidity and a photoperiod of 16 h light.

The mycotoxin DON (purity >99%) was kindly provided by M. Lemmens (BOKU, Vienna, Austria). A stock solution (1000 mg L^{-1}) was prepared in sterile water and stored at -20 °C.

3.2.2 Experimental setup to examine the lethal effects of deoxynivalenol on S. avenae and A. ervi

Aphids were dietary exposed to DON by using an aphid feeding apparatus (Fig. 3.2) as described in Sadeghi *et al.* (2009). The feeding apparatus contained 200 µL of artificial diet based on formulation A of Prosser and Douglas (1992) amended with DON to a final concentration of 0.25, 1, 3 and 5 mg L^{-1} DON or sterile water (0 mg L^{-1} DON). During the experiment, the diet was changed every other day and amended with the different concentrations of the persistent molecule DON. Adult *S. avenae* aphids were randomly selected from the stock culture and put on wheat seedlings to produce neonates (nymphal stage 1). The age of these neonates was between 0 and 24 h (day 0). Five neonates were transported from the seedlings to each aphid feeding apparatus. At day 4 the aphids were exposed to one naive *A. ervi* female per aphid feeding apparatus. At this time the aphids were in the second or third nymphal stage. Subsequently, aphids remained in the feeding apparatus until mummification. The number of mummies was counted daily. Each mummy was then put in a small Petri dish in order to evaluate the time until emergence of the parasitoid progeny.

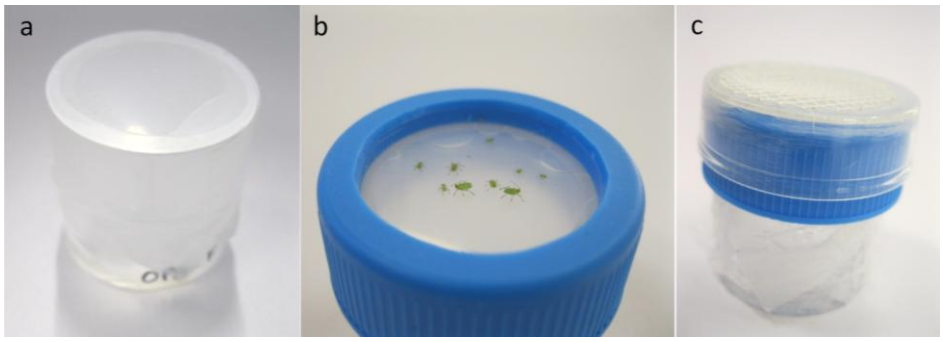

Fig. 3.2 Experimental setup of aphid feeding apparatus: a parafilmsachet (**a**) exists of two stretched parafilm layers with artificial diet in between from which aphids can feed (**b**). Aphids are enclosed using a lid with netting on top (**c**).

In total, 60, 30, 45, 45 and 30 aphids divided into groups of five individuals per feeding apparatus were used as starting population in this experiment and exposed to 0, 0.25, 1, 3 and 5 mg L^{-1} DON, respectively. During the experiment the percentage of aphid populations was calculated at different stages: % surviving aphids in the DON treatment at

the moment of exposure to the parasitoid female and % surviving aphids at the beginning of mummification. Moreover, the percentages of aphids that turned into mummies (% mummification) and newly emerged parasitoids (% successful parasitism) were calculated.

3.2.3 Experimental design to examine sublethal effects of deoxynivalenol on S. avenae aphids

S. avenae neonates were exposed to DON in a final concentration of 0.25 or 1 mg L^{-1} DON or sterile water (0 mg L^{-1} DON) using aphid feeding apparatus as described above. Aphids were examined daily to examine the nymphal development and mortality. The presence of exuvia was used for the determination of a molt. To examine reproduction, the progeny of surviving females was counted daily and carefully removed using a fine brush. Progeny of females who died during nymphal development was considered zero. The intrinsic rate of increase (r_m) (Wyatt & White, 1977) was calculated by following formula: $r_m = [0.738 \ln(Md)]/D$, with Md = numbers of nymphs produced by one female during its whole adult life (D). In total, ten aphids divided into ten aphid feeding apparatus (one per apparatus) were monitored daily for each concentration of DON (0, 0.25 and 1 mg L^{-1} DON).

3.2.4 Dual-choice assay to test preference of A. ervi for control and DON-contaminated aphids

In the preference test, naive female *A. ervi* were given the choice between aphids fed with DON and control aphids. A repeat consisted of two aphids in a small Petri dish (3.5 cm diameter), one fed with 3 mg L^{-1} DON and one control aphid (fed with 0 mg L^{-1} DON). Both aphids were synchronized to the second or third nymphal stage (Pan & Liu, 2014). In the Petri dish, one naive female was introduced and allowed to choose between the DON-contaminated aphid and the control aphid. The female was monitored for a maximum of 10 minutes. The aphid that was first stabbed by the female and the time until this first stab (*i.e.* parasitoid touching the aphid with its ovipositor) occurred was recorded.

Females that required more than 10 minutes to stab an aphid were labeled as 'no choice' (Daza-Bustamante *et al.*, 2003). In total, the choice of 60 females was tested.

3.2.5 Statistical analyses

Data were analyzed using SPSS (Statistical Package for Social Sciences) Statistics 22. Statistical differences of (sub)lethal effects by different DON concentrations against *S. avenae* and *A. ervi* (Fig. 3.3 and Table 3.2) and the duration of *A. ervi* development (Table 3.4) were analyzed using a nonparametric Kruskal-Wallis test. In case of significant differences between the treatments, nonparametric Mann-Whitney U tests were used to compare two treatments. Data in Fig. 3.3 were analyzed using one-sided Mann-Whitney U tests corrected with the Benjamini and Hochberg False Discovery Rate (B&H FDR) at a level of 0.05 for controlling the type I error rate (Benjamini & Hochberg, 2000). All tests were conducted with a significance level of $\alpha = 0.05$.

3.3 Results

3.3.1 (Sub)lethal effects of deoxynivalenol on S. avenae and A. ervi

In this experiment *S. avenae* aphids were dietary exposed to five different concentrations of DON (0, 0.25, 1, 3 and 5 mg L^{-1} DON). The lethal effects of DON were measured by calculating the percentage of surviving *S. avenae*, and for *A. ervi* by calculating the percentage of aphids that turned into mummies and resulted into newly emerged parasitoid progeny. When the aphids that were feeding on the different concentrations of DON, were exposed to the female parasitoid, a negative effect of 5 mg L^{-1} DON on the survival of S. avenae was found (Fig. 3.3 and Table 3.1). The surviving aphid population feeding on 5 mg L^{-1} DON was significantly lower than the other treatments. Treatment of the aphids with 3 mg L^{-1} DON also caused a decrease in the aphid population but at a later time (when mummification started).

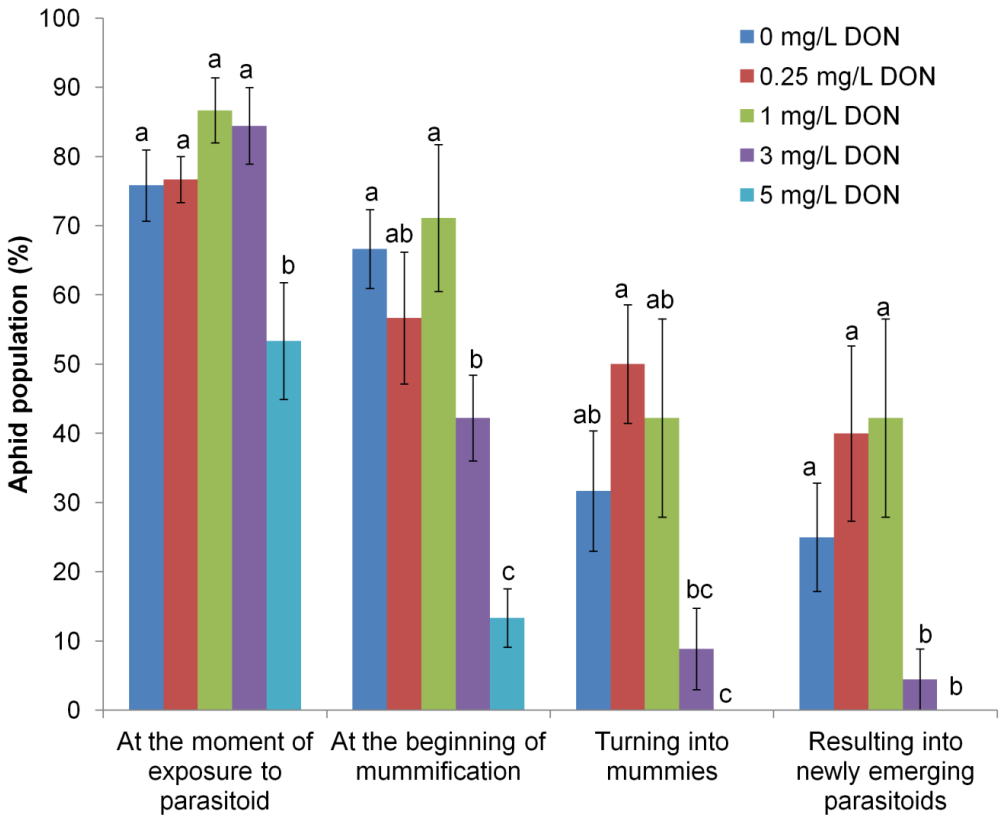

Fig. 3.3 The percentage of aphid populations (mean ± SE) at different stages: % surviving deoxynivalenol (DON) at the moment of exposure to the parasitoid female, % surviving at the beginning of mummification, % aphids becoming mummies and % aphids that produced newly formed parasitoid progeny. Different letters represent significant differences ($P < 0.05$) between treatments with different concentrations of DON using one-sided Mann-Whitney U tests corrected with B&H FDR. Starting populations (100%) consisted of 60, 30, 45, 45 and 30 aphids divided into groups of five individuals per aphid feeding apparatus exposed to 0, 0.25, 1, 3 and 5 mg L^{-1} DON, respectively.

Table 3.1 P-values according to one-sided Mann-Whitney U tests associated with Fig. 3.3. The concentrations of 0, 0.25, 1, 3 and 5 mg L^{-1} deoxynivalenol are represented as numbers 1 to 5 respectively. P-values in bold are significant after B&H FDR correction.

P-values	At the moment of exposure to parasitoid	At the beginning of mummification	Turning into mummies	Resulting into newly emerging parasitoids
1-2	0.456	0.384	0.090	0.145
1-3	0.075	0.071	0.316	0.232
1-4	0.135	**0.008**	0.027	**0.019**
1-5	**0.017**	**0.001**	**0.007**	**0.013**
2-3	0.061	0.076	0.382	0.476
2-4	0.135	0.098	**0.002**	**0.004**
2-5	**0.012**	**0.004**	**0.001**	**0.004**
3-4	0.406	**0.013**	0.039	**0.018**
3-5	**0.003**	**0.004**	**0.017**	**0.017**
4-5	**0.006**	**0.002**	0.116	0.207

Sublethal effects of DON were measured by investigating the aphids nymphal development and reproduction while feeding on 0, 0.25 and 1 mg L^{-1} DON (Table 3.2 and Table 3.3). The duration of the last three nymphal stages (days) of aphids dietary exposed to 1 mg L^{-1} DON was significantly longer ($P < 0.05$) compared to 0 or 0.25 mg L^{-1} DON. When aphids were fed with 0, 0.25 and 1 mg L^{-1} DON, Md (*i.e.* the number of nymphs produced per female during its whole adult life), D (*i.e.* the whole adult life of the aphid in days) and rm (*i.e.* the aphid population intrinsic rate of increase) were calculated. Md, D and r_m showed no significant differences between 0 and 0.25 mg L^{-1} but both treatments were significantly higher ($P < 0.05$) than 1 mg L^{-1} DON, except for r_m where the effect by 0.25 and 1 mg L^{-1} DON was not significantly different ($P = 0.057$).

Table 3.4 presents the duration of *A. ervi* development in days inside *S. avenae* aphids contaminated with different concentrations of DON (0, 0.25, 1 and 3 mg L^{-1} DON). No significant differences were found between treatments 0, 0.25, 1 and 3 mg L^{-1} DON ($P = 0.349$ and 0.340 for oviposition until mummification and from mummification until emergence respectively, according to Kruskal-Wallis tests). No mummies were found for the 5 mg L^{-1} DON treatment. Aphids feeding on 3 mg L^{-1} DON produced significantly fewer mummies compared to aphids feeding on 0.25 mg L^{-1} DON (Fig. 3.3 and Table 3.1 continued). Significantly less parasitoid progeny emerged from aphids fed on 3 mg L^{-1} DON compared to the lower concentrations tested. Table 3.5 presents the amount of mummies, amount of parasitoid progeny and sex ratio of the progeny.

3.3.2 Preference of parasitic wasp to attack control and DON-contaminated S. avenae aphids

In our binary-choice experiment, female *A. ervi* did not discriminate between DON-contaminated and control aphids. Out of a total of 60 wasps, 21 stabbed the control aphid first, 21 stabbed the DON-contaminated aphid first, and 18 were recorded as 'no choice'. The mean time (± SE) needed by females to make a choice was not significantly different between both treatments (2.9 ± 0.5 minutes for control aphids and 3.3 ± 0.6 minutes for DON-contaminated aphids).

Table 3.2 Sublethal effects of different concentrations of deoxynivalenol (DON, mg L^{-1}) on *S. avenae* aphids nymphal development and reproduction.

