Detoxification and metabolism of maize benzoxazinoids by lepidopteran herbivores

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1. Introduction

1.1. The evolutionary arms race between plants and insect herbivores

Plants and insects have coexisted for at least 350 million years (Gatehouse, 2002). During this time, both partners established relationships that greatly influenced each other. While some of these relationships are mutually beneficial, for example pollination, others are not. The most common interaction between these partners is the consumption of plants by insects, linked to adaptations by plants to defend themselves from insect herbivory. According to the coevolutionary theory, insect herbivory represents a major driving force of diversification for both insect and plant hosts (Ehrlich and Raven, 1968). Plants can go through occasional mutations of defensive traits that protect them from herbivory, entering a new adaptive zone and undergoing evolutionary radiation. Insects, on the other hand, can also develop adaptations to these new plant defenses, avoiding competition from other herbivores and thus undergoing diversification. These events are thought to be repeated and to drive the evolution of both plant and their insect counterparts, establishing the so-called evolutionary arms race. Indeed, insects are by far the most diverse group of organisms on Earth, comprising as many as 30 million species, most of which feed on plants and form clades that are consistently more diverse than their non-phytophagous sister groups (Jaenike, 1990). Additionally, plants have developed the ability to synthesize more than 200,000 secondary metabolites (also called “specialized metabolites”) in order to respond to ecological challenges such as insect herbivory (Pichersky and Lewinsohn, 2011).

1.2. Diversity of plant chemical defenses

As sessile organisms, plants do not have the ability to move in order to escape herbivory, and must rely on defensive strategies in order to fend off herbivore damage. These strategies include physical adaptations such as thorns and cuticular waxes, as well as chemical adaptations involving the production of toxic, antinutritive and deterrent allelochemicals (Mithöfer and Boland, 2012; Pentzold *et al.*, 2014). The high diversity of plant secondary metabolites is considered to enhance their biological activities, favoring synergistic effects, and to provide protection from a wide range of organisms (Jones and Firn, 1991; Wittstock and Gershenzon, 2002). However, the production of defensive metabolites incurs physiological, ecological, and evolutionary costs, some of which might limit growth and reproduction (Steppuhn and Baldwin, 2008). In order to maximize defensive potential while minimizing costs, plants have developed resource allocation strategies and a high plasticity of metabolic capabilities (Mithöfer and Boland, 2012). In this context, plant defenses can be produced constitutively or be induced upon herbivore attack. Induced defenses are produced specifically when they are needed, locally and/or systemically, which minimizes costs but leaves the plant unprotected

during the time lag between attack and defense deployment. Constitutive defenses, on the other hand, require constant investment but might be preferred by plants or tissues that are attacked frequently or more severely (Wittstock and Gershenzon, 2002). Plant defenses can also be classified between direct and indirect. Direct defenses exert detrimental effects directly on the herbivore, while indirect defenses are used to attract organisms from an additional trophic level, such as predators and parasitoids of the attacking herbivore (Mithöfer and Boland, 2012).

Chemically, plant defensive metabolites show remarkable diversity and modes of action. While primary metabolites are practically universal constituents of cells, tissues and organs, plant secondary metabolites are often restricted in their distribution, both taxonomically and ontogenetically (Berenbaum, 1995). Plant chemical defenses are not restricted to small organic molecules, but also include inorganic compounds such as silica (SiO_2), which accelerates mandibular wear in insects, and toxic proteins that can exert antinutritional effects and toxicity towards herbivores (Dang and Van Damme, 2015; Mithöfer and Boland, 2012). Among the small organic molecules, plant chemical defenses are divided into several classes, such as terpenoids, steroids, alkaloids, phenolics, glucosinolates, cyanogenic and iridoid glycosides, and benzoxazinoids. Their modes of action include membrane disruption, inhibition of nutrient and ion transport, inhibition of signal transduction processes, inhibition of metabolism, and disruption of hormonal control of physiological processes (Mithöfer and Boland, 2012). Some defenses have well defined biochemical targets (yet are toxic towards a broad spectrum of organisms), such as cyanogenic glycosides, which release HCN and affect cellular respiration in general by inhibiting the oxygen binding to cytochrome-*c*-oxidase in mitochondria (Mithöfer and Boland, 2012); while other defenses are more untargeted, such as benzoxazinoids, which generate highly electrophilic intermediates that react in a non-specific manner with nucleophilic amino acid residues in proteins (Niemeyer, 2009).

While developing defensive metabolites that are highly toxic to a broad spectrum of organisms, plants must protect themselves from autotoxicity. One way to achieve this is to synthesize and store defensive chemicals as inactive precursors (typically glycosides), and keep them spatially separated from enzymes that are able to activate such compounds when required. In this way, these chemical defenses are only activated and become toxic upon herbivore damage. Such activated defenses are known as phytoanticipins or two-component defenses, and represent a widespread strategy in plants, including cyanogenic and iridoid glycosides, glucosinolates, and benzoxazinoids (Pentzold *et al.*, 2014; Wittstock and Gershenzon, 2002). The compartmentalization of inactive precursors and activating enzymes can vary among these classes of compounds. While glucosinolates and their activating myrosinase enzymes are kept in separate specialized cells, cyanogenic glucosides and benzoxazinoids and their corresponding glucosidases are stored in separate organelles inside the plant cell (Wittstock and Gershenzon, 2002).

1.3. Strategies for insect resistance towards plant chemicals

In order to counteract the detrimental effects of plant chemical defenses, insect herbivores have developed a diverse range of strategies that allow them to feed on well-defended plants. Similarly to plant chemical defenses, some strategies used by insects to cope with plant toxins can be constitutively expressed or induced only upon contact with plant chemicals. Insect herbivores can also be classified according to their foraging preferences into specialists (which feed on a few, often related, host plant species) and generalists (which use a broad range of host plants, generally from different families), and the entire spectrum between these two extremes. Specialist herbivores normally encounter high levels of a narrow and predictable range of chemical defenses, while generalists face a wide variety of defenses differentially distributed among potential host plants (Li *et al.*, 2007). Accordingly, the specialist/generalist status of a given insect species influences the strategies it employs to deal with chemical defenses, as well as how they are regulated. In general, specialists possess constitutive resistance mechanisms towards plant defenses, while generalists rely on induction of these mechanisms upon exposure to individual host plants (Pentzold *et al.*, 2014). Furthermore, insects normally utilize a combination of resistance strategies discussed below in order to successfully feed on well-defended host plants.