	Duration of nymphal development (days)				Reproduction		
DON	N1	N2	N3	N4	Md	D	r_m
0	1.1 ± 0.1[a]	1.4 ± 0.2[a]	1.4 ± 0.4[a]	1.9 ± 0.1[a]	16.6 ± 4.6[a]	12.8 ± 3.6[a]	0.108 ± 0.033[a]
0.25	1.0 ± 0.1[a]	1.8 ± 0.2[a]	1.6 ± 0.2[a]	1.9 ± 0.1[a]	14.3 ± 3.3[a]	14.6 ± 3.4[a]	0.076 ± 0.017[ab]
1	1.5 ± 0.3[a]	2.6 ± 0.2[b]	3.0 ± 0.4[b]	3.5 ± 0.5[b]	0.9 ± 0.7[b]	1.7 ± 1.5[b]	0.035 ± 0.026[b]

N1-N4: aphid nymphal stage 1 to 4, Md: number of nymphs produced by females during their whole adult life, D: whole adult life of the aphid in days, r_m: aphid population intrinsic rate of increase. Data are presented as means ± SE. Levels of significance between treatments (0, 0.25 and 1 mg L^{-1} DON) were derived by comparing treatments two by who using Mann-Whitney U tests. Different letters indicate significant differences between treatments ($P < 0.05$).

Table 3.3 P-values according to Mann-Whitney U tests associated with Table 3.2. The concentrations of 0, 0.25 and 1 mg L^{-1} deoxynivalenol are represented as numbers 1 to 3, respectively.

	Duration of nymphal development (days)				**Reproduction**		
P-values	N1	N2	N3	N4	Md	D	r_m
1-2	0.563	0.195	0.677	1	0.818	0.646	0.566
1-3	0.194	0.008	0.033	0.015	0.008	0.016	0.039
2-3	0.114	0.042	0.016	0.015	0.008	0.008	0.057

Table 3.4 Duration of *A. ervi* development (mean ± SE) inside *S. avenae* aphids contamined with different concentrations of deoxynivalenol (DON, mg L^{-1}).

DON	**oviposition → mummification (days)**	**mummification → emergence (days)**
0	8.1 ± 0.3	5.2 ± 0.2
0.25	8.6 ± 0.3	5.5 ± 0.3
1	8.4 ± 0.3	5.4 ± 0.2
3	8.0 ± 0.7	4.5 ± 0.5

Data are presented as means ± SE. There were no significant differences between treatments according to Kruskal-Wallis tests.

Table 3.5 Parasitoid development parameters for *A. ervi* developing inside aphids feeding from different concentrations of deoxynivalenol (DON, mg L^{-1}).

DON	**Amount of mummies**	**Amount of progeny**	**Sex ratio (% females)**
0	19	15	54,5
0.25	15	12	40
1	19	19	35,7
3	4	2	-

3.4 Discussion

Survival, nymphal development and reproduction of *S. avenae* aphids were negatively influenced by DON**.** In our experiment, female *A. ervi* did not discriminate between control and DON-contaminated aphids, but successful parasitism of the aphids by *A. ervi* (*i.e.* emergence of parasitoid progeny) on 3 mg L^{-1} DON decreased. This could mean that the parasitoid eggs or larvae were directly or indirectly susceptible to DON or DON derivatives inside the aphid body. Directly, DON causes inhibition of mitochondrial function, has effects on cell division and membrane integrity, induces apoptosis and inhibits protein, DNA- and RNA synthesis (Rocha *et al.*, 2005, Pestka, 2010). Possibly these toxic effects can lead to mortality of the parasitic wasp eggs or larvae inside the aphid body. Indirectly, it is possible that DON-contaminated aphids were feeding less efficiently than control ones (indeed, their nymphal development is slower), leaving the parasitoid larvae with fewer nutritional resources for its development. Nevertheless, there was a negative effect on the parasitoid developing inside DON-contaminated aphids. Aphids feeding from 3 mg L^{-1} DON were less likely to be successfully parasitized. Even though the aphid population dropped because of the negative effects of DON, the remaining aphids (tolerant to DON) would have higher surviving chances because there were also less likely to be successfully parasitized (worse development of parasitoid inside DON-contaminated aphid). These aphids have a dual advantage: they are tolerant to DON and are less successfully parasitized by *A. ervi*. This could suggest that DON causes a decreased efficiency of biological control agents such as parasitoids through food chain contamination.

In our experiment we used concentrations of DON ranging from 0 to 5 mg L^{-1}. DON is a water soluble compound and is translocated in ears of cereal crops through phloem sieve tubes (Kang & Buchenauer, 1999). Concentrations found in wheat samples from fields all over Flanders (Belgium) fluctuate around 0.1-10 mg kg^{-1} DON (Audenaert *et al.*, 2009, Isebaert *et al.*, 2009, Landschoot *et al.*, 2013). These concentrations are present in grains at harvest and thus do not necessarily represent concentrations in the phloem during the time period that aphids are feeding from the phloem sieve tubes. It is not known which DON concentrations are present in the phloem during the moment that aphids are present on the ear although we assume these can rise to high concentrations. Indeed, when a field has an

average concentration of *e.g.* 5 mg kg^{-1}, this means that individual infected spikelets might be exposed to *e.g.* 10 fold higher concentrations. Moreover, not all spikelets in the field are infected. Exact concentrations of DON and its derivatives in the phloem sap that impair both the aphids and their parasitoids are unknown. To cope with xenobiotics, *S. avenae* has several enzyme families which show increased activities upon contamination. P450s (cytochrome P450 mono-oxygenases, phase I metabolic enzymes) and GSTs (glutathion-S-transferases, phase II metabolic enzymes) activities increased with the secondary plant compound hydroxamic acid levels in wheat (Castaneda *et al.*, 2010) and GST activities also increased when *S. avenae* was dietary exposed to phenol catechol, alkaloid gramine and non-protein amino acid L-ornithine-HCl (Cai *et al.*, 2009, Zhang *et al.*, 2013). Lu and Gao (2009) suggested the involvement of P450s and GSTs in the susceptibility to the insecticide pirimicarb. More specifically for the role of these mechanisms in detoxifying mycotoxins, studies with aphids remain scarce. In contrast to the aphids, hymenopteran insects such as honeybees, bumblebees and the parasitoid *Nasonia vitripennis*, for which the whole genomes are sequenced, are known to have substantially fewer genes coding for detoxification enzymes (Claudianos *et al.*, 2006, Oakeshott *et al.*, 2010, Werren *et al.*, 2010, Sadd *et al.*, 2015) compared to other insects, leaving them more vulnerable for xenobiotics. Exposure of Hymenoptera parasitoids, such as *A. ervi*, to mycotoxins through food chain contamination is thus another important parameter when assessing the risk against natural enemies.

In literature, evidence of fungus-infected plants affecting the parasitoids is present. Preference of parasitoid *Cotesia marginiventris* was influenced by plant volatiles upon infection with beet armyworms (*Spodoptera exigua*). The wasps were even more responsive to these volatiles when plants were infected with both catterpillars and white mold fungus. This means that parasitoid behaviour is also influenced by the effect of pathogen-induced biochemical changes in plants (Cardoza *et al.*, 2003). In our model system, we did not observe host preference by *A. ervi* and *A. ervi* females did not discriminate between DON contaminated aphids and control aphids for oviposition. A possible explanation might come from the fact that besides volatiles, visual cues like aphid color, shape and movement are important features in preference of parasitoids (Mackauer *et al.*, 1996). Fuentes-Contreras and Niemeyer (1998) showed that reduction in *S. avenae* size was related to a decreased

success in avoiding parasitoid oviposition of *A. rhopalosiphi*. Though we noticed in the experiments that aphids feeding from 3 mg L^{-1} DON were smaller, female *A. ervi* did not discriminate between treated and control aphids. In contrast to the study of Cardoza *et al.* (2003) who included the plant into the experiment, our study exclusively focuses on DON. It would be advisable that future experiments include the plant in order to get the bigger picture (*e.g.* preference of parasitoids can be influenced by plant volatiles produced upon pathogen attack). In that case, field studies including *Fusarium*-infected versus healthy grain ears would be necessary to investigate the performance of *A. ervi*. In this chapter, we focused on the mycotoxin itself. This implicates that the concentrations of DON must be known. In field studies, DON concentrations can't be controlled and thus it isn't known to which concentrations the aphids are exposed. Moreover, other factors like rain, natural *Fusarium* infections with a blend of *Fusarium* species and other mycotoxins can influence field experiments. In addition, FHB is caused in the field by a species complex all producing a different set of mycotoxins.

The *in planta* presence of fungal endosymbionts triggers plants to produce herbivore-toxic substances. Harri et al. (2009) investigated life-history traits of the parasitoid *A. ervi* when it was exposed to the endophyte-tolerant aphid *Metopolophium festucae* feeding from plants infected with a mycotoxin-producing endophyte. The presence of endophytes significantly increased the development time of *A. ervi*. The authors concluded that extended parasitoid development should ultimately reduce the population growth of *A. ervi* and thus endophyte presence may represent an advantage for endophyte-tolerant aphid species. Similarly, Bultman et al. (2009) demonstrated that the survival of parasitoid *Euplectrus comstockii* parasitizing fall armyworms (*Spodoptera frugiperda*) was also negatively influenced in caterpillars fed with plants infected with a fungal endophyte, although these results were varying when different isolates of the fungus were used. The above mentioned studies cannot directly link the negative effect on the parasitoids to the toxic substances inside the plants produce upon fungal attack. In contrast, our study is able to provide that direct link by using only the toxin without the interactions of the plant.

The study of van Nouhuys and Laine (2008) provides also interesting insights in tritrophic interactions when plants are infected by fungi. Their study is supported by a six years analysis of population dynamics of the parasitoid *Cotesia melitaearum* that parasitizes *Melitaea cinxia* butterflies. Similar to the previous studies mentioned, a negative effect on

the parasitoid is demonstrated when they are parasitizing hosts fed with *Podosphaera plantaginis*-infected plants. The progeny of these parasitoid weighed less, indicating that the fungus causes the hosts to be of poor quality. In our study, we didn't weigh the parasitoid progeny to compare this parameter between DON-contaminated and uncontaminated aphids. We also didn't take into account the post-emergence reproduction of the parasitoid progeny. In future experiments (in the lab, in the field and with or without the use of plants as mediators) these parameters should be tested because they are good indicators of the parasitoid's fitness. van Nouhuys and Laine (2008) also noticed that the parasitoids reared from hosts fed fungal-infected diet tended to be female, a characteristic that is associated with high host quality. As a result, the probability of colonization of a host population by the parasitoid increased more than twofold in a six year analysis of the parasitoid population dynamics. The authors were not able to determine a causal relationship between the plant pathogen and the increasing fraction of female offspring. During the experiment in this chapter, mummies were put separately in small petri dishes until the parasitoid emerged. This progeny was then sexed. We observed a shift in sex ratio from more female parasitoid progeny to more males. Although this result was consistent, it was not statistically significant. This might point to developing females being more susceptible to DON than developing males. To give a decisive answer about the effect of DON on the sex ratio of *A. ervi*, more biological replicates of higher DON concentrations might be included in future experiments. Also Joseph *et al.* (2011) noticed a shift towards male progeny in aphids contaminated with insecticide compared to the controls. Further research should investigate the DON concentration inside the aphids that is toxic for the different stages of both sexes of *A. ervi* and elucidate the molecular mechanism behind this parasitoid susceptibly to DON.

In conclusion, chapter 3 emphasized the importance of DON in food chain contamination from plant to insects (insect-plant interactions; grain aphids *S. avenae*) and their natural enemies (higher trophic interactions; parasitic wasp *A. ervi*). Based on our data we believe that DON can cause a decreased efficiency of biological control agents such as parasitoids through food chain contamination. Therefore, it is important to consider the presence of mycotoxins in environmental risk assessments when insect pest control systems are based on biological control with natural enemies, such as the use of parasitic wasps against aphids.

Chapter 4:

Aphids transform and detoxify deoxynivalenol via a type II bio-transformation mechanism yet unknown in animals

Redrafted from De Zutter N*, Arroyo-Manzanares N*, Audenaert K*, De Boevre M, Van Poucke C, De Saeger S, Haesaert G* and Smagghe G*.

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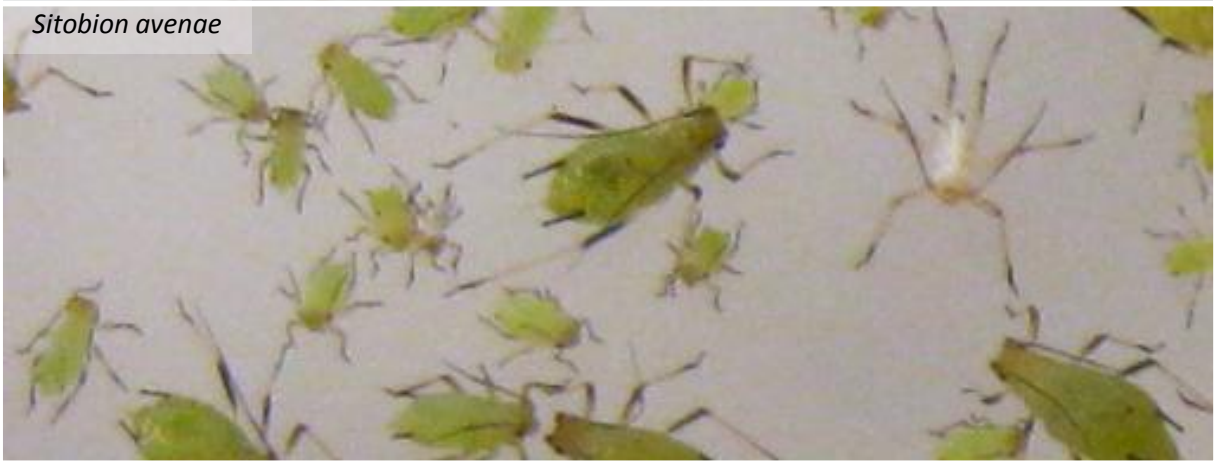

Abstract

Biotransformation of mycotoxins in animals comprises phase I and phase II metabolization reactions. For the trichothecene deoxynivalenol (DON), several phase II biotransformation reactions have been described resulting in DON-glutathiones, DON-glucuronides and DONsulfates made by glutathione-S-transferases, uridine-diphosphoglucuronyl transferases and sulfotransferases, respectively. These metabolites can be easily excreted and are less toxic than their free compounds. Here, we demonstrate for the first time in the animal kingdom the conversion of DON to DON-3-glucoside (DON-3G) via a model system with plant pathogenic aphids. This phase II biotransformation has solely been reported in plants. As the DON-3G metabolite was less toxic for aphids than DON this conversion is a detoxification reaction. Remarkably, English grain aphids (*Sitobion avenae*) which co-occur with the DON producer *Fusarium graminearum* on wheat during the development of fusarium symptoms, tolerate DON much better and convert DON to DON-3G more efficiently than pea aphids (*Acyrthosiphon pisum*), the latter being known to feed on legumes which are no host for *F. graminearum.* Using a non-targeted high resolution mass spectrometric approach, we detected DON-diglucosides in the insects probably as a result of sequential glucosylation reactions. Data are discussed in the light of a co-evolutionary adaptation of *S. avenae* to DON.