The strategies insects use to cope with plant chemical defenses can have a **behavioral**, **molecular**, and/or **biochemical** basis, and are summarized in Figure 1. **Behavioral** strategies include (*i*) the aspects innate to the type of feeding guild, (*ii*) the avoidance of plant defenses, and (*iii*) switching between host plants. (*i*) Different feeding guilds possess anatomical adaptations that affect the amount of damage caused on the plant tissue, which in turn can modulate the exposure to plant toxins, especially in the case of activated plant defenses. In this context, piercing-sucking herbivores such as aphids minimize plant cell damage and activation of two-component defenses, whereas leaf-chewing herbivores cause extensive plant damage and are therefore exposed to high levels of activated plant defenses (Pentzold *et al.*, 2014). (*ii*) The avoidance of plant chemicals involves foraging patterns that minimize exposure by selecting plant tissues and developmental stages that possess lower concentrations of defensive metabolites (Després et al., 2007), as well as more complex behaviors such as vein cutting of latex-rich leaves by specialist caterpillars feeding on plants with laticifers (Bernays *et al.*, 2004). (*iii*) Lastly, switching between different host plants that differ in their defensive chemistry allows generalist herbivores to “dilute” individual toxic compounds, limiting their intake and detrimental effects (Singer et al., 2002). **Molecular** strategies employed by insect herbivores include (*i*) target mutation and (*ii*) differential regulation of enzymes. (*i*) Target mutations are associated with plant defenses that possess a specific molecular target in the insect, for example cardenolides, which target Na+-ATPases. Some insects specialized in cardenolide-containing plants have developed point mutations in Na^+/K^+-ATPases that impair the binding of cardenolides and render them less effective toxins (Agrawal et al., 2012). (*ii*) Changes in enzyme regulation include the down-regulation of insect enzymes that enhance toxicity of plant defenses, such as insect endogenous β-glucosidases able to hydrolyze and activate plant glucosydes, in addition to the overexpression of

existing enzymes that metabolize plant defenses and diminish their toxicity (Li *et al.*, 2007; Pentzold *et al.*, 2014).

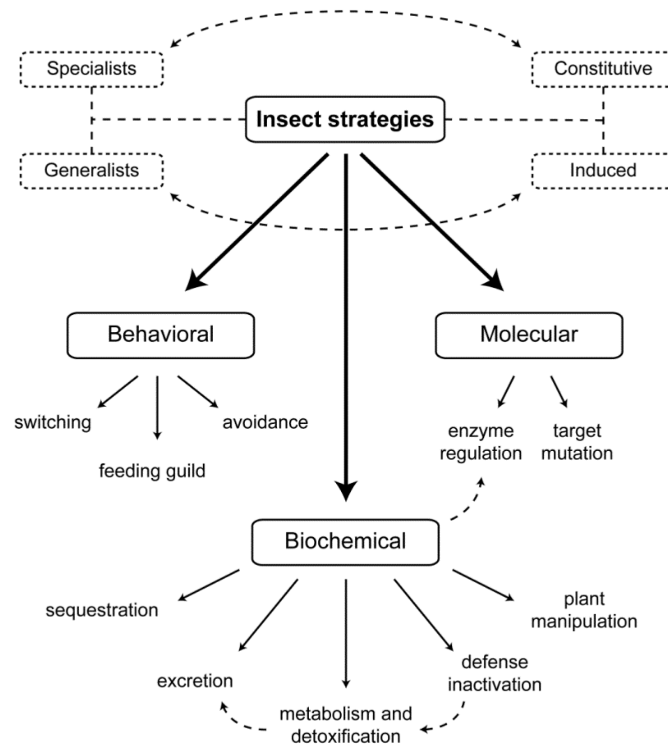

Figure 1. Summary of the main insect strategies for resistance towards plant chemical defenses (dashed lines represent mechanisms that might be associated)

Biochemical adaptations of insect herbivores include (*i*) the manipulation of plant defenses, (*ii*) inactivation of plant defenses, (*iii*) rapid excretion, (*iv*) sequestration, and (*v*) metabolism and detoxification. (*i*) The manipulation of plants by the insect involves the suppression or reduction of chemical defenses caused by elicitors present in oral secretions, such as salivary glucose oxidase enzymes from caterpillars (Després et al., 2007). Other examples are complex modifications such as gall formation in willow leaves by sawflies, which are associated with lower local concentrations of phenolic defenses (Nyman and Julkunen-Tiitto, 2000). (*ii*) The inactivation of plant defenses include the enzymatic inhibition of plant glucosidases required for toxin activation inside the alkaline gut of lepidopterans (Fitzgerald, 2008). Other examples involve the modification of inactive precursors of activated defenses by insects to avoid their activation by the plant's own enzymes, as is the case for glucosinolate modification by sulfatases (Falk and Gershenzon, 2007; Ratzka *et al.*, 2002). (*iii*) The excretion of plant toxins is typically associated with metabolism and detoxification strategies, but can also act on unmetabolized xenobiotics, effectively pumping them out of cells before they cause damage and thus representing the first line of defense (Brattsten, 1988; Kennedy and Tierney, 2013). (*iv*) The sequestration of plant chemical defenses is a specialized strategy that involves their

differential metabolism, transport, and storage in specific tissues in the insect, where they can be used as defenses against natural enemies, or as precursors for insect secondary metabolites. Some highly specialized insects that rely on *de novo* synthesized chemical defenses against predators nevertheless can also sequester these compounds from host plants, as is the case for the six-spot burnet moth larvae *Zygaena filipendulae*, which sequesters cyanogenic glucosides from plants (Zagrobelny and Møller, 2011). (*v*) Finally, the metabolism and detoxification of plant chemicals are major adaptations developed by insect herbivores, and these are the subject of this thesis. Insect metabolism and detoxification strategies are covered in detail in the following section.

1.4. Insect metabolism and detoxification of xenobiotics

The metabolism and detoxification of xenobiotics in insects include pathways used to minimize the toxicity of both plant allelochemicals and synthetic pesticides. Indeed, insect metabolic pathways associated with resistance towards plant defenses might contribute to the development of resistance towards pesticides (Després *et al.*, 2007), but the pathways for detoxification of these two types of xenobiotics reflect their differences in biological effects, dose, diversity, and distribution (Li *et al.*, 2007). Moreover, the pathways by which insects metabolize xenobiotics also present some similarity with equivalent reactions in humans, which are implicated in drug metabolism (Kennedy and Tierney, 2013).

Traditionally, the metabolism of xenobiotics is divided in three phases (I to III, summarized in Figure 2). The reactions of phase I metabolism introduce or expose a reactive functional group in the molecule, normally a nucleophilic group such as –OH, –SH, $–NH_2$, and –COOH. Reactions from phase I metabolism increase the polarity and hydrophilicity of the original xenobiotic, increasing its excretion potential, while also rendering it more reactive towards phase II reactions. Phase II metabolism consists of conjugation reactions that minimize toxicity and greatly enhance hydrophilicity, facilitating the active transport of metabolites. Xenobiotics that possess reactive functional groups, as is the case for two-component defenses, can skip phase I metabolism and be directly conjugated by phase II reactions. Notably, such phase I and II reactions involved in xenobiotic metabolism also play an important role in modifying endogenous metabolites such as signaling molecules and biosynthetic precursors, controlling their bioactivities and distribution and contributing to homeostasis. Although they normally stabilize xenobiotics and diminish their toxicity, phase I and II metabolism reactions can also generate products with enhanced biological activities (metabolic activation or bioactivation), similarly to a prodrug activation mechanism. Finally, phase III encompasses the excretion of metabolites by active transport through membranes (Ioannides, 2002; Kennedy and Tierney, 2013).

Phase I metabolism reactions are promoted by cytochrome P450 enzymes (P450s) and esterases. P450s are versatile heme-thiolate enzymes best known for their monooxygenase role, transferring one atom from molecular oxygen to the substrate and reducing the other to water. Strikingly, P450s possess a wide range of activities, including oxidase, reductase, desaturase, and

isomerase, being able to catalyze at least 60 chemically distinct reactions (Feyereisen, 2012). Due to their extraordinary catalytic versatility and broad substrate specificity, P450s can catalyze aliphatic and aromatic hydroxylation, *N*-hydroxylation, epoxidation, *O*-, *N*- and *S*- dealkylation, deamination, sulfoxidation, desulfuration, and oxidative dehalogenation reactions. Esterases, on the other hand, are responsible for hydrolysis of xenobiotics containing ester and amide groups, generating carboxylic acids, alcohols and amines that are suitable substrates for phase II metabolism (Kennedy and Tierney, 2013). Esterases also act on endogenous metabolites of insects, being important for juvenile hormone metabolism and nerve impulse transmission mediated by acetylcholinesterases (Brattsten, 1988).

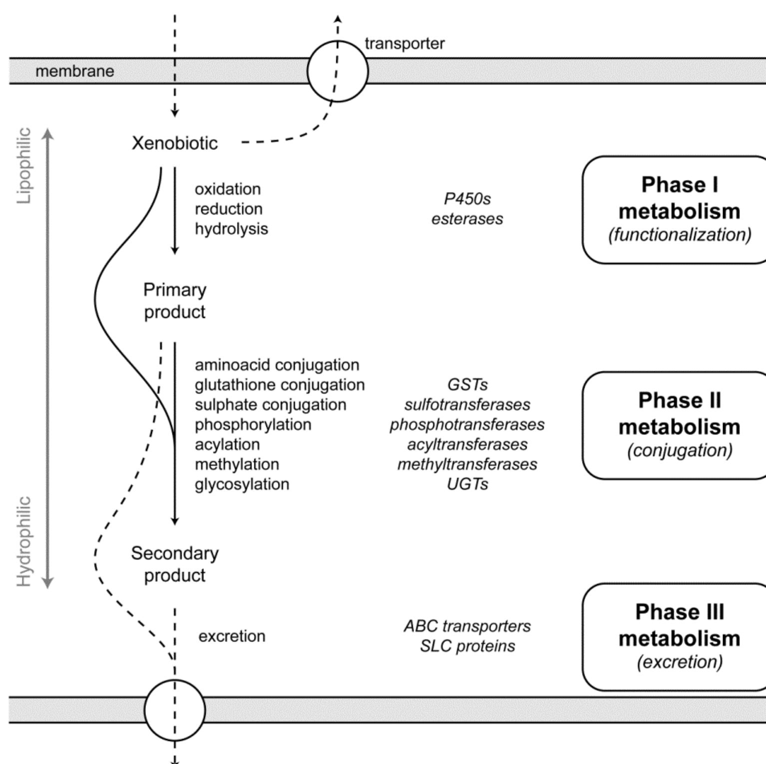

Figure 2. Summary of the metabolism of xenobiotics by insects and representative reactions and enzymes (full lines represent biotransformations; dashed lines represent transport)

Conjugation reactions of **phase II metabolism** involve a wide range of transformations. Depending on the reactivity of the metabolite and the conjugating agent, the reactions of phase II metabolism can occur without enzymatic catalysis, as is the case of some conjugation reactions with aminoacids and glutathione. In most cases, however, the conjugating agent must be present in an active donor form such as uridine diphosphate glucose (for glucose) and 3'-phosphoadenosine-5'-phosphosulfate (for sulfate). Conjugation with amino acids in insects has been observed for metabolites containing carboxylic acid groups, such as benzoic acid and 3-nitropropanoic acid

(Novoselov *et al.*, 2015; Stauber *et al.*, 2012), possibly involving two steps: the formation of their acyl coenzyme A thioesters and their reaction with the amino group of amino acids (mainly glycine) mediated by *N*-acyltransferases (Steventon, 2002). This reaction can also be spontaneous, as the glycine conjugation of iridoid glycoside metabolites, which prevents the reactive aglucones to denaturate insect proteins (Konno *et al.*, 1998). The conjugation with reduced glutathione represents a major detoxification mechanism of electrophilic xenobiotics in insects, such as isothiocyanates resulting from glucosinolate hydrolysis (Gloss *et al.*, 2014; Jeschke *et al.*, 2016; Schramm *et al.*, 2012). Glutathione is a tripeptide that contains cysteine, glutamic acid, and glycine, and its conjugation to xenobiotics occurs via the cysteine thiol group, generating a thioether, typically catalyzed by glutathione-*S*-transferase enzymes (GSTs). The glutathione adduct can be further metabolized by the enzymatic removal of the glutamic acid and glycine moieties, generating a cysteine *S*-conjugate that can be finally acylated to its *N*-acetyl derivative, mercapturic acid (Kennedy and Tierney, 2013). The conjugation with sulfate consists in the transfer of a SO_3^- group to alcohols, phenols and amines catalyzed by sulfotransferases. In mammals, this metabolic pathway seems to complement glucuronidation (Kennedy and Tierney, 2013). Phosphorylation seems to be more common in insects than in vertebrates, and despite little being known about it, this pathway is suggested to contribute to xenobiotic detoxification (Wilkinson, 1986). Acylation reactions of xenobiotics are represented mostly by *N*-acetylation of amides forming more hydrophilic amides. Such acetylation reactions are promoted by *N*-acetyltransferases and require acetyl-CoA as a cofactor (Ioannides 2001). Methylation reactions use *S*-adenosyl methionine and are promoted by *O*-, *N*- and *S*-methyltransferases, which catalyze common reactions of endogenous compounds. In contrast to the other conjugation reaction discussed here, methylation masks functional groups and reduces water solubility, possibly impairing metabolite excretion. Therefore, methylation reactions are usually not quantitatively relevant to the detoxification of xenobiotics (Kennedy and Tierney, 2013). Lastly, glycosylation constitutes the major conjugation reaction of both xenobiotics and endogenous compounds in most organisms (Kennedy and Tierney, 2013). These reactions involve the conjugation with any sugar moiety, catalyzed by UDP-glycosyltransferases (UGTs). The character of the sugar donor varies according to the organism, with the conjugation of glucuronic acid (by UDP-glucuronosyltransferases) being the preferred pathway in mammals, and the conjugation with glucose (by UDP-glucosyltransferases) being the preferred pathway in insects and plants. Glycosylation reactions of xenobiotics are an important topic covered in this thesis, and are discussed in more detail in the next subsection.

Phase III metabolism involves the active transport of metabolites out of the cell and their final excretion. Since all cells and subcellular compartments are separated from their environment by lipid membranes, the transport of polar compounds, both endogenous metabolites and xenobiotics, is mediated by specific proteins associated with the membrane. The two main types of proteins recruited for this function are ATP-binding cassete (ABC) and solute carrier (SLC) proteins (Kennedy and Tierney, 2013). ABC transporters have been extensively studied in humans for their important role in multidrug resistance. Despite not being well explored in insects, ABC transporters are expected to

play an important role in xenobiotic resistance, as they have been implicated in tolerance towards Bt toxin in lepidopterans (Dermauw and Van Leeuwen, 2014; Heckel, 2012).