Keywords

Aphids · Deoxynivalenol · Glucosylation · Survival · Tolerance

4.1 Introduction

Trichothecenes are a class of mycotoxins produced by several fungal species of the genus *Fusarium* and related genera in agricultural crops. They belong to the structural group of sesquiterpenoids, all bearing a common tricyclic 12,13-epoxytrichothec-9-ene core structure. Type A, B, C and D trichothecenes can be distinguished based on substitutions at position C-4, C-7, C-8 and/or C-15 (Bottalico & Perrone, 2002, McCormick *et al.*, 2011). Worldwide deoxynivalenol (DON, Fig. 4.1) is the most important trichothecene (type B) because of its omnipresence in many cereal-based matrices (Bottalico & Perrone, 2002, Goswami & Kistler, 2004, De Boevre *et al.*, 2012a). DON provokes acute and chronic disease symptoms in humans and animals (Bennett & Klich, 2003). Its toxic effects range from diarrhea, vomiting, gastro-intestinal inflammation, necrosis and apoptosis of the intestinal tract, the bone marrow and the lymphoid tissues. DON causes inhibition of the mitochondrial function and has effects on cell division and membrane integrity (Pestka, 2010). Finally, it also inhibits protein, DNA- and RNA synthesis in eukaryotic cells (Jimenez *et al.*, 1975, Grant *et al.*, 1976, Rocha *et al.*, 2005). The toxicity of these sesquiterpenes can be explained by their chemical structure containing an epoxide at the C-12 and C-13 position (Fig. 4.1) (Desjardins *et al.*, 1993). Although most eukaryotic organisms are to a certain level prone to trichothecenes, many of them have developed strategies to arm themselves against the detrimental effects of these mycotoxins, and examples are present throughout the fungal, animal and plant kingdom.

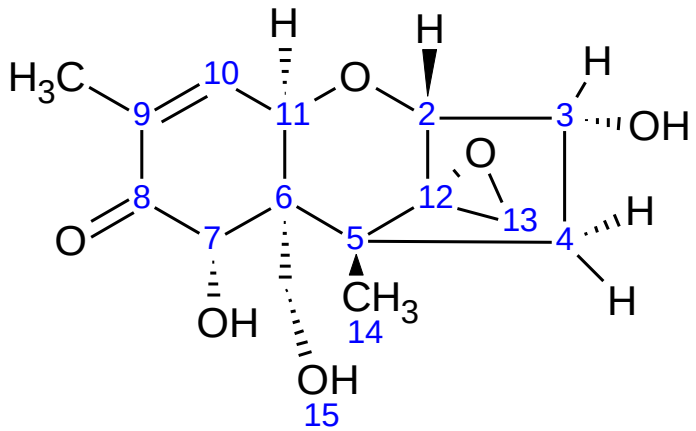

Fig. 4.1 Chemical structure of the mycotoxin deoxynivalenol.

In the yeast *Saccharomyces cerevisiae* a spontaneous mutant which was tolerant to the trichothecene trichodermin was isolated (Jimenez *et al.*, 1975). The tolerance was shown to be based on alteration of the target side of trichothecenes. The gene responsible for the trichodermin resistance was called *tcm1* (Grant *et al.*, 1976) and was suggested to encode for the ribosomal protein L3 (RPL3) (Fried & Warner, 1981), which is the target of trichothecenes. The DNA sequence of *tcm1* was determined (Schultz & Friesen, 1983) and a mutation in this gene did not only cause tolerance to trichothecenes, but also affected the maturation of either 40S or 60S ribosomal subunits (Fernandez-Lobato *et al.*, 1990). Similarly, Mitterbauer *et al.* (2004) depicted several mutations in *Rpl3* conferring semi-dominant resistance to trichothecenes. Transgenic tobacco plants expressing a modified *Rpl3* cDNA were shown to be able to adapt to DON. Nevertheless, the tolerance was not constitutive because the engineered RPL3 protein was not utilized in the presence of the native RPL3 due to a lower affinity of the engineered RPL3 for the ribosome assembly factor Rrb1p (Mitterbauer *et al.*, 2004).

In animals, two major metabolic pathways for detoxification of trichothecenes have been reported. Deepoxidation of the trichothecene DON to deepoxidated DON (DOM-1) is a well known example. In addition, several so called type II biotransformation reactions have been reported in which DON is conjugated with glucuronides, sulphonates or glutathione (Berthiller *et al.*, 2013, Wen *et al.*, 2016). Remarkably, in contrast to the vast amount of data on higher animals, information on transformation and detoxification strategies in insects remains scarce. Nevertheless, these animals often live in close proximity of trichothecene producing fungi and the toxicity has been reported in a few studies: trichodermin and other 12,13-epoxytrichothecenes have been shown to have larvicidal activity against mosquitoes of *Aedes aegypti* (Grove & Hosken, 1975). DON has toxic effects on lepidopteran *Spodoptera frugiperda* cells (Fornelli *et al.*, 2004). The trichothecenes (type A) diacetoxyscirpenol and neosolaniol were demonstrated to be potent anti-feedants against larvae of *Galleria mellonella* (Mule *et al.*, 1992).

Finally, trichothecenes are also prone to metabolization in plants. Cereals have developed mechanisms to detoxify trichothecenes by conjugation to endogenous metabolites (*e.g.* glucosylation) or de-epoxidation (Boutigny *et al.*, 2008). With respect to DON, the probably most important detoxification reaction to reduce the toxicity of DON in planta is its conjugation to glucose reported for naturally *F. graminearum*-inoculated and

contaminated wheat (Berthiller et al., 2005). Glucosyltransferases insert a glucose on the free toxins that renders the toxin more water soluble (Berthiller *et al.*, 2013). Such metabolites are DON-3G, DON-di-hexoside, 15-acetyl-DON-3-glucoside, DON-malonylglucoside (Kluger *et al.*, 2015). Recently, many other derivatives of DON have been identified in wheat. DON is also conjugated to glutathione (DON-S-glutathione (DON-GSH), "DON-2H"-S-glutathione, DON-S-cysteinyl-glycine and DON-S-cysteine) (Schroder *et al.*, 2007, Gardiner, SA *et al.*, 2010, Kluger *et al.*, 2015). Warth *et al.* (2015a) identified DON-3-sulfate and DON-15-sulfate. After metabolization of DON, the compartmentation phase takes place: the resulting products are either transported to the vacuole and stored there, or further modified and deposited in the cell wall (Coleman *et al.*, 1997, Berthiller *et al.*, 2007). Walter *et al.* (2015) reported on an wheat ABC transporter that contributes to mycotoxin tolerance. ATP-binding cassette (ABC) transporters are transmembrane proteins that use the energy from ATP hydrolysis to transport substances across the cell membrane (Jones *et al.*, 2009).

Finding new detoxification strategies for mycotoxins is a growing field of interest and a first crucial step in order to implement this knowledge in future mycotoxin remediation strategies. In this light, the present study aimed to assess the ability of plant-pathogenic aphids to cope with the trichothecene DON.

More in particular, we conducted a comparative study between grain aphids *S. avenae* and pea aphids *A. pisum*. *S. avenae* are known to colonize cereals (monocots) and feed from the cereal ear's phloem. They form a unique tripartite evolutionary relationship with wheat and *Fusarium* spp. Piercing-sucking insects like aphids ingesting plant phloem sap come into contact with all components in the transport fluid (Kehr, 2006). Thus these insects can also come in contact with secondary metabolites of pathogens that are being translocated in the plant through the phloem system when the pathogen is residing on the same plant part as the insect. Grain aphids reside on wheat ears and can come into contact with DON when this ear is infected by *F. graminearum.* DON is being translocated in ears through xylem vessels and phloem sieve tubes (Kang & Buchenauer, 1999). In contrast, *A. pisum* has a host specificity for several species of legumes (dicots) and thus, under natural conditions, never encounters DON. This unique tripartite relationship (grain aphids – wheat – *Fusarium*) occurred in nature for many generations. We believe that this co-evolution

could have resulted in adapted survival mechanisms of aphids in presence of toxins produced by pathogens, whereas this is not the case for pea aphids. To underpin this idea, we formulated several hypotheses: (i) *S. avenae* is more tolerant for DON compared to *A. pisum* when dietary exposed to this mycotoxin, (ii) mutations in the DON target molecule RPL3 is a possible tolerance mechanism, (iii) less toxic derivatives of DON as a result of type II biotransformation mechanisms will be found inside the *S. avenae* aphids using (non)targeted analyses.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is a separation/detection technique, and is particularly useful for the simultaneous determination of multiple mycotoxins. The technique involves the use of reference standards (targeted approach). Nowadays, only three DON derivatives (DON-3G, 3-acetyl-DON (3-ADON) and 15-acetyl-DON (15-ADON)) are commercially available.

Liquid chromatography (LC) is a dynamic separation technique that is able to separate mixtures of chemical compounds *e.g.* mycotoxins. For LC analysis, the compounds should be soluble in or miscible with the mobile phase. The basis of separation of target molecules is the difference in affinity between the mobile and stationary phase. To achieve the desired separation of target compounds the composition and nature of these two phases are crucial. A mass spectrometer (MS) converts sample molecules into ions in the gas phase (ionization). In the next step the formed ions are separated according to their mass-to-charge ratio (m/z) and finally the individual ions are recorded according to the current intensities at each mass in a mass spectrum (detection). Electrospray ionization (ESI) is a type of atmospheric pressure ionization: it operates by a process called 'ion evaporation'. During this process, ions are emitted from a droplet into the gas phase. The formed ions are transferred into the mass analyzer. Mass spectrometers use the difference in m/z of the ions to distinguish them. In this study, a Quattro Premier XE® MS/MS was used, consisting of two mass analyzers or quadrupoles and a T-wave collision cell. In quadrupole instruments the potentials are adjusted, so that only ions of a selected mass go through the rods. At the end of the mass spectrometric process the selected ions have to be transformed into a usable signal (detection). Because the number of ions leaving the mass analyzer is quite small, significant signal amplification is necessary (Monbaliu, 2011, De Boevre, 2013).

We also used a full-scan mode high-resolution mass spectrometry (HRMS) because of reference standards of conjugated mycotoxins are not always available and because unknown forms can occur. HRMS is able to analyze compounds for which only the molecular formulae are known, without the availability of reference standards (non-targeted approach). Compared to LC-MS/MS, the preselection of targeted compounds is not necessary (we do no longer have to pre-suppose which mycotoxins might be anticipated), and compound detection depends on a predefined full-scan over a wide mass range (to screen for a much wider group of metabolites). Another advantage of HRMS (Synapt G2-Si MS system) is that acquired data can even be evaluated retrospectively for additional compounds. In this study we used the Time-of-Flight (TOF) technology that provides high-resolution data of the molecular and fragment ions, being thus particularly powerful for structure elucidation of unknown compounds. During TOF, ions are accelerated by an electrical field to equal kinetic energy with the velocity of the ion depending on *m/z*. This instrument is superior to quadrupole mass spectrometers in terms of full-scan sensitivity and mass accuracy (Monbaliu, 2011, De Boevre, 2013).

4.2 Experimental procedures

4.2.1 Insects and chemicals

Laboratory stock cultures of cereal aphids *S. avenae* and pea aphids *A. pisum* were maintained on respectively wheat seedlings and young broad bean plants under standard conditions of 22 °C and a photoperiod of 16 h light, stimulating parthenogenesis (De Zutter *et al.*, 2012).

DON was kindly provided by M. Lemmens (BOKU, Vienna, Austria). Purity of the provided stock standard was > 99%. A stock solution was prepared by dissolving 5 mg DON in 5 mL (1 mg mL^{-1}) sterile water and stored at -20 °C.

4.2.2 Survival of S. avenae and A. pisum when feeding from DON and DON-3G

To determine the effect of DON and DON-3G on the survival of *S. avenae* and *A. pisum*, DON and DON-3G were added to the artificial aphid diet based on formulation A from Prosser and Douglas (Prosser & Douglas, 1992) to a final concentration of 0.5, 1 or 3 and 100 mg L^{-1}. Sterile water was added to the artificial diet as control (0 mg L^{-1} DON or DON-3G). For both aphid species, there were three aphid feeding apparatus prepared as described by Sadeghi, et al. (Sadeghi *et al.*, 2009) for all treatments and control. Each apparatus contained ten randomly picked nymphs who could feed on a parafilm sachet containing 200 µL of the mixture. Over a period of three days the surviving nymphs were counted. Abbott's formula (Abbott, 1925) was used to correct the survival rates: (nTa / nCa) * 100 with nTa the number of survivors after treatment and nCa the number of survivors in the control treatment. Statistical differences ($P < 0.05$) between aphid survival when feeding from different DON concentrations were analyzed by using non-parametric Kruskal Wallis analysis followed by a Dunn's test to perform pairwise comparisons using IBM SPSS (SPSS Statistics 22). These experiments were repeated at least two times.