1.4.1. The importance of UGTs in insect metabolism of xenobiotics

UGTs are enzymes that catalyze the transfer of a sugar moiety to a wide range of acceptors, using activated sugar donors. The most common donors are nucleotide diphosphate sugars, typically uridine diphosphate glucose (UDP-glucose) and UDP-glucoronic acid, but others such as UDP-galactose, UDP-rhamnose, and UDP-xylose can also be used. The acceptor substrates utilized by glycosyltransferases can be other sugars, lipids, proteins, or small molecules, such as hormones, secondary metabolites, and xenobiotics. Sugar transfer most frequently occurs to the oxygen atom of hydroxyl groups, but can also occur to nitrogen, sulphur, and carbon nucleophiles (Lairson *et al.*, 2008). Addition of one or more sugar moieties to lipophilic small molecules increases their polarity. As a result, diffusion across lipid bilayers and intracellular compartments is restricted, and the glycosylated products can access membrane-bound transport systems that recognize glycosyl residues (Bowles *et al.*, 2006). Therefore, glycosylation of small molecules can alter their stability, subcellular localization, bioavailability, and activity, and constitutes a strategy for regulating cell homeostasis and adapting to dynamic environmental conditions (Meech *et al.*, 2012).

In plants, bacteria, and animals, UGTs are presumably derived from the same ancestral gene, and evolved to promote protection against toxic chemicals and to control the activity and distribution of various endogenous metabolites and signaling compounds (Meech *et al.*, 2012). However, animal UGTs are membrane-bound enzymes located in the endoplasmic reticulum, whereas plant UGTs are presumed to be cytosolic (Ross *et al.*, 2001). Mammalian UDP-glucuronosyltransferases are the best characterized UGTs, and related enzymes found in insects are likely to have equally important functions (discussed in **Manuscript IV**). However, while UGTs found in vertebrates typically catalyze the transfer of glucuronic acid, insects show a preference for UDP-glucose as sugar donor (Meech *et al.*, 2012).. In this thesis, I focus on the metabolism of plant defenses by insects, and therefore glucose is the most relevant sugar moiety used for conjugation reactions. When referring to insects, the term UGT is used as a synonym of UDP-glucosyltransferase.

1.5. Benzoxazinoids in the plant-insect interface

In order to avoid herbivory, one of the strategies adopted by many grasses (Poaceae) is the production of benzoxazinones and their benzoxazolinone degradation products, collectively called benzoxazinoids (BXDs). They can be found in important crops, such as maize, wheat, and rye, as well as in a few dicot species (Frey *et al.*, 2009). Benzoxazinones possess a 2-hydroxy-*2H*-1,4-benzoxazin-3(*4H*)-one skeleton and are stored in plants as stable glucosides (Figure 3). Upon cell

damage caused by herbivory, they come into contact with β-glucosidases present in other plant cell compartments and are hydrolyzed to toxic aglucones, thus representing an example of two-component defenses. Benzoxazinone aglucones are cyclic hemiacetals that undergo a fast and reversible ring opening reaction by oxo-cyclo tautomerism, generating reactive electrophilic species. The basis of their toxicity is considered to be their unspecific reactions with biological nucleophiles, such as thiol, hydroxy and amino groups in amino acid residues from proteins (Niemeyer, 2009). The substitution patterns of the core benzoxazinone skeleton give rise to a diverse family of compounds that can be generally divided into lactams (R^1=H), hydroxamic acids (R^1=OH), and *N*-*O*-methylated derivatives (R^1=OMe). Furthermore, hydroxamic acids and *N*-*O*-methylated derivatives undergo spontaneous degradation yielding benzoxazolinones, which are toxic towards a wide range of organisms. The structural features of benzoxazinoids greatly influence their stability, reactivity, and biological activities. A comprehensive review on the chemistry of BXDs and how it modulates their potential as plant chemical defences is presented in **Manuscript I** of this thesis.

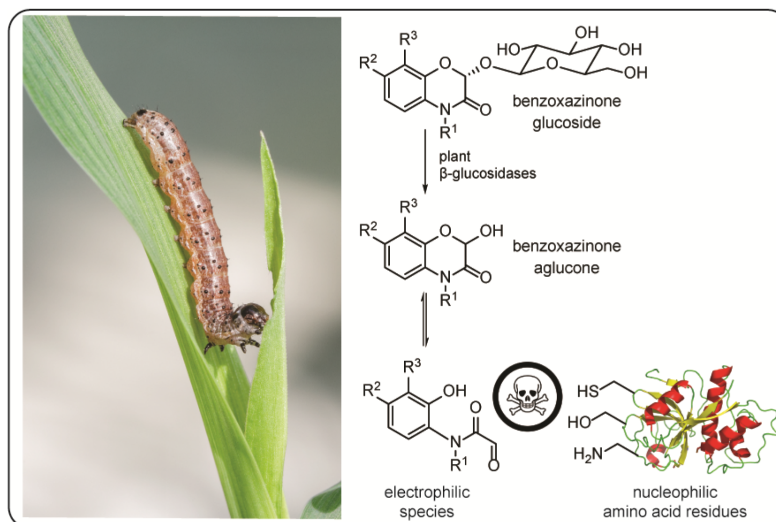

Figure 3. Larvae of *Spodoptera frugiperda* (Noctuidae) are serious agricultural pests of maize. Plants defend themselves by producing benzoxazinoids, whose mode of action is considered to be unspecific reactivity with biological nucleophiles. (Photo: A. Schroll)

The chemical versatility of BXDs grants them diverse biological effects against a broad range of organisms, including insecticidal, antifeedant, antimicrobial, and allelopathic activities. The detrimental effects of BXDs towards insects have been implicated in the resistance towards insect herbivory in several maize and wheat lines. Moreover, the combined constitutive production and differential induction of BXDs towards herbivore attack and their dynamic allocation likely contribute to their important role as a defense against insect herbivores with diverse feeding behaviors. Despite the potential of BXDs as effective defensive secondary metabolites in plants, some insect herbivore species possess remarkable resistance towards these compounds. The strategies presented in the

previous sections are expected to play a role in insect resistance towards BXDs, being responsible for their status as agricultural pests. In **Manuscript II**, I discuss the biosynthesis and induction of BXDs in plants, explore the different layers of their biological activity towards insect herbivores depending on their feeding guilds, and summarize current knowledge about the metabolism of BXDs by insect herbivores.

Maize (*Zea mays*) is one of the most important food crops in the world, being cultivated over an area of around 180 million hectares with annual production of over 900 million tons (USDA, 2016). It is an important staple food crop, being responsible for 15%-56% of the total daily calorie intake by people from 25 developing countries, especially in Africa and Latin America (Dhillon *et al.*, 2014). Modern maize (*Zea mays* ssp. *mays*) has originated from domestication of the Balsas teosinte (*Zea mays* ssp. *parviglumis*) initiated around 9,000 years ago in the south of present-day Mexico (Maag *et al.*, 2015). In general, modern maize has decreased both direct and indirect defenses against insect herbivores during domestication, and teosinte appears to be more resistant to certain pests and pathogens (de Lange *et al.*, 2014). One of the most important agricultural pests of maize is the fall armyworm, *Spodoptera frugiperda* (Smith). It is a migratory pest originally from the Western hemisphere and occurs from Argentina to Southern Canada. Besides attacking maize, larvae of *S. frugiperda* cause considerable economic losses in crops such as sorghum, rice, cotton, alfalfa and forage grasses. Two morphologically indistinguishable host strains have been identified: the so-called corn and rice strains (Clark et al., 2007). Larvae of *S. frugiperda* feed on all growth stages of maize, but most commonly feed in the whorl of young plants up to 45 days old. Reduction of maize yields due to *S. frugiperda* feeding has been reported to be as high as 34% (Cruz et al., 1999).

The main topic of this thesis is the metabolism of maize BXDs by lepidopteran herbivores. Knowledge about how insects detoxify these defense compounds is crucial in order to understand the metabolic basis of their resistance towards BXDs. I focused my work on *S. frugiperda* and its metabolism of DIMBOA, the major BXD in maize aerial tissues. However, the overall results described here could be extended to other insect species, BXD derivatives, and plant species. In **Manuscript III**, I describe a metabolic detoxification pathway of DIMBOA by *S. frugiperda*, *S. littoralis*, and *S. exigua*: reglucosylation with controlled stereochemistry in order to generate (2*S*)-DIMBOA-Glc. I propose that the formation of this compound, an epimer of the plant original (2*R*)-DIMBOA-Glc, represents an effective way to stabilize and deactivate this abundant plant defense. In **Manuscript IV**, I explore the molecular basis of such BXD detoxification reactions by identifying, expressing, and functionally characterizing UGTs from *S. frugiperda* that have the potential to glucosylate DIMBOA and MBOA. Finally, in **Manuscript V**, I investigate the overall metabolism of DIMBOA, MBOA, (2*R*)-DIMBOA-Glc and (2*R*)-HMBOA-Glc by controlled feeding of *S. frugiperda* larvae, setting a quantitative framework for the metabolic fates of these compounds in which the initial glucosylation reactions can be inserted and from which new detoxification strategies can be speculated. The data presented in the five manuscripts included in this dissertation are critically assessed in the general discussion section at the end of this dissertation.

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2. Manuscript Overview

Manuscript I

Benzoxazinoids: Reactivity and Modes of Action of a Versatile Class of Plant Chemical Defenses

Felipe C. Wouters, Jonathan Gershenzon, Daniel G. Vassão

Submitted to *Journal of the Brazilian Chemical Society*

We were invited to write this review article by the organizers of the 5th BCNP (Brazilian Conference on Natural Products) after my presentation of a short lecture. This article will be featured in a special edition of the *Journal of the Brazilian Chemical Society* dedicated to this conference.

In this manuscript I summarize the knowledge about the stability and chemical reactivity of BXDs and how these properties are modulated by stereoelectronic effects derived from the diverse substitution patterns observed in natural and non-natural BXD structures. I discuss the different mechanisms by which benzoxazinoids are considered to exert their biological activities and interpret structure-activity relationship studies from the literature in order to evaluate the relative importance of individual mechanisms for different biological activities, including antifeedant, insecticidal, antimicrobial, and allelopathic activities. The critical assessment of the chemical properties of BXDs and the influence of their structures over stability and reactivity is essential in order to explore how these compounds differentially exert their biological activities over a range of target organisms. This work establishes the chemical basis that supports the investigations included in this thesis and future research on BXDs, not limited to the context of chemical ecology.

Felipe C. Wouters reviewed the literature and wrote the manuscript draft, Jonathan Gershenzon revised the manuscript, and Daniel G. Vassão revised and submitted the manuscript.

Manuscript II

Biological activities and metabolism of benzoxazinoids on insect herbivores

Felipe C. Wouters, Jonathan Gershenzon, Daniel G. Vassão

Manuscript in preparation for submission to *Phytochemistry Reviews*

We were invited to write this review article by the editors of *Phytochemistry Reviews*.

In this manuscript I explore the differential biological activites of BXDs towards insect herbivores from different ecological guilds: chewing, piercing-sucking, and root herbivores. I summarize and update the literature covering the contribution of BXDs to plant resistance towards insect herbivores via toxicity, digestibility-reduction, and antifeedant activites. I compare the concentrations in which BXDs were shown to cause detrimental effects to insects in order to facilitate the interpretation of laboratory and field experiments using the same insect species and assess the spectrum of resistance levels towards BXDs observed in different insect species. Finally, I discuss recent works on BXD metabolism by different insect herbivores and mammals and suggest future research directions. In addition to reviewing the literature, I also present two datasets obtained during my doctoral studies, exploring the influence of MBOA on food consumption and utilization by *S. frugiperda*, and comparing the activities of MBOA detoxification by *N*-glucosylation in *S. frugiperda* and *Ostrinia nubilalis* (univoltine and bivoltine strains). Within this manuscript, I discuss how the feeding behavior of insects and other ecological aspects of plant-insect interactions moderate their exposure to BXDs and the resulting biological activities. The assessment of data from the literature also helps to draw the important distinction between toxic, digestibility-reducing, and antifeedant activities of BXDs, and contributes to a more detailed understanding of the physiological mechanisms underlying the overall biological effects of BXDs towards insect herbivores. The literature review of BXD metabolism by insects summarizes the knowledge in this field and establishes the state of the art relative to the broad theme of the present thesis.

Felipe C. Wouters reviewed the literature, designed and performed the experiments, analyzed the data, and wrote the manuscript draft, Jonathan Gershenzon revised the manuscript, and Daniel G. Vassão designed the experiments and revised the manuscript.

Manuscript III

Reglucosylation of the Benzoxazinoid DIMBOA with Inversion of Stereochemical Configuration is a Detoxification Strategy in Lepidopteran Herbivores

Felipe C. Wouters, Michael Reichelt, Gaétan Glauser, Eugen Bauer, Matthias Erb, Jonathan Gershenzon, and Daniel G. Vassão

Published in *Angew. Chem. Int. Ed.* **2014**, *53*, 11320-11324

DOI: 10.1002/anie.201406643

In this manuscript we describe a novel strategy employed by *Spodoptera* species to metabolize DIMBOA, the most abundant BXD present in maize aerial parts, by glucosylating it to (2*S*)-DIMBOA-Glc. We show that the insect-derived glucoside is not hydrolyzed by plant β-glucosidases, therefore representing an inert detoxification product. This detoxification strategy is potentially involved in the relatively high tolerance of *Spodoptera* species to BXD-containing plants. This work represents our first results on the detoxification strategies employed by *Spodoptera* species, and led to many further questions in this project, some of them addressed in **Manuscript IV** and **Manuscript V**.

Felipe C. Wouters designed and performed the feeding assays, *in vitro* enzymatic assays, HPLC analyses, and synthesis of DIMBOA-Glc, performed and interpreted the NMR analyses, and wrote the manuscript. Michael Reichelt designed and performed HPLC analyses, contributed with helpful discussions, and revised the manuscript. Gaétan Glauser provided samples of (2*R*)-DIMBOA-Glc isolated from maize plants, contributed with helpful discussions, and revised the manuscript. Eugen Bauer performed microelectrode pH measurements of the gut lumen of *S. frugiperda* larvae. Matthias Erb contributed with helpful discussions, and revised the manuscript. Jonathan Gershenzon contributed with helpful discussions, and revised the manuscript. Daniel G. Vassão designed experiments, and revised and submitted the manuscript.