To determine the long-term DON effect on the survival of *S. avenae* and *A. pisum,* aphids were gradually exposed to increasing concentrations: 0 → 0.5 → 1 → 3→ 5 mg L^{-1}. For both aphid species, there were six aphid feeding apparatus prepared for the treatment and for the control (0 mg L^{-1} DON). Each apparatus contained five neonates produced by adult aphids within 24 h (day 0). In these experiments the diet was changed every two days. Statistical differences ($P < 0.05$) between treatments at different time points in the long-term survival experiment were computed using one-sided t-tests (SPSS Statistics 22).

For non-targeted and targeted LC-MS/MS analyses *S. avenae* and *A. pisum* aphids were taken from the laboratory stock cultures and put in aphid feeding apparatus containing 0 or 100 mg L^{-1} DON. After 40 h the surviving aphids were stored at -20 °C until analysis. For each aphid species there were five repeats of aphids that were fed with 0 and 100 mg L^{-1} DON. Also, the artificial diet after 40 h of feeding of all repeats were analyzed.

4.2.3 Analysis of the ribosomal protein L3

RNA from *S. avenae* and *A. pisum* aphids was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. The extracted RNA was quantified using a Quantus Fluorometer (Promega, Madison, WI, USA). With a GoScript Reverse Transcription System (Promega) first-strand cDNA was synthesized. The PCR reactions were performed in a total reaction volume of 25 µL, consisting of 0.125 µL goTaq DNA polymerase (Promega), 5 µL of 5 x goTaq buffer colorless (Promega), 1.25 µL dNTPs, 1 µL of each primer (5 µM), 14.625 µL nuclease-free water (Promega) and 2 µL of the cDNA. The RPL3 sequence was picked up in two parts (p1 and p2) using following primers (5'-3'): GCACATCCACTTTCGTCAAG (p1_F), CTAGGATGCCATGCTCCAAT (p1_R), ACCAAGGGTCGTGGATACAA (p2_F) and CGCTGTGGCTTTCTCTTCTT (p2_R). PCR analysis was done with a Bio-Rad T100 Thermal Cycler and following thermocycle profile was used: 5 min at 95 °C followed by 35 cycles of 95 °C for 30s, 59.7 °C (p1) or 60 °C (p2) for 20s and 72 °C for 60s. Finally 72 °C continued for 10 min and cooled down until 15 °C. The remaining product was purified using the E.Z.N.A. Cycle-Pure Spin kit (VWR) and send to LGC Genomics for single sample DNA sequencing.

4.2.4 Sample preparation and targeted LC-MS/MS analysis

Individual mycotoxin solid standards (1 mg) of DON, 3-ADON, 15-ADON and DOM-1 (internal standard) were purchased from Sigma-Aldrich NV/SA (Bornem, Belgium). DON-3G (50.2 ng μL^{-1} in acetonitrile) was obtained from Biopure Referenzsubstanzen GmbH (Tulln, Austria). All mycotoxin solid standards were dissolved in methanol (1 mg mL^{-1}), and were stored at -18 °C. Working solutions of DON, 3-ADON, 15-ADON and DOM (10 ng μL^{-1}) were prepared in methanol and stored at -18 °C, while DON-3G (50.2 ng μL^{-1}) was dissolved in acetonitrile and stored at 4 °C. The targeted LC-MS/MS analysis was performed using a Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray interface in positive mode (ESI^+). Following multiple reaction monitoring (MRM)-traces were monitored: DON (297>203.3 ; 249.4), 3-ADON (339.2>231.2 ; 203.1), 15-ADON (339.1>137.1 ; 321.2) and DON-3G (476.1>248.6 ; 296.9). LC-MS/MS parameters are described in detail by De Boevre *et al.* (2012b). MassLynx™ version 4.1. and

QuanLynx® version 4.1. software (Waters, Milford, MA, USA) were used for data acquisition and processing.

Aphid samples were collected, crushed and individually weighed in recipients. According to their weight, 500 ng g^{-1} of DOM internal standard (10 ng μL^{-1}) was added. A matrix-matched calibration curve with a linear range of 0 ng g^{-1} to 1500 ng g^{-1} for DON, 3-ADON, 15-ADON and DON-3G with non-contaminated grain aphids was prepared. The reference standards were allowed to equilibrate for 15 min. An extraction with 1.5 mL acetonitrile/water/acetic acid (79/20/1, v/v/v) was performed, and the samples were vigorously vortexed for 1 min. The sample extract was centrifuged at 4307 g for 1 min, afterwards, the supernatant was collected in a small test tube using a glass Pasteur pipette with a bulb. This process was repeated twice. The organic mycotoxin-mixture was evaporated until dryness under N_2 at 60 °C using the TurboVap® LV (Biotage, Dusseldorf, Germany), and redissolved in 150 µL of injection solvent (50/50 v/v, H_2O/MeOH (95/5, v/v), 0.1% of HCOOH + 10 mM of $HCOONH_4$ [solvent A]; MeOH/H_2O (95/5, v/v), 0.1% of HCOOH + 10 mM of $HCOONH_4$ [solvent B]). Finally, the redissolved sample was vortexed for 3 minutes, collected in an Ultrafree-MC centrifugal device (0.22 µm, Millipore, Bedford, MA, USA) and centrifuged for 10 minutes at 10,000 g.

To confirm the presence of DON, 3-ADON, 15-ADON and DON-3G, two transitions between precursor and fragments were monitored. According to the Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC, 2002), a system of identification points was applied to interpret the data (European Commission, 2002). The first criterion is that the relative retention time, relative to the internal standard DOM-1, should not exceed 2.5%. The second identification point involved that the relative abundance of both transitions should not exceed the range of 20% to 50%, depending on the relative intensity between the transitions. Also, all MRM-transitions should possess a signal-to-noise (s/n) ratio higher than 3:1 (2002/657/EC, 2002).

Statistical differences ($P < 0.05$) between concentrations of DON, DON-3G and total titer retrieved in aphids were analyzed by using One-way ANOVA post-hoc Tukey (SPSS Statistics 22).

4.2.5 Sample preparation and non-targeted LC-MS^E analysis

To an exact amount of the aphid sample (individually checked), 750 µL of extraction solvent acetonitrile/water/acetic acid (79/20/1, v/v/v) was added. Using a glass spatula, the aphids samples were crushed until a homogeneous mass was obtained. The spatula was rinsed with 750 µL of extraction solvent. The organic mixture was vigorously vortexed for 1 minute. Next, the sample was centrifuged at 4307 g for 1 minute. The obtained supernatant was transferred into a small test tube. To extract the maximum amount of mycotoxins, 1.5 mL of extraction solvent was additionally added to the centrifuged residue. The vortex and centrifugation step were repeated, and the remaining supernatant was transferred into the same test tube. The organic mycotoxin-mixture was evaporated until dryness under N_2 at 60 °C using the TurboVap® LV (Biotage, Dusseldorf, Germany). The residue was redissolved with 150 µL of MeOH/CAN/H_2O (30/30/40, v/v/v) and centrifuged in a Ultrafree®-MC centrifugal device (0.22 µm) for 5 minutes at 14,000 g.

DON and its derivatives (DON-3G, 3-ADON, 15-ADON, DON-GSH, DON-diglucosides, DON-triglucosides and DON-tetraglucosides) were investigated using UPLC/QTOF-MS with the MS^E data acquisition strategy. The LC instrument used was an Acquity UPLC^{TM} system (Waters Milford, MA, USA) with a ZORBAX RRHD Eclipse Plus C18 (1.8 µm, 2.1 x 100 mm) from Agilent Technologies (Diegem, Belgium). The mobile phase consisted of H_2O/MeOH (95/5, v/v) containing 0.1% of HCOOH and 10 mM of $HCOONH_4$ [solvent A] and MeOH/H_2O (95/5, v/v) containing 0.1% of HCOOH and 10 mM of $HCOONH_4$ [solvent B]. The following gradient elution program was applied: 0-0.5 min: 0% B, 0.5-20 min: 0-99% B, 20-21 min: 99% B, 21-24 min: 0% B, 24-28 min: 0% B. The flow rate was 0.4 mL min^{-1}. The column temperature was set at 30 °C, and the temperature of the autosampler was 10 °C. Five µL of the sample was injected. Instrument control and data processing were carried out by MassLynx^{TM} version 4.1. software (Waters, Milford, MA, USA). The Q-TOF MS instrument used was a Synapt G2-Si MS system (Waters, Milford, MA, USA). The data acquisition mode was TOF MS^E in ESI$^+$ mode. The data acquisition range was from 50 Da to 1200 Da with a 0.1 s scan time. The MS source temperature was set at 150 °C, and the desolvation temperature was set at 500 °C with a desolvation gas flow set at 800 L h^{-1} and a cone gas flow at 100 L h^{-1}. The capillary voltage was 2.8 kV and the sampling cone voltage was 30 V. The collision energy was set as 45 eV - 60 eV ramp (trap) for the high-energy scan. Data was collected in

continuum mode and the mass was corrected to ensure accuracy during the MS analysis after acquisition using leucine enkephaline (200 pg μL^{-1}) at a flow rate of 100 $\mu L\ min^{-1}$ as lock mass compound. HRMS data were processed using MassLynx^{TM} and compounds were identified after applying lockspray correction, extracting the chromatogram and generating the molecular formula from the exact mass.

4.3 Results

4.3.1 Survival of S. avenae and A. pisum upon exposure to DON

S. avenae and *A. pisum* aphids were fed for 3 days on a diet containing different concentrations of DON (0, 0.5, 1, and 3 mg L^{-1}) using an aphid feeding apparatus. The survival of *S. avenae* aphids was not affected by DON up to concentrations of 3 mg L^{-1} compared to the control, while survival of *A. pisum* aphids was significantly reduced. The lowest concentration of 0.5 mg L^{-1} DON significantly reduced the survival rate of *A. pisum* (Table 4.1 part 1). As we wanted to assess the tolerance of *S. avenae* more in detail, we exposed both aphid species to a concentration of 100 mg L^{-1} DON for 3 days. Remarkably 43% ± 8 of the *S. avenae* aphids survived this dose while for the *A. pisum* aphids survival rates dropped to 4% ± 2.

Table 4.1 Percentage survival of *S. avenae* aphids and *A. pisum* aphids feeding from diet containing different concentrations of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3G). Different letters indicate significant differences ($P < 0.05$) between treatments using a two-sided non-parametric Kruskall wallis test followed by a Dunn's for pairwise comparisons. Standard errors are indicated in the tables.

		Sitobion avenae	***Acyrthosiphon pisum***
control		97 ± 3 a	100 ± 0 a
DON	0.5 mg L^{-1}	93 ± 3 a	62 ± 6 bc
	1 mg L^{-1}	93 ± 2 a	48 ± 9 c
	3 mg L^{-1}	93 ± 4 a	50 ± 11 c
DON-3G	0.5 mg L^{-1}	88 ± 6 a	88 ± 5 ab
	1 mg L^{-1}	89 ± 5 a	96 ± 2 ab
	3 mg L^{-1}	81 ± 7 a	88 ± 6 ab

4.3.2 Involvement of RPL3 in the tolerance of S. avenae to DON

Previous research has reported on increased tolerance to DON by amino acid modifications in the RPL3 protein which is the target of DON. The nucleotide sequence of the gene encoding for RPL3 of *S. avenae* and *A. pisum* was sequenced. After converting the nucleotide sequence to amino acids, no differences were found between the RPL3 sequence of *S. avenae* when aligned with 60S RPL3 of *A. pisum* (NCBI Reference Sequence: XP_001951042.1). The typical amino acid changes observed in *Saccharomyces cerevisiae* which were associated with DON tolerance were not reported in any of the aphid species. From experiments with transformed plants, it is known that an eventual modified DON insensitive RPL3 protein can be present heterozygously. In this case, the insensitive RPL3 protein is not used by the translation machinery in the presence of the native RPL3 protein due to a lower affinity of the mutant RPL3 for the ribosome assembly factor Rrb1p. In this scenario, the mutant RPL3 protein only accumulates when organisms are gradually exposed to DON which allows the mutant RPL3 to push out the native RPL3 protein (Mitterbauer *et al.*, 2004). In order to investigate whether a similar adaptation mechanism was present in *A. pisum* aphids, they were exposed to an increasing concentration of DON in a time-lapse experiment. However, feeding *A. pisum* aphids with increasing concentrations of DON during a longer period of time did not result in an increased survival (Fig. 4.2). As expected, the survival of *S. avenae* was not negatively influenced by increasing DON concentrations.