Manuscript IV

Screening of UDP-glycosyltransferases responsible for benzoxazinoid detoxification in *Spodoptera frugiperda*

Felipe C. Wouters, Seung-Joon Ahn, Katrin Luck, Heiko Vogel, Matthias Erb, Jonathan Gershenzon, Daniel G. Vassão

Manuscript in preparation for submission to *Insect Biochemistry and Molecular Biology*

In this manuscript we report the identification and functional screening of *S. frugiperda* UDP-glucosyltransferases (UGTs) in order to determine the enzyme(s) responsible for BXD detoxification via glucosylation. Taking advantage of RNA sequencing technology, we identified and heterologously expressed 25 UGTs from *S. frugiperda*. Five of these candidates showed activity towards BXDs and might be involved in the glucosylation reactions of DIMBOA (**Manuscript III**), HMBOA (**Manuscript V**), and MBOA by *S. frugiperda*. Additionally, we investigated the induction and tissue localization of UGT activities towards DIMBOA and MBOA, which contributes to understanding the compartmentalization and temporal aspects of the metabolic pathways described in **Manuscript III** and **Manuscript V**. This works also provides sequence data that greatly facilitate further studies on BXD glucosylation by *S. frugiperda* on a molecular level, as well as candidates for functional screening studies of insect UGTs.

Felipe C. Wouters designed and performed feeding assays, dissections, and *in vitro* enzymatic assays, performed the amplification, cloning, heterologous expression and screening of UGT candidates, and wrote the manuscript. Seung-Joon Ahn analyzed the assembled sequence data, contributed with helpful discussions, and wrote the manuscript. Katrin Luck assisted on the amplification, cloning, heterologous expression and screening of UGT candidates, contributed with helpful discussions, and revised the manuscript. Heiko Vogel analyzed the raw transcriptome sequence data. Matthias Erb contributed with helpful discussions. Jonathan Gershenzon designed experiments, contributed with helpful discussions, and revised the manuscript. Daniel G. Vassão designed experiments, analyzed the raw transcriptome sequence data, and wrote the manuscript.

Manuscript V

Metabolic fate of benzoxazinoids in *Spodoptera frugiperda*

Felipe C. Wouters, Christian Paetz, Matthias Erb, Jonathan Gershenzon, Daniel G. Vassão

Manuscript in preparation for submission to *Journal of Chemical Ecology*

In this manuscript I report on a quantitative exploration of the BXD metabolism of *S. frugiperda* through oral administration of individual BXDs followed by screening of metabolites in frass and hemolymph. I describe the glucosylation of HMBOA to (2*S*)-HMBOA-Glc, similarly to that observed with DIMBOA (**Manuscript III**). The quantitative evaluation of the multiple metabolic pathways helps us to assess the contribution of the *in vitro* glucosylation reactions described in **Manuscript III** and **Manuscript IV** in a natural physiological context. The identification of metabolites resulting from feeding of individual BXDs reveals the connections in the network of BXD metabolic routes in *S. frugiperda* and contributes to the exploration of the biochemical basis of BXD resistance by lepidopteran agricultural pests in general.

Felipe C. Wouters synthesized and purified BXDs, designed and performed feeding and *in vitro* enzymatic assays, performed and analyzed the HPLC data, and wrote the manuscript. Christian Paetz performed and interpreted the NMR analyses and wrote the manuscript. Matthias Erb contributed with helpful discussions. Jonathan Gershenzon contributed with helpful discussions and revised the manuscript. Daniel G. Vassão contributed with helpful discussions and revised the manuscript.

3. Manuscripts

Note: the formatting of the manuscripts that were not yet published was partially modified in order to better match the formatting of the rest of the thesis.

Manuscript I

Benzoxazinoids: Reactivity and Modes of Action of a Versatile Class of Plant Chemical Defenses

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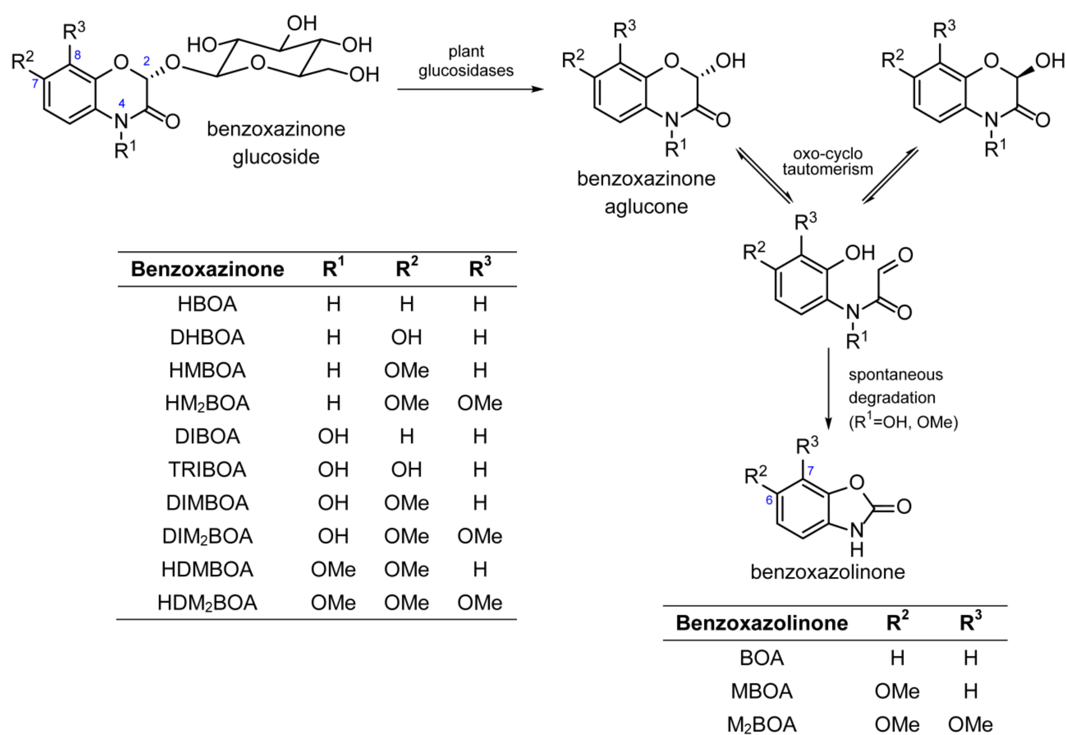
Abstract

In order to protect themselves from biotic stresses, including enemy and competing organisms, many plants recruit defensive secondary metabolites. Compounds containing a 2-hydroxy-*2H*-1,4-benzoxazin-3(*4H*)-one skeleton and their derivatives, collectively known as benzoxazinoids, are common secondary metabolites in many grasses, including important cereal crops such as maize, wheat, and rye, as well as several dicot species. This diverse class of compounds is known for their broad range of antifeedant, insecticidal, antimicrobial, and allelopathic activities. However, the mechanisms underlying such biological activities are not yet completely understood. The present review aims to summarize current knowledge on the biological reactivity of benzoxazinoids and associate it to their proposed modes of action. Structure-activity relationships for a wide spectrum of biological effects are critically discussed and directions for future research are addressed.

Keywords: benzoxazinoids, plant defenses, chemical ecology, natural products, mode of action

1. Introduction

Compounds with the 2-hydroxy-*2H*-1,4-benzoxazin-3(*4H*)-one skeleton (benzoxazinones) and their benzoxazolinones degradation products have been recognized as general defense metabolites from plants, being involved in antifeedant, insecticidal, antimicrobial, and allelopathic activities.[1] Given the variety of biologically relevant structures in this group, we will refer to benzoxazinones (both acetal glucosides and hemiacetal aglucone forms) and benzoxazolinones collectively as benzoxazinoids (BXDs) in this article. These compounds are given acronyms based on their official names, such that 2,4-**di**hydroxy-7-**m**ethoxy-2*H*-1,4-**b**enz**o**x**a**zin-3(4*H*)-one is normally referred to as DIMBOA, and its glucoside, (2*R*)-2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one, as DIMBOA-Glc. The most common naturally occurring BXD structures and their relationships are shown in Scheme 1.



Benzoxazinone	R^1	R^2	R^3
HBOA	H	H	H
DHBOA	H	OH	H
HMBOA	H	OMe	H
HM_2BOA	H	OMe	OMe
DIBOA	OH	H	H
TRIBOA	OH	OH	H
DIMBOA	OH	OMe	H
DIM_2BOA	OH	OMe	OMe
HDMBOA	OMe	OMe	H
HDM_2BOA	OMe	OMe	OMe

Benzoxazolinone	R^2	R^3
BOA	H	H
MBOA	OMe	H
M_2BOA	OMe	OMe

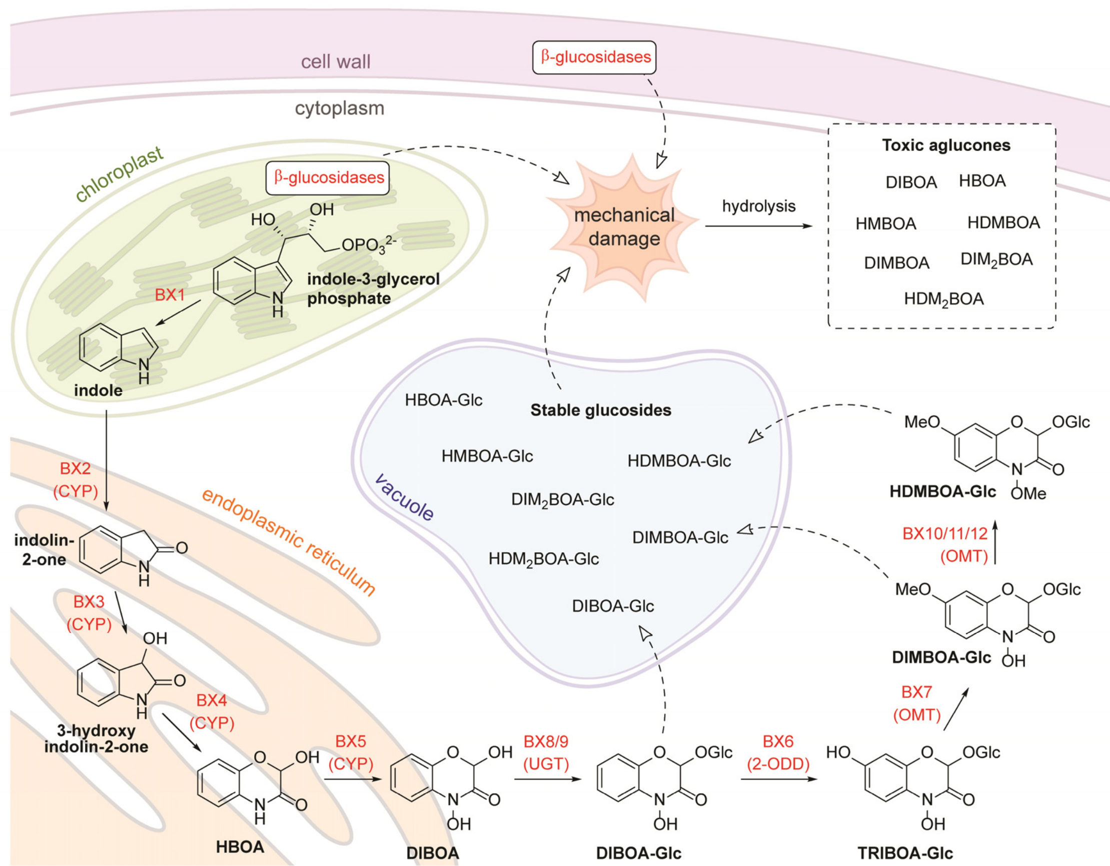
Scheme 1. Hydrolysis of naturally occurring benzoxazinone glucosides and degradation to benzoxazolinones via oxo-cyclo tautomerism.

This group of nitrogen-containing secondary metabolites is present in many grasses (Poaceae), including economically important crops such as maize, wheat, and rye (but not rice, oat,

sorghum, and cultivated barley).[1] BXDs are also found in a few species within the dicot families Acanthaceae, Ranunculaceae, Plantaginaceae, and Lamiaceae.[2,3]

The BXD biosynthetic pathway has been mostly established in maize[2,4] and is summarized in Scheme 2. The biosynthesis of BXDs, their evolution in plants, and related genetic aspects have been recently comprehensively reviewed.[2,3,5] Bx1 catalyzes the first committed step of the pathway, converting indole-3-glycerol phosphate into indole in the chloroplasts. This enzyme, which also produces free indole released by plants, is a homolog of the subunit α of tryptophan synthase, which performs the same reaction but is coupled to a β subunit that further converts indole to tryptophan without releasing it from the enzyme complex. Free indole produced by Bx1 is then oxidized by four cytochrome P450-dependent monooxygenases, Bx2-Bx5, which are located in the endoplasmic reticulum and are substrate-specific and regioselective for the introduction of oxygen atoms. The resulting DIBOA is the first toxic intermediate in the pathway and is glucosylated by UDP-glucosyltransferases Bx8 and Bx9, presumably to minimize autotoxicity and provide a stable intermediate for further modifications in the cytoplasm. DIBOA-Glc can be hydroxylated by the 2-oxoglutarate-dependent dioxygenase Bx6, and *O*-methylated by *O*-methyltransferase Bx7, yielding DIMBOA-Glc. Recently, another three homologous *O*-methyltransferases, Bx10, Bx11, and Bx12, were implicated in the reaction from DIMBOA-Glc to HDMBOA-Glc.[4] The biosynthesis of DIM_2BOA-Glc, HDM_2BOA-Glc, and other BXDs is being studied, and the general pathway involve redundancy among the enzymes. (Handrick, pers. comm.) The stable glucosides are considered to be transported to and stored in the vacuole.[6] Since β-glucosidases that use BXD glucosides as substrates are present in chloroplasts, cell walls, and cytoplasm, no hydrolysis normally occurs in intact tissue.[7]

Upon disruption of the cell structure (by herbivore feeding or pathogen attack), BXD glucosides come into contact with β-glucosidases and are hydrolyzed to reactive aglucones, which are implicated in the toxicity of BXDs. A similar pattern is also observed for other plant defenses, such as glucosinolates and cyanogenic and iridoid glycosides, that are also activated only upon damage.[8,9] In roots, BXDs are actively exuded from plant tissue to the soil and thus can exert direct effects on root herbivores, soil microorganisms, and other plants.[10,11] Benzoxazinone aglucones can spontaneously degrade to benzoxazolinones at rates that depend on their chemical nature and environmental conditions, as will be discussed in more detail below.