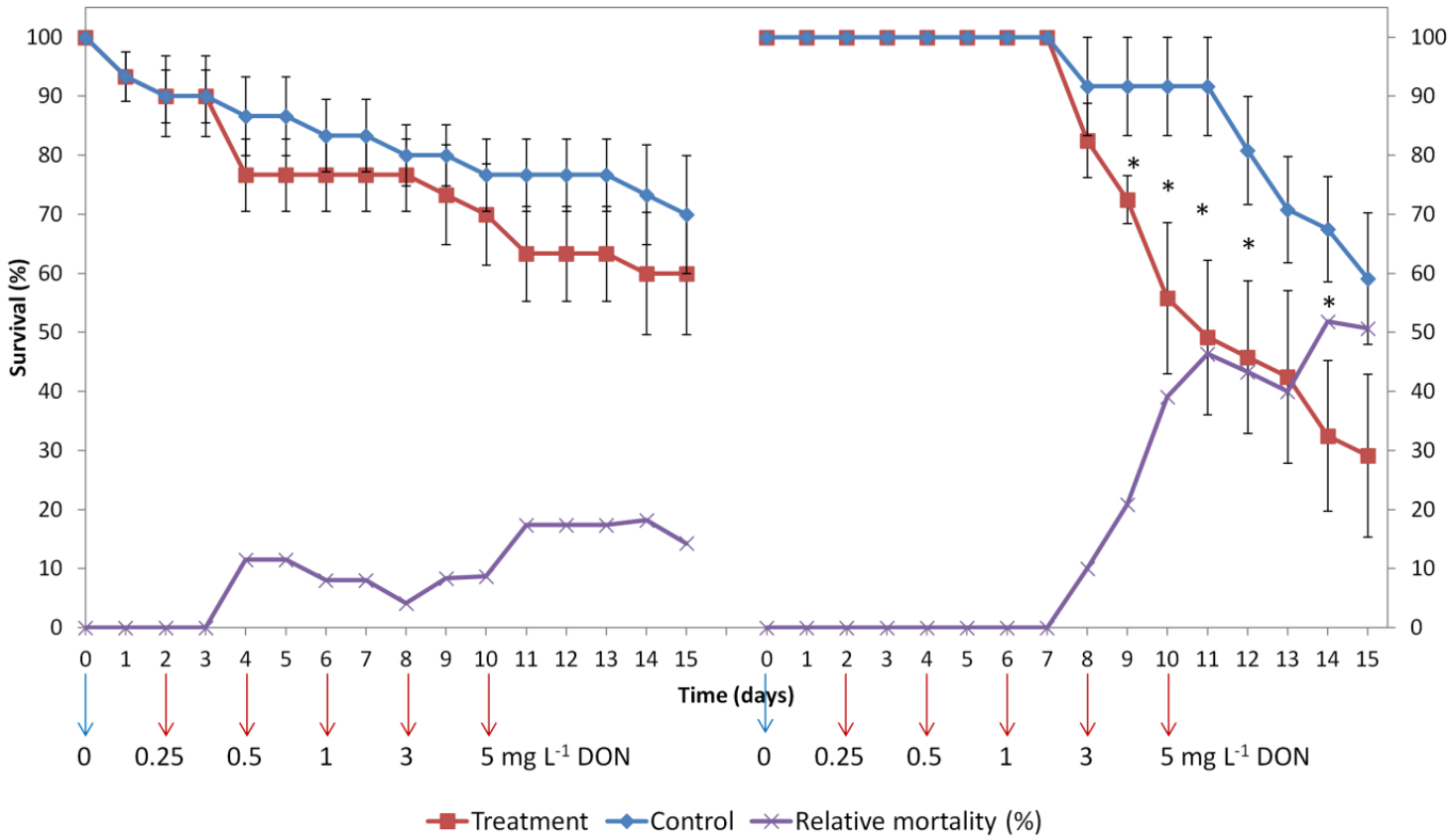

Fig. 4.2 Long-term survival of *S. avenae* aphids (left) and *A. pisum* aphids (right) (means ± SE) feeding from diet containing concentrations of deoxynivalenol (DON) that increased every two days (from 0, 0.25, 0.5, 1, 3 up to 5 mg L^{-1}). Significant differences ($P < 0.05$) between treatment and control using one-sided t-tests are depicted with an asterisk. The purple line indicates the mortality of the aphids relative to the surviving fraction at each time point.

4.3.3 Conversion of DON to DON-3G in aphids results in a detoxification

In order to get an insight into the ability of the aphids to detoxify DON, aphids fed on 100 mg L^{-1} of DON in an aphid feeding apparatus. In the control aphids which fed on artificial diet only, no DON or DON derivatives were detected. In the artificial diet amended with 100 mg L^{-1} DON, we found 99.37 ± 0.83 mg L^{-1} DON illustrating that DON was not chemically degraded in the feeding apparatus during the course of the experiment. Using a targeted liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) approach, aphids which were exposed to DON were analyzed for DON (limit of detection (LOD), 45 µg kg^{-1}; limit of quantification (LOQ), 89 µg kg^{-1}), DON-3G (LOD, 34 µg kg^{-1}; LOQ, 67 µg kg^{-1}), 3- and 15-acetyldeoxynivalenol (3-ADON: LOD, 47 µg kg^{-1}; LOQ, 94 µg kg^{-1}) and 15-ADON (LOD, 33 µg kg^{-1}; LOQ, 67 µg kg^{-1}).

Remarkably, DON-3G, a type II-conjugate of DON that is normally solely reported in plant detoxification pathways, was detected. Moreover, there was a clear difference in the metabolization efficiency between both aphid species. The *S. avenae* aphids which were shown in Table 4.1 to be tolerant to DON and efficiently converted DON to DON-3G, whereas in *A. pisum* only 55% of the total DON titer consisted of DON-3G (Fig. 4.3). In view of these results, the toxicity of DON-3G in aphids was assessed by dietary exposing both *S. avenae* and *A. pisum* to DON-3G concentrations of 0.5 mg L^{-1}, 1 mg L^{-1}, and 3 mg L^{-1}. These experiments clearly demonstrate that DON-3G was no longer toxic for either of both aphid species (Table 4.1 part 2).

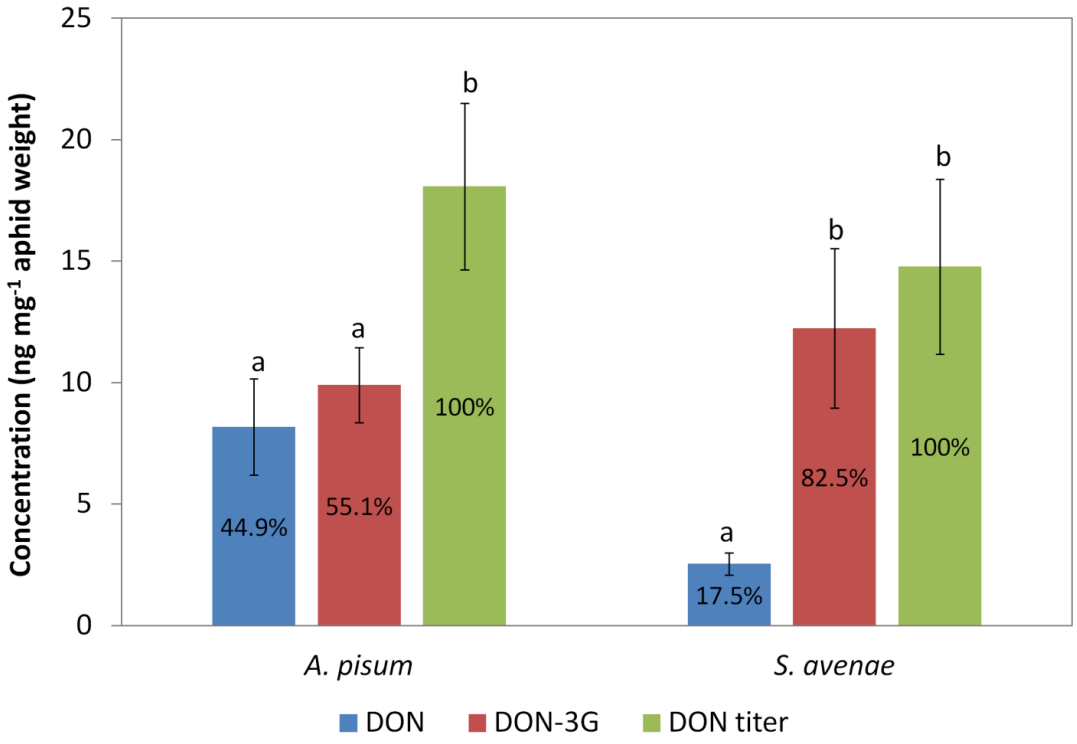

Fig. 4.3 Concentrations of deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3G) and total titer (means ± SD, ng mg^{-1} aphid weight) retrieved in *S. avenae* and *A. pisum* aphids after two days feeding from artificial diet amended with 100 mg L^{-1} DON. Different letters indicate significant differences ($P < 0.05$) between treatments using One-way ANOVA posthoc Tukey.

4.3.4 DON-3G can be further metabolized: diglucosides of DON

Using a non-targeted high resolution HR-MS^E approach in the two aphid species fed with DON (100 mg L^{-1}), we were able to pick-up DON-diglucosides, for which unfortunately reference standards are not available. Other possible conjugates such as DON-GSH which are known to be produced in other organisms as type II biotransformation products were investigated, however, we were not able to detect DON-GSH in any of the aphid samples. The DON-fed aphid samples were analyzed and discrepancies between aphid species were checked. Three peaks at retention times of 3.98 min, 4.25 min and 4.42 min were observed, corresponding to different structural isomers of DON-diglucoside (Fig. 4.4). For these specific retention times, the measured and theoretical masses were investigated via software-analysis. The obtained molecular formula was $C_{27}H_{40}O_{16}Na^+$ with a mass of 643.2214

(theoretical) and 643.2202 (measured), resulting in a mass error of -1.9 ppm. The chemical structure of this diglucoside is proposed in Fig. 4.5. In addition, in view of the presence of DON-3G, we hypothesize here that the presence of the diglucoside form results from a sequential process in which the conversion of DON to DON-3G is the primary step. In order to understand the insertion of the glucose molecules of the three structural isomers, a study via nuclear magnetic resonance (NMR) is interesting but limited by the extremely small amounts that can be isolated from aphid samples.

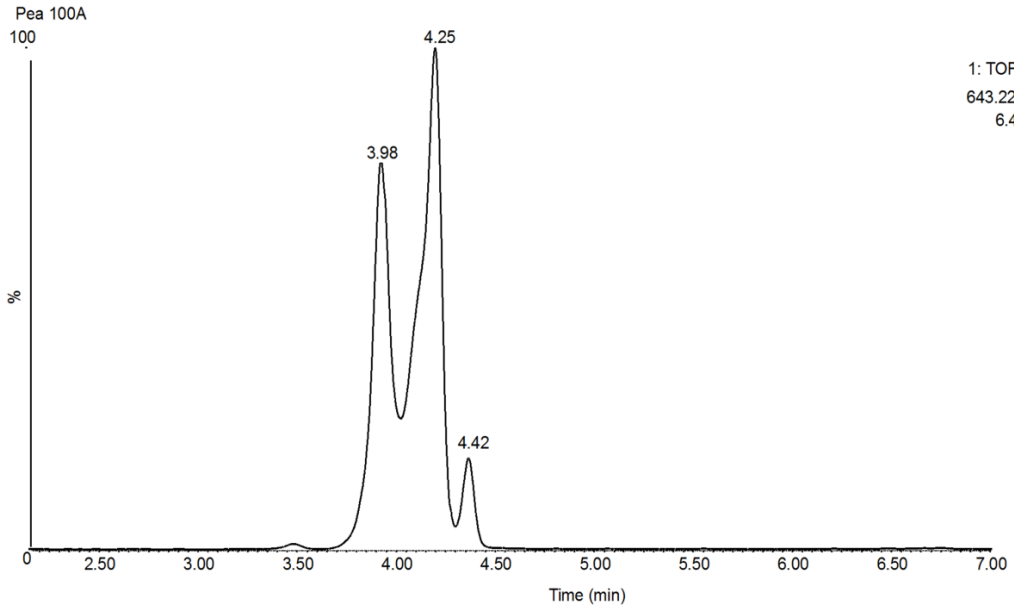

Fig. 4.4 The isomeric pattern of deoxynivalenol-diglucosides in pea aphid sample one of five (A) exposed to 100 mg L^{-1} deoxynivalenol (*pea 100 A*).

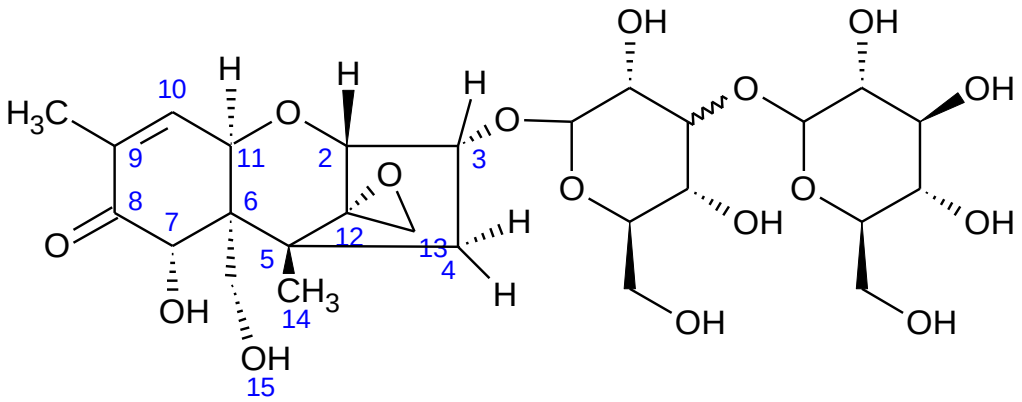

Fig 4.5 Proposed chemical structure of deoxynivalenol-diglucoside.

4.4 Discussion

The trichothecene DON is a sesquiterpenoid mycotoxin produced by several *Fusarium* species and is toxic for most eukaryotic cells. In the present study, we assessed the toxicity of DON for two aphid species: the English grain aphid *S. avenae* and the pea aphid *A. pisum*. Dietary exposure of both aphids to DON showed that *S. avenae* were tolerant to DON compared to *A. pisum*. To explain this unique difference in toxicity of DON between the two aphid species three hypotheses were verified.

4.4.1 RPL3 is not involved in the tolerance of S. avenae to DON

Firstly, we examined the amino acid sequence of the gene encoding RPL3, the target molecule of DON, in both aphid species. Trichodermin and other sesquiterpenoids of the same group are known inhibitors of the peptidyltransferase center of eukaryotic ribosomes, and thereby block protein synthesis (Carrasco *et al.*, 1973, Barbacid & Vazquez, 1974). Research showed that RPL3 plays an essential role in the formation of this peptidyltransferase center (Fried & Warner, 1981, Schulze & Nierhaus, 1982, Meskauskas & Dinman, 2007). One of the resistance mechanisms to DON identified in yeast is the modification of this ribosomal target by amino acid changes in RPL3 (Mitterbauer & Adam, 2002). Mitterbauer *et al.* (2004) used yeast as a model system to identify several mutations in the gene encoding RPL3 (*e.g.* W255C, a change of tryptophan into cysteine at position 255), which confer resistance to trichothecenes, in particular to DON. However amino acid sequence of *S. avenae*'s RPL3 showed none of these mutations. In addition, no functional aberrations were observed between the amino acid sequence of RPL3 from *S. avenae* and the predicted RPL3 from *A. pisum*. Amino acids at places 190 and 382 are valine (V) for *A. pisum*, but isoleucine (I) for *S. avenae*, however, when comparing the chemical structure of these two amino acids we assume this cannot explain the better survival of *S. avenae* in presence of DON compared to *A. pisum*. Not the whole nucleotide sequence of *S. avenae*'s gene encoding for RPL3 was picked up, leaving seven amino acids undetermined at the end. As our sequencing data of the RPL3 of *S. avenae* did not show the mutations as reported to be reason for tolerance in yeast, we believe that we can conclude that the target of DON, the gene encoding RPL3, is not the reason of the tolerance in grain aphids.

Secondly, we investigated the hypothesis of Mitterbauer *et al.* (2004) stating that organisms might be heterozygous for the RPL3 locus. In this hypothesis, native ribosomes originating from one allele could be preferentially dismantled and degraded *in vivo* upon DON exposure, so that in turn the remaining fraction of resistant ribosomes on the second allele could allow the synthesis of new ribosomal proteins, eventually leading to a higher steady-state level of modified RPL3 protein in ribosomes. This hypothesis was validated by these researchers (Mitterbauer & Adam, 2002) via integrating an engineered tomato RPL3 containing mutations of yeast RPL3 in tobacco which resulted in an adaptation but not in a constitutive tolerance against DON pointing to the semi-dominant nature of this tolerance. The aberrant RPL3 protein (rendering tolerance to DON) was not utilized when wild-type RPL3 protein was present, unless the yeast transformants or the transgenic tobacco plants were challenged with sub-lethal amounts of DON. Indeed, after toxin treatment in a dose-dependent manner, they noticed an accumulation of the modified protein due to the selection pressure in the presence of DON (Mitterbauer *et al.*, 2004). We investigated this hypothesis also with our two aphid species in an experimental setup where we fed the aphids increasing DON-concentrations over a longer period of 2 weeks. However, we did not detect any augmented tolerance especially not in the *A. pisum* aphids when gradually exposed to increasing DON doses.

4.4.2 Aphids convert DON into the less toxic DON-3G

Finally, we investigated whether DON was subject to a type II biotransformation process in aphids. Remarkably, we were able to demonstrate the presence of DON-3G in both aphid species. Moreover, the tolerant *S. avenae* species converted DON to DON-3G more efficiently than the susceptible *A. pisum* species. To our knowledge, this is the first time that the conversion of DON to DON-3G is reported in animal species. To date, DON glucosylation has solely been reported in plant cells. In plants, a vast number of genes that code for putative UDP-glycosyltransferases (UGTs) has been revealed (Berthiller *et al.*, 2013). In barley and *Brachypodium distachyon,* genes from the UGT family with potential relevance for DON tolerance have functionally been characterized (Schweiger *et al.*, 2010, Schweiger *et al.*, 2013). Although DON glucosylation has never been reported in animals, genes encoding

for UGTs are known to be present in insects. They catalyze the conjugation of a range of diverse small lipophilic compounds with polar compounds (*i.e.* carbohydrates) to produce glucosides, and as such they play an important role in type II detoxification processes of xenobiotics in insects (Ahn *et al.*, 2012). However, the presence of these UGTs have never been linked with mycotoxin glucosylation.

Although we provide valuable evidence for a role of glucosylation in DON detoxification in aphids, several other detoxification enzymes have been described in aphids for coping with xenobiotics (*e.g.* secondary compounds of the plant or insecticides); examples are cytochrome P450 mono-oxygenases (P450s), glutathion-*S*-transferases, esterases and oxidoreductases (Figueroa *et al.*, 1999, Cai *et al.*, 2009, Castaneda *et al.*, 2009, Lu & Gao, 2009, Castaneda *et al.*, 2010, Zhang *et al.*, 2013). Some of these enzymes are known to be involved in the detoxification of mycotoxins (Gardiner, SA *et al.*, 2010). With the use of piperonyl butoxide (PBO), a known P450 inhibitor, it was evidenced that P450s (phase I detoxification enzymes) were involved in bioactivation of aflatoxin B1 (AFL B1) produced by *Aspergillus* spp. by corn earworms *Helicoverpa zea* (Zeng *et al.*, 2006). In contrast, P450s in honeybees (*Apis mellifera*) are able to detoxify ALF B1 (Niu *et al.*, 2011). In some animal species P450s are responsible for bioactivation of these compounds, catalyzing the epoxidation of the terminal furan ring of AFL B1 resulting a highly genotoxic metabolite. Its toxicity originates from its ability to bind to DNA, RNA and proteins (Lequesne, 1983, Iyer *et al.*, 1994, McLean & Dutton, 1995, Wild & Turner, 2002). In others, P450s metabolize AFL B1 to hydroxylated metabolites, including AFL M1 and AFL Q1, that have lower genotoxic or toxic activities than AFL B1 (Eaton *et al.*, 1988, Ramsdell & Eaton, 1990). Also conjugation of ALF B1 to glutathione (mediated by glutathione S-transferase) is regarded as an important detoxification pathway in animals. Resistance to AFL B1 toxicity has been interpreted in terms of levels and activities of these detoxifying pathways (McLean & Dutton, 1995).

To elaborate, a possible involvement of multiple detoxification mechanisms in the aphids of this study cannot be excluded. It is possible that other degradation products of DON were present in the aphid bodies, but that were not (yet) discovered.

4.4.3 Formation of DON-diglucosides from DON-3G

Using a non-targeted HR-MS^E approach, we were able to detect DON-diglucosides (via HR-MS^E) in both *S. avenae* and *A. pisum* aphids. To date, the only report on the presence of DON-diglucosides was in beer; oligoglucosylated DONs with up to four bound hexose units were present (Zachariasova *et al.*, 2012). Remarkably, although detoxification of DON through conjugation with glutathione (DON-GSH) has been observed in plants (Gardiner, SA *et al.*, 2010, Kluger *et al.*, 2013) and in many animal species (Wen *et al.*, 2016) no glutathione derivatives of DON were observed during the HR-MS^E analyses of our aphid samples.

4.4.4 DON detoxification as a possible result from co-evolution

The question remains why *S. avenae* is able to convert DON to DON-3G more efficiently than *A. pisum*. Insights might come from the knowledge that *S. avenae* occurs on cereal ears which are often colonized by *Fusarium* spp. producing DON while *A. pisum* occurs on plant species that are no hosts for DON producing *Fusarium* spp. It is remarkable that *A. pisum* although it disposes of a very large arsenal of UGTs compared to other insects (Ahn *et al.*, 2012), does not convert DON efficiently to DON-3G which points to the substrate specificity of these enzymes. Consequently, we might speculate on adaption by co-evolution in *S. avenae*. It has been reported before that insects are capable to develop tolerance when exposed to a toxin over many generations. *Drosophila melanogaster* larvae which were exposed to *Aspergillus nidulans* over 26 generations displayed higher survival rates in the presence of *A. nidulans* and a higher tolerance to the mycotoxin sterigmatocytin (*i.e.* an aflatoxin precursor) compared to control lines (Trienens & Rohlfs, 2011).

Finally, it is tempting to argue on the origin of the glucosyltransferase in aphids. Although we do not provide firm evidence in the present study, one of the possibilities of acquiring this specific glucosyltransferase is through horizontal gene transfer. Indeed this idea is realistic as the gene encoding for the enzyme to detoxify the toxic hydrogen cyanide that is a plant defense toxin, has also been horizontal transferred so that plant feeding mites can survive (Wybouw *et al.*, 2014). This might explain why it has never been encountered in higher animals. We believe our study increases the awareness of the importance of laterally transferred genes in the genomes of higher organisms.

Contributions:

(Non)targeted analyses were done by Department of Bioanalysis, Laboratory of Food Analysis

Chapter 5:

Discussion and future perspectives

5.1 Introduction to the tripartite pathosystem

When pathogens and herbivores inhabit the same niche on a plant they can interact. These interactions are called tripartite as they involve three parties. In this thesis we focused on the grain aphid *Sitobion avenae* and the pathogen *Fusarium graminearum*, who both live on the ears of wheat *Triticum aestivum*. *F. graminearum* is a well-known toxigenic fungus which produces the mycotoxin deoxynivalenol (DON) as a virulence factor during its infection of the wheat ear (Bai *et al.*, 2002, Langevin *et al.*, 2004, Jansen *et al.*, 2005). *S. avenae* are aphids that feed from the phloem sap in the ears (Kehr, 2006).

5.2 Aphids smooth the path for *F. graminearum*

Cereal aphids infest wheat plants soon after emergence. *S. avenae* are known to be ear-feeders and rapidly move from the leaves to the ears when ears emerge (Wratten, 1975, Watt, 1979). At this moment they feed from the phloem sap of the ears and initiate plant responses. In order to establish a long-term interaction with the plant, the aphids must avoid or even suppress this response (Tjallingii, 2006). In **chapter 2**, we studied the response in wheat ears after an attack by aphids during a time period of twelve days using quantitative real time polymerase chain reaction (RT-qPCR) analysis (De Zutter *et al.*, 2016a). We demonstrated that genes involved in early steps of defense gene activation were not upregulated. Indeed, it is suggested that aphids are able to suppress or avoid activation of potentially effective plant defensive machinery to allow optimal feeding from the plants phloem sap (Zhu-Salzman *et al.*, 2004, Walling, 2008, Elzinga *et al.*, 2014). Several other genes (*e.g.* pathogenesis-related (PR) genes) were induced upon aphid attack, but only for a short period of time. At the moment of anthesis, *F. graminearum* infects the ears of wheat as well. From this moment on, both the grain aphids and the pathogen live together on wheat ears. Infection with solely *F. graminearum* showed a typical sensitive plant response. Remarkably, genes that were upregulated during aphid attack, showed clear overlap with genes typically induced during successful infection with *F. graminearum*. It is known that insects are able to deceive the plant (triggering non-effective defenses) in order to enhance their success on host plants. The defense genes in ears that were infected with aphids and *F.*

graminearum together, showed earlier and/or enhanced transcription after exposure to both aphids and *Fusarium* compared to a sole *Fusarium* infection. We cannot directly link these enhanced expressions to the aphids but it seemed that if plant genes were already upregulated previously (because the plants were deceived by the aphids), they were able to react faster/better to a subsequent *Fusarium* attack.

It must be highlighted that we only tested a small number of defense genes that are considered hallmarks of defense responses in wheat. This gives us a general though limited view on the plant response in wheat ears. A whole genome approach using a micro-array or RNAseq analysis to measure expression levels of large numbers of genes simultaneously might be a valuable more holistic approach.

Moreover, it is important to highlight that in this chapter, solely gene expression was considered. There are many posttranscriptional processes that might affect the outcome of changes in gene expression. Measurement of enzymatic activity or monitoring accumulation of end products are additional techniques that can be included in future work. We can exemplify this by the observed induced expression of NADPH oxidase genes. This result suggests that H_2O_2 is formed and thus might play a role in this interaction. However, there are many enzyme activities involved in H_2O_2 formation such as NADPH oxidases, superoxide dismutases, catalases and peroxidases all influencing the resulting H_2O_2 concentration. Moreover, the end product itself H_2O_2 might also be monitored using NBT (nitro blue tetrazolium) or DAB (diaminobenzidine) staining procedures.

Finally, it is known that both insects and pathogens influence the primary metabolism of plants. This has been demonstrated for *F. graminearum* (Audenaert et al., 2014) but also for aphids. These changes in primary metabolism upon pathogen attack have been elaborated for the N-metabolism in several model systems (Seifi *et al.*, 2013). For *F. graminearum* it has been shown that upon infection, arginine and polyamines which are in turn triggers for DON biosynthesis pointing to the fact that the pathogen hijacks the plants primary metabolism to its benefit. Also in the interaction of plants with aphids, the amino acid (AA) metabolism has been shown to be involved. Changes in the plant's AA composition can alter the fitness of *S. avenae*. Changes to AA composition of plants have already been suggested as a mechanism behind the altered fitness of *Euceraphis betulae* aphids on birch leaves infected with *Marssonina betulae* (Johnson *et al.*, 2003) and *Aphis fabae* aphids when feeding on bean plants infected with *Botrytis fabae* (Zebitz & Kehlenbeck, 1991). Further

studies should investigate the aphid's honeydew to get a clue about the host plant AA composition (Leroy *et al.*, 2011).

5.3 The induced plant defense response by aphids triggers the toxic secondary metabolism in *F. graminearum*

In present study we could confirm findings of Drakulic *et al.* (2015) demonstrating more symptoms and increased production of DON by the fungus when ears were predisposed to aphids. However, we also found higher concentrations of DON derivatives, namely DON, 3-acetyl deoxynivalenol (3-ADON) and 15-acetyl deoxynivalenol (15-ADON). In search for an explanation of these increased DON levels, which could not be attributed to a higher fungal biomass in ears exposed to both *Fusarium* and aphids, we hypothesize that NADPHOX induction results in the biosynthesis of H_2O_2. H_2O_2 is known to induce DON production once the fungus becomes necrotrophic (Ponts *et al.*, 2006, Audenaert *et al.*, 2010, Audenaert *et al.*, 2014). This could explain the higher DON concentrations demonstrated in ears containing both aphids and *F. graminearum* compared to ears without aphids. Higher contents of DON in wheat ears can lead to serious problems regarding human and animal health. It is advisable for farmers to intervene timely when aphids are being spotted before the crucial flowering period. Moreover, 3-ADON and 15-ADON are two metabolites of particular importance as they augment the total DON titer in the plant and as they also display toxic effects to eukaryotic cells. From a legislative point of view, they are of particular interest as there are no maximum threshold concentrations yet but the European commission is gathering data on an eventual extension of the DON-legislation with these DON derivatives.

5.4 Grain aphids are adapted to living in proximity of DON producing fungi

As we observed high concentrations of DON in ears co-infected with aphids and *F. graminearum,* we contemplated on an eventual detrimental effect of DON on *S. avenae* who are feeding from the phloem sap in the *Fusarium*-infected ears. In our experiments, we

noticed that after 6 and 12 days of co-inhabiting the ears together with *F. graminearum*, aphid populations dropped. This could have two reasons: first, DON is toxic for the aphids (see **chapter 3**) and when the disease worsens the concentrations of DON in the phloem rise, and second, when *F. graminearum* colonizes the ear, the ear bleaches and sap streams shut down, leaving the aphids without food. Because we demonstrated in **chapter 4** that grain aphids are able to efficiently (approximately 80%) convert DON into the less toxic DON-3-glucoside (DON-3G) we think that toxicity of DON is not the limiting factor for aphid growth. In this context we must mention that a population of aphids was tested for DON derivatives inside their bodies, containing adults and nymphs. This means that we don't know if aphids in different developmental stages have different converting efficiencies. We therefore believe that the second hypothesis (no sap stream and thus starvation) is an explanation for the decreasing aphid population at later time points. However, it is obvious that under natural conditions (in the field) the aphids will get winged and move away from the diseased ears towards healthier ears. At this time point they will not act as a vector of fungal particles because experiments in the beginning of the whole research demonstrated that the aphid's stylet is too narrow for internal uptake and their body to smooth for external uptake of particles (mentioned in 'thesis outline and research hypotheses'). When moving towards other ears, we believe they show no preference for *F. graminearum* infected ears (De Zutter, unpublished data). In contrast, other studies evidenced attraction or repellence of cereal insects (*e.g.* cereal leaf beetles *Oulema* spp.) to volatiles emitted by *Fusarium* infected cereals (Piesik *et al.*, 2011a, Piesik *et al.*, 2013). Drakulic *et al.* (2015) showed that 2-pentadecanone produced by ears containing *F. graminearum* symptoms were repellent for *S. avenae*. As we didn't see such a repellency in our study, we suggest that this volatile is only produced after symptom development. Our ears were not yet symptomatic when exposed to the aphids. This could mean that 2-pentadecanone is not a reliable component for early detection of *F. graminearum* infection. Probably, symptomless ears or ears with beginning symptoms do not yet trigger the aphid's preference behavior by avoiding poor quality hosts. Under field conditions, no significant differences were found in choice behavior of the aphids. This implicates that *S. avenae* was not attracted nor repelled by the treatments. Their response was 'neutral'. Probably, in our experiment external factors like wind could dilute the volatiles produced by ears with beginning disease symptoms, and thereby negate the aphid's response. Further studies should elucidate the volatile blend

emitted by symptomatic ears and response by *S. avenae* aphids in a time-dependent manner. Moreover, because pathogen-induced plant volatiles can affect the behavior of herbivorous insects and discourage infestation, it would be interesting to explore this mechanism as a potential aphid control or prevention strategy in agriculture. After landing on a potential host, aphids probe the plant several times before deciding to accept or reject it. As severely *Fusarium*-infected ears loose phloem sap stream, these ears are no longer a favorable site to linger (we noticed decreased aphid populations on totally diseased ears in **chapter 2**) and aphids scatter towards better and less diseased ears.

To reveal the effect of DON on *S. avenae* aphids, we used aphid feeding apparatus to expose the aphids to different concentrations of DON (from 0 to 5 mg L^{-1}). Concentrations found in wheat samples from fields all over Flanders (Belgium) fluctuate around 0.1-10 mg kg^{-1} DON (Audenaert *et al.*, 2009, Isebaert *et al.*, 2009, Landschoot *et al.*, 2013). These concentrations are present in grains at harvest and thus do not necessarily represent nor concentrations in an infected ear nor concentrations in the phloem during the time period that aphids are feeding from the phloem sieve tubes. It is known that on the level of infected spikelets, DON concentrations can mount to 50–100 mg kg^{-1}. It is not known which DON concentrations are present in the phloem during the moment that aphids are present on ears. However, it is tempting to calculate this based on some assumptions. If an aphid is feeding from a wheat spikelet (approximately 100 mg) containing 10 mg kg^{-1} DON, the aphid is exposed to 1 µg DON. In comparison, when the aphid is feeding from 200 µL artificial diet containing 5 mg L^{-1} DON, it is also exposed to that same amount of DON (1 µg). When feeding the aphids (starting from neonates) with the different DON concentrations we noticed that there was a detrimental effect on their survival, on their nymphal development and their reproduction. Indeed, in **chapter 2** we demonstrated that aphid populations dropped on ears infected with *F. graminearum*. Further aims should delve into the determination of DON concentrations in the phloem of wheat ears during *Fusarium* colonization. This will reveal the (accumulating) concentration of DON to which aphids are exposed during feeding. Kang and Buchenauer (1999) already demonstrated that toxins can be translocated upwards through the xylem vessels and phloem sieve tubes, and downwards through the phloem sieve tubes. Aphids are known phloem-feeders. Concentrations of DON in the phloem could be determined by using stylectomy (*i.e.* cutting of the stylet of the phloem feeding insect and collecting the exudates (Gaupels *et al.*, 2008)). To elaborate on

the limitation that in our experiments DON concentrations were checked inside aphids that were feeding from artificial diet with a known DON concentration (100 mg L^{-1}) (**chapter 4**), further studies should analyze the aphid's stylet when feeding from *Fusarium*-infected wheat ears, over a long period of time (from the moment of *Fusarium* infection until phloem sap stream is stalled). In addition, in future experiments honeydew from aphids feeding on DON (in artificial diet and on infected plants) can be collected although this approach will give only a limited view on DON concentrations in the phloem of infected ears because it was proven that aphids can convert DON into other derivatives (**chapter 4**). A similar approach was pursued in order to analyze sugars in the phloem: although the sugar composition of honeydew reflects the composition of the phloem sap, a number of other mono-, di-, and oligo-saccharides are also synthesized by the sap feeder (through the action of gut enzymes on plant derived sucrose) (Hendrix *et al.*, 1992, Wackers, 2000, Sabri *et al.*, 2013).

5.5 Effect of DON on a hypertrophic level: one insect's breath is another insect's death

The detrimental effect of a long-term exposure to DON led us wonder what the effect would be on parasitoids living inside the aphid bodies. The experiment in **chapter 3** is pioneering work. No other studies ever tried to elucidate the direct effect of mycotoxins on the survival of parasitoids inside the host. Other studies did examine the effect of a fungus-infected plant on parasitoids (Cardoza *et al.*, 2003, Harri *et al.*, 2008, van Nouhuys & Laine, 2008, Bultman *et al.*, 2009) but could only draw indirect conclusions. For more direct evidence, we studied the effect of DON on successful parasitism of *S. avenae* aphids by the parasitoid *Aphidius ervi* (**chapter 3**). Survival of *A. ervi* wasps inside DON-contaminated aphids was deteriorated even though the females did not discriminate between DON-contaminated and uncontaminated aphids (De Zutter *et al.*, 2016b).

Aphids feeding from 3 mg L^{-1} DON were less likely to be successfully parasitized. Although their population size already dropped because of the negative effects of DON, the remaining aphids (tolerant to DON) would have higher surviving chances because there were also less likely to be successfully parasitized (detrimental effects of DON on parasitoid

developing inside DON-contaminated aphid). At this point they would now have a dual advantage (tolerance to DON + less parasitism by *A. ervi*) which they could possibly pass on to their next generations, especially aphids that are reproducing parthenogenetically (producing clones of themselves). Therefore, **chapter 3** emphasizes the importance of mycotoxins in food chain contamination from the plant to insects and their natural enemies. Until today information on this issue remains scarce.

Because we didn't know what happened with DON inside the aphid bodies, and thus we didn't know to which DON concentration or DON metabolites the parasitoid was exposed we performed targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) and non-targeted LC-MS^{E} analysis to detect DON derivatives inside the aphid's bodies (**chapter 4**). This revealed that *S. avenae* aphids could convert DON into DON-3G. Thus, when the parasitoid developed inside the aphid's body, they came in contact with DON as well as DON-3G. Although we demonstrated in **chapter 4** that DON-3G was indeed less toxic to *S. avenae* aphids than DON itself, but we do not know if this is also the case for *A. ervi*.

In a higher trophic level, natural enemies (predators and parasitoids) feeding on or developing in contaminated herbivores can be directly exposed to secondary (fungal) metabolites or indirectly be affected by the host's reduced growth. In contrast to predation, parasitoids kill their host only after the larval development is completed. During this interaction, the host's immune system may prevent successful parasitism by encapsulating and killing the parasitoid eggs or larvae. This immune response can be reduced by the plant's quality (Gols, 2014). For example, *Pieris rapae* hosts developing on cabbage (*Brassica oleracea*) plants with less secondary metabolites were able to encapsulate more parasitoids eggs compared to hosts on plants with high levels of secondary metabolites (Bukovinszky *et al.*, 2009). In contrast, De Zutter *et al.* (2016b) showed that higher concentrations of the mycotoxin DON could have a negative impact on the endoparasitoid *Aphidius ervi* developing inside DON-contaminated *S. avenae* aphids through food chain contamination (see chapter 3). These studies demonstrate that secondary metabolites (no matter the origin) can affect the immune response of herbivores to parasitism.

5.6 Aphids convert DON via a plant-type of detoxification mechanism

In **chapter 4**, we delved into the possible tolerance mechanisms that aphids can have against mycotoxins. More in particular, we reported on a unique observation that *S. avenae*, being an important insect pest of wheat, could tolerate higher DON concentrations than pea aphids *Acyrthosiphum pisum*, known to feed on legumes worldwide and under natural conditions never encounter DON.

The effects of DON seemed to more detrimental to *S. avenae* aphids in the experiments in **chapter 3** compared to the results in **chapter 4**. This can be explained by the experimental setup. In chapter 3 the aphids were exposed to DON from the moment they were born (neonates). In chapter 4 nymphs of different stages were exposed to DON and in long-term experiments they became adults. It is tempting to assume that the survival of DON is depending on the aphid's age.

Also in **chapter 4**, assessing the aphid's tolerance mechanisms, we reported that *S. avenae* were more efficient in converting DON into its glucosylated form, DON-3G than *A. pisum*. This is a phase II biotransformation implying a covalent binding of more polar endogenous compounds with the mycotoxin. We described for the first time in the animal kingdom that plant-pathogenic aphids are able to convert DON into DON-3G. We could retrieve DON-3G by the use of targeted LC-MS/MS. Unfortunately, there are only standards available for some DON metabolites but not for all (*e.g.* not for DON-glutathione (DON-GSH)) and this limits the study. As a result, we also conducted non-targeted analysis that detected DON-diglucosides in the insects, probably as a result of a sequential multiple glucosylation reaction. Earlier analyses of aphids fed with 1 and 10 mg L^{-1} DON did not detect DON. Probably the DON concentrations were lower than the limit of detection. Therefore, we performed the experiment again with the unusual high concentration of 100 mg L^{-1} DON, since it was the purpose to investigate if the aphids could convert DON into other derivatives (whether or not less toxic). In this experiment we were also unable to detect DON-GSH. There is a possibility that the concentration of this metabolite (or others) was also beneath the limit of detection. DON-GSH has already been observed in plants (Gardiner, SA *et al.*, 2010, Kluger *et al.*, 2013) and in many animal species (Wen *et al.*, 2016).

Future studies should further elucidate which DON derivatives are present inside the

aphid's body. We reported on DON-3G and DON-diglucosides but there can be other DON derivatives inside the aphids that are not yet discovered. In addition, examining the aphid's honeydew (Hogervorst *et al.*, 2007, Sabri *et al.*, 2013) can help with the identification of the enzyme mechanisms behind tolerance to DON. Revealing such mechanisms can provide us insights into how aphids or other insects adapt to changing environments and food sources.

Results in **chapter 4** suggested a co-evolutionary adaptation of *S. avenae* to DON but this is a hypothesis and also needs further exploration. Although it has not (yet) been demonstrated for trichothecenes, it is possible that over many generations insects develop a certain tolerance when exposed to a toxin. This is the case for *Drosophila melanogaster* larvae which were exposed to *Aspergillus nidulans* over several generations. They became less susceptible to the mycotoxin sterigmatocytin (Trienens & Rohlfs, 2011). We hypothesize the possibility of an adaptation of *S. avenae* to DON through co-evolution, resulting in aphids with an increased tolerance to DON by evolving toxin-tolerating metabolic mechanisms. Future research should focus on a comprehensive analysis of genes encoding for UDP-glucosyltransferase (UGTs) present in *S. avenae* grain aphids and search for potential horizontal transfers of UGT genes from *e.g.* endosymbionts to the aphid.

S. avenae aphids used in the experiments were not all originating from the same stock culture (new cultures were regularly purchased). It is possible that the cultures of obligate bacteria like *Buchnera* - that are essential for the survival of the aphids - were varying between the aphid stock cultures. We do not think that this had an impact on the results of the experiments, because the biological observations were very consistent throughout the four research years, especially when looking at aphid survival.

Still, another interesting hypothesis that was not addressed in this PhD thesis is the role of endosymbionts in the detoxification of DON inside aphids. There are several categories of endosymbionts inside aphids. The primary symbionts (*Buchnera aphidicola* in aphids) consists of obligate mutualists required to support normal host development (supply nutrients to hosts). They are typically restricted to a specialized organ, called a bacteriome. The facultative (or secondary) symbionts are able to invade various cell types, including reproductive organs, and may reside extracellularly in the hemolymph. These facultative symbionts consist of two categories. The first ones are the facultative mutualist who provide fitness benefits to their hosts by allowing the hosts to live longer and reproduce more. As a result, there are increasing frequencies of mutualists in the infected hosts. The benefits for

the insects include protection against natural enemies, heat or other mortality factors. In aphids they are represented by *Hamiltonella defense*, *Regiella insecticola* and *Serratia symbiotica*. The second facultative category are the reproductive manipulators: parasites that spread by increasing host reproduction through daughters at the expense of reproduction through sons. In many arthropods *Cardinium hertigii* and *Wolbachia* species are present (Moran *et al.*, 2008). One of the major differences between obligate and facultative symbionts is that obligate symbionts show no horizontal transfer compared to the facultative symbionts who show horizontal transfer within and between host species (Moran *et al.*, 2008). For example, the pea aphid *A. pisum* acquired genes from bacteria via lateral gene transfer and these genes are used to maintain the obligately mutualistic bacterium, *Buchnera* (Nikoh & Nakabachi, 2009, Richards *et al.*, 2010). These functional genes in *A. pisum* were acquired from bacteria other than its primary endosymbiont *B. aphidicola* (Nikoh *et al.*, 2010). The horizontal DNA transfers from *Wolbachia* to different insect species are described (Nakabachi, 2015).

Although it has not (yet) been demonstrated that endosymbiotic bacteria inside aphids can contribute to detoxification of mycotoxins or horizontal transfer of certain genes from bacteria to aphid can contribute to detoxification, it would be an interesting future research line. Mycotoxin-degrading bacteria have already been isolated from agricultural soils (Shima *et al.*, 1997, Islam *et al.*, 2010), infested plant material (Sato *et al.*, 2012) and animal digestive tracts (Binder *et al.*, 1997, Guan *et al.*, 2008, Guan *et al.*, 2009, Berthiller *et al.*, 2011, Meca *et al.*, 2012). But the effect of DON on obligate or facultative endosymbionts is to our knowledge until today not known.

5.7 Aphids transmitting viruses can impact the tripartite interaction

The tripartite interaction (plant-insect-fungus) can possibly also be affected by the aphids that are able to carry and transmit viruses. Most research conducted over the past years has deepened into vectoring of viruses by herbivores. This brought forward a classification according to their mode of transmission. Persistently transmitted viruses require the vector upon feeding on the infected host for several hours before acquiring the virus for life and

dispersing it to new healthy hosts. Non-persistently transmitted viruses are incorporated in seconds or minutes upon feeding on the infected host but do not retain by the vector for more than a few hours. Semi-persistently transmitted viruses are vectored by a transmission mode in between the former two (Gray & Banerjee, 1999, Mauck *et al.*, 2012). For example, barley yellow dwarf virus (BYDV) is persistently vectored by cereal aphids *R. padi* (Jimenez-Martinez *et al.*, 2004b) and *S. avenae* (Fereres *et al.*, 1989, Liu, XF *et al.*, 2014). Leaf hoppers *Graminella nigrifrons* (Hemiptera: Cicadellidae) transmit maize chlorotic dwarf virus in a semi-persistent manner (Childress & Harris, 1989). Host selection behavior of vectors can change after the acquisition of a virus. *R. padi* aphids vectoring BYDV preferred non infected wheat plants after acquiring the virus while non infective aphids preferred infected wheat plants (Ingwell et al., 2012). Perception of BYDV infected plants by aphids was influenced by volatile cues (Jimenez-Martinez et al., 2004a). In general we can hypothesize that virus-infected aphids can affect the tripartite interaction. Not only does their preference for certain plants change, it is also known that the physiology of a plant is greatly affected by viruses. This can, on its turn, result in a differential colonization by fungi. For example, the barley stripe mosaic virus causes chlorosis, leaf curling and growth inhibition in wheat. The symptoms are accompanied by induction of defense genes implicated in the defense against pathogens, namely PR1, PR4, PR5, PR10 and PAL. Inoculation of wheat with the virus resulted in decreased susceptibility against the blast pathogen *Magnaporthe oryzae* due to a reduction in penetration of epidermal cells and cell colonization but did not affect the development of *Blumeria graminis f. sp. tritici* (powdery mildew) (Tufan *et al.*, 2011). Further research should also investigate the role of viruses (*e.g.* BYDV) in the tripartite interaction between aphids and *F. graminearum* on wheat .

5.8 The conclusion

In **conclusion**, this thesis emphasizes the importance of studying interactions with other organisms developing on the same plant part in order to gain knowledge about the epidemiology of each and every individual participator, herbivore or pathogen. The dynamic three-way interactions of insects, pathogens and plants can constantly change and can be subject to influences from the environment like the weather. Further research on these

unique complexities is necessary to provide more insights into the cereal's ecosystem. This research should tend towards a time-specific approach. Through the growing season of wheat, the interactions between pathogens and insects can change. The tripartite interaction in this thesis started at anthesis (= infection period of Fusarium head blight (FHB)), where the wheat response towards *Fusarium* infection was altered by aphids that were already feeding from the ears. When feeding from infected wheat ears, the aphids are indirectly able to modulate epidemiology of *F. graminearum* through activation of host plant responses. A structural insight into tripartite interactions is warranted to acquire a comprehensive view of the array of defenses that wheat can use against fungal and insect invaders. While aphids can provide a benefit for the fungus, the fungus creates a detrimental environment for the aphids, either directly by the production of toxic DON or indirectly by turning ears in a unfavorable environment (lack of nutritional value for the aphids). Increasing concentrations of DON during disease development did not only impact the performance of the aphids, but also the parasitoids developing inside the aphids. On high concentrations of DON *S. avenae* experience negative effects on their performance, but are still more tolerant to the mycotoxin than pea aphids due to the reason (and possibly other reasons that we don't know about) that they were able to convert DON into DON-3G more efficiently. Aphids developed tolerance mechanisms to cope with this toxin, probably due to generations of co-existence. Future experiments investigating tripartite interactions should contain such a time lapse principle (from the moment one of the two organisms interact with the plant till the time both pathogen and insect go their separate ways (or one or the other dies)). Further research on these unique complexities is necessary to provide more insights into the cereal's ecosystem. Moreover, little work has been done to evaluate the potential role of insects in FHB epidemiology while cereals are growing in the vegetative stage.

Beside the interactions between wheat ears, cereal aphids *S. avenae* and the toxin producing pathogen *F. graminearum*, many other tripartite interactions are present in cereals (and other crops) that still remain to be uncovered. For example, on wheat leaves an interaction between cereal aphids *Metopolophium dirhodum* (the rose-grain aphid) and *Septoria* spp. (causing septoria leaf blotch) can occur. Other organisms with other feeding mechanisms (chewing insects instead of piercing-sucking insects and biotrophic pathogens instead of hemibiotrophic or necrotrophic pathogens) probably result in totally different

interactions with different outcomes and results. This makes the research of investigating three (or four in case of parasitoids) organisms at once interesting and intriguing.

If we take human impact or climate change into account when studying these interactions the experiments and possible outcomes become unlimited as these interactions are continuously subject to changes (the life and performance of each organisms on its own can be changed and on their turn impact the interaction). This broadens the research topic and makes room for many more experiments with many more hypotheses as a result.

Although this thesis gives more insight into the interactions that can occur between cereal aphids, *F. graminearum* and wheat, many questions and topics remain unanswered.

Summary

Cereal crops are a very important food source in the world. With the human population increasing rapidly, a higher demand for cereals arises to satisfy the human nutritional needs. Unfortunately, wheat is prone to many diseases. An important disease in wheat is Fusarium head blight that is caused by a complex of *Fusarium* species that attack wheat during anthesis and produce a plethora of mycotoxins. *Fusarium graminearum* is especially feared because of its aggressive nature and production of the mycotoxin deoxynivalenol (DON). DON can cause serious health problems for humans and animals upon consumption of contaminated wheat-derived foods.

In this thesis we tried to elucidate the interaction with aphids present on *Fusarium*-infected wheat ears. To learn more about the infection process of *F. graminearum* and grain aphid epidemiology, it is imperative to investigate every interaction between fungus and aphid inhabiting the same plant tissue. The English grain aphid *Sitobion avenae* is a known ear-feeder and thus inhabits the same ears as *F. graminearum* during flowering.

At the moment that both the fungus and grain aphids inhabit the wheat ears, we tried to elucidate plant defense responses that are triggered by *S. avenae* and *F. graminearum*. Wheat ears infected with *F. graminearum* showed more disease symptoms and higher DON levels when ears were predisposed to aphids compared to a sole inoculation with *F. graminearum*. Aphids induced defense genes that are typically induced upon a *F. graminearum* infection. Our study suggests that predisposal of wheat ears to aphids can affect the plant response which plays a role in the subsequent attack of *F. graminearum*, enabling the fungus to colonize the ears faster. Higher contents of DON in wheat ears can lead to serious problems regarding human and animal health.

The effect of DON on *S. avenae* aphids and their parasitoid *Aphidius ervi* was tested. DON had a negative impact on the development of *S. avenae* but also on their parasitoid *A. ervi*. This part emphasizes the importance of mycotoxins in food chain contamination from the plant to insects (insect-plant interactions) and their natural enemies (higher trophic interactions), which until today remains scarce.

On high concentrations of DON *S. avenae* experience negative effects on their performance, but are still more tolerant to the mycotoxin than pea aphids *Acyrthosiphon*

pisum because they were able to convert DON into its glucosylated form DON 3-glucoside more efficiently.

This thesis emphasizes the importance of studying interactions with other organisms developing on the same plant part in order to gain knowledge about the epidemiology of each and every individual participator, herbivore or pathogen. The dynamic three-way interactions of insects, pathogens and plants can constantly change and can be subject to influences from the environment like humans, weather, climate change, etc. Further research on these unique complexities is necessary to provide more insights into the cereal's ecosystem.

Samenvatting

Graangewassen zijn een zeer belangrijke voedselbron in de wereld. Door de snel toenemende menselijke bevolking ontstaat er een hogere vraag naar graan om aan de nutritionele behoeften van de mens te voldoen. Helaas, tarwe is gevoelig aan vele ziekten. Een belangrijke ziekte bij tarwe is aarfusarium. Deze wordt veroorzaakt door een complex van verschillende *Fusarium*soorten die tarwe tijdens de bloei infecteren en een overvloed aan mycotoxines produceren. De pathogeen *Fusarium graminearum* is vooral gevreesd vanwege zijn agressieve aard en productie van het mycotoxine deoxynivalenol (DON). DON kan tot ernstige gezondheidsproblemen leiden bij mens en dier wanneer deze voedsel consumeren die van besmette tarwe afkomstig is.

In dit proefschrift bestuderen we de interactie van herbivore bladluizen met *Fusarium*-besmette tarwe-aren. Om meer kennis te vergaren over het infectieproces van *F. graminearum* en graanluis epidemiologie, is het noodzakelijk om elke interactie tussen de schimmel of graanluis die hetzelfde plantenweefsel benutten te onderzoeken. De grote graanluis *Sitobion avenae* voedt zich ook op tarwe-aren. Diezelfde aren worden tijdens de bloei geïnfecteerd met *F. graminearum*.

Op het moment van bloei, wanneer zowel *F. graminearum* als *S. avenae* de aren bewonen, hebben we getracht om de verdedigingsreacties van tarwe tegen bladluizen en *F. graminearum* op te helderen. Tarwe-aren besmet met *F. graminearum* vertoonden meer symptomen van aarfusarium en hogere DON niveaus wanneer de aren eerder blootgesteld werden aan *S. avenae* bladluizen in vergelijking met aren zonder bladluisinfestatie. Bladluizen induceerden verdedigingsgenen die doorgaans ook worden opgewekt door een *F. graminearum* infectie. Deze studie suggereert dat blootstelling van tarwe-aren aan bladluizen een impact heeft op de plantrespons bij een daaropvolgende aanval van *F. graminearum*, waardoor de schimmel de aren sneller kan koloniseren. Dit leidt tot hogere concentraties aan DON in de tarwe-aren, wat ernstige problemen met betrekking tot de gezondheid van mens en dier kan teweeg brengen.

Het effect van DON op *S. avenae* bladluizen en hun natuurlijke vijand, de sluipwesp *Aphidius ervi*, werd ook getest. DON had een negatief effect op de ontwikkeling van *S. avenae*, maar ook op hun sluipwesp *A. ervi*. Dit deel benadrukt het belang van onderzoek

naar mycotoxinen in de voedselketen in insect-plant interacties en interacties met hogere trofische niveaus, wat tot op heden schaars is.

Op hoge concentraties van DON ervaren *S. avenae* negatieve effecten op hun ontwikkeling maar ze zijn nog steeds toleranter aan het mycotoxine dan erwtenbladluizen *Acyrthosiphon pisum* onder andere omdat ze DON efficiënter kunnen convergeren naar zijn geglucolyseerde vorm DON-3-glucoside.

Dit proefschrift benadrukt het belang van onderzoek naar interacties met andere organismen op hetzelfde plantendeel om kennis te vergaren over de epidemiologie van elke individuele deelnemer in de interacties, zowel herbivoor als pathogeen. De dynamische driedelige interacties tussen insecten, pathogenen and planten kunnen continue veranderen en zijn onderhevig aan invloeden vanuit de omgeving zoals de mens, het weer, klimaatsveranderingen enz. Verder onderzoek naar deze unieke complexe interacties is noodzakelijk om meer inzicht te verwerven in het ecosysteem van graangewassen.

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Curriculum vitae

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