Scheme 2. BXD biosynthesis and model of compartmentalization in a plant cell.

The abundance of BXDs and their proportion vary between plant species and varieties and within plants among tissues, developmental stages, and after induction by biotic factors. The total concentrations of BXDs can reach >0.1% of fresh weight as in maize leaves after caterpillar attack.[12,13] The main BXD in rye is DIBOA-Glc,[14] whereas DIMBOA-Glc is the major BXD in aerial parts of wheat and maize,[14,15] and HDMBOA-Glc is dominant in maize roots.[16] In maize, BXDs reach the highest total concentrations in seedlings less than 10 days after germination, and then decline as the plant grows[15] being differently allocated to leaves according to their age.[17] Different compounds show different profiles. For example, HDMBOA-Glc is especially induced after herbivory,[12,13] fungal attack[18] and upon jasmonic acid treatment[14,19] in maize.

Despite the relatively well documented effects of BXDs on insects, microorganisms and plants, little is known about their mode of action in biological systems. As will be discussed throughout this paper, BXDs show a range of chemical reactivities that could be responsible for their biological effects. Moreover, the mechanisms by which they exert toxicity or repellence are not necessarily the same, but may vary depending on factors such as pH and the physiology of the target organism.

Given the important role of BXDs, interactions with insects, microorganisms, and other plants, understanding more about this class of compounds is valuable from the viewpoint of pest control, plant breeding, ecology, and evolution of chemical defenses. Several authors have reviewed different aspects of BXDs, including their biological activities,[1,20] synthetic strategies,[21,22] chemical reactivities,[23] and biosynthetic evolution and genetics.[2,3] The present review aims to summarize and update the current knowledge about BXD chemistry with emphasis on the properties that may explain their wide range of biologically relevant activities. Studies on structure-activity relationships are discussed and mechanisms for biological activities are suggested, taking into account the specific features of each of the target organisms.

2. Chemical properties of BXDs

The benzoxazinones shown in Scheme 1 can be divided according to the nature of group R^1 as lactams (R^1=H), hydroxamic acids (R^1=OH), or *N*-*O*-methylated derivatives (R^1=OMe). These functional groups, together with the substituents R^2 and R^3 in the aromatic system, greatly influence the stability and reactivity of each compound and therefore their biological activities. The presence of a nitrogen heteroatom in the core structure of benzoxazinones is also considered to lower the stability of these molecules, which is essential to their reactivity.[24]

The acetal group, a feature common to all naturally occurring benzoxazinone glucosides, is remarkably stable[25] and requires the action of glucosidases for its hydrolysis. The resulting aglucones are cyclic hemiacetals, or lactols, that undergo oxo-cyclo tautomerism involving a fast, reversible ring opening reaction.[26] Proton NMR of HMBOA aglucone confirms that the closed form is predominant, as shown by a singlet at 5.60 ppm corresponding to the lactol proton, as well as the ratios between signals from the open and closed form in the aromatic region. No aldehyde proton is visible, probably because of the low intensity of the signal and hydration-dehydration equilibrium.[27] As discussed in detail below, the open form of benzoxazinone aglucones presents different reactivities than the closed tautomer and is involved in their degradation to benzoxazolinones.

As another consequence of oxo-cyclo tautomerism, the aglucones exist as racemic mixtures in solution. Capillary electrophoresis and liquid chromatography do not separate DIBOA and DIMBOA enantiomers, since their interconversion occurs rapidly and continuously during the timescale of these analytical procedures.[28,29] However, discrimination of DIBOA and DIMBOA enantiomers was achieved by NMR using chiral solvating agents, which form diastereoisomeric solvation complexes.[30] On the other hand, racemic mixtures of synthetic BXD methyl acetals have been successfully resolved by all three methods, confirming their stability towards hydrolysis and racemization by ring opening. It is interesting to note that all known benzoxazinone glucosides produced by plants are (2*R*)-2-β-D-glucosides[31-34] that upon glucosidase activity yield racemic mixtures of (2*R*) and (2*S*) aglucones.

The high activity of BXD β-glucosidases and the instability of aglucones make the extraction and quantitative analysis of BXDs in natural samples quite challenging. Once plant material is mechanically disrupted for the extraction process, plant β-glucosidases quickly hydrolyze benzoxazinone glucosides to aglucones. Furthermore, these aglucones spontaneously degrade to benzoxazolinones at different rates depending on the original compound. In general, different extraction methods yield different products.[35,36] Extraction with water followed by incubation at room temperature and heating allows the hydrolysis of BXD glucosides and further degradation to benzoxazolinones, which are recovered by this method. Maceration of plant material in water followed by acidification to pH 3 yields mostly benzoxazinone aglucones, which are more stable in such conditions. Extraction with boiling methanol[35] or grinding under liquid nitrogen followed by extraction with methanol and acidified water[12] avoids the enzymatic hydrolysis of glucosides, which are then the main compounds extracted by these methods.

Detection and quantitation of BXDs have been achieved by different techniques. Colorimetric methods based on the complexation of hydroxamic acids with Fe(III) have been developed, but are limited to this class of compounds and do not distinguish between different hydroxamic acids in extracts. GC-MS has also been used to analyze BXDs, but requires a derivatization step in order to increase the volatility and stability of the analytes. More recent studies rely on HPLC methods using C18 columns and detection by UV, MS, or MS^2. Fragmentation patterns of BXD derivatives studied using electrospray time-of-flight mass spectrometry (ESI-TOFMS) have revealed differences in the stability of BXDs in the mass spectrometer.[37] Higher sensitivity is achieved with triple quadrupole instruments operating in multiple reaction monitoring (MRM) mode, but transitions should be carefully chosen, as many BXDs have highly similar fragmentation patterns and share identical transitions and fragments. Extraction and analytical methods for BXDs have been thoroughly evaluated and compared.[36]

Large scale isolation from natural sources has been reported for abundant BXDs in plants. For example, DIMBOA can be extracted from maize seedlings,[38] while DIBOA-Glc and DIBOA can be obtained from rye.[22] Maize seedlings grown in dark conditions accumulate more DIMBOA(-Glc) than when illuminated, and therefore this method can be used to improve yields.[39] Semi-preparative LC on maize extracts can yield pure DIMBOA-Glc, DIMBOA, HMBOA-Glc, HMBOA, and HDMBOA-Glc.[12] BXDs have also been obtained synthetically, both as their natural structures and analogs with various substitution patterns and functional groups.[27,40,41] Chemical glucosylation of BXDs, which is particularly challenging due to the control of two stereogenic centers, has also been achieved in a stereoselective fashion.[42,43] Synthetic approaches to BXDs have been summarized elsewhere.[21,22] Such methodologies enable the access to standards, materials for bioassays, analogs for structure-activity studies, as well as labeled compounds for tracing experiments and mechanism elucidation.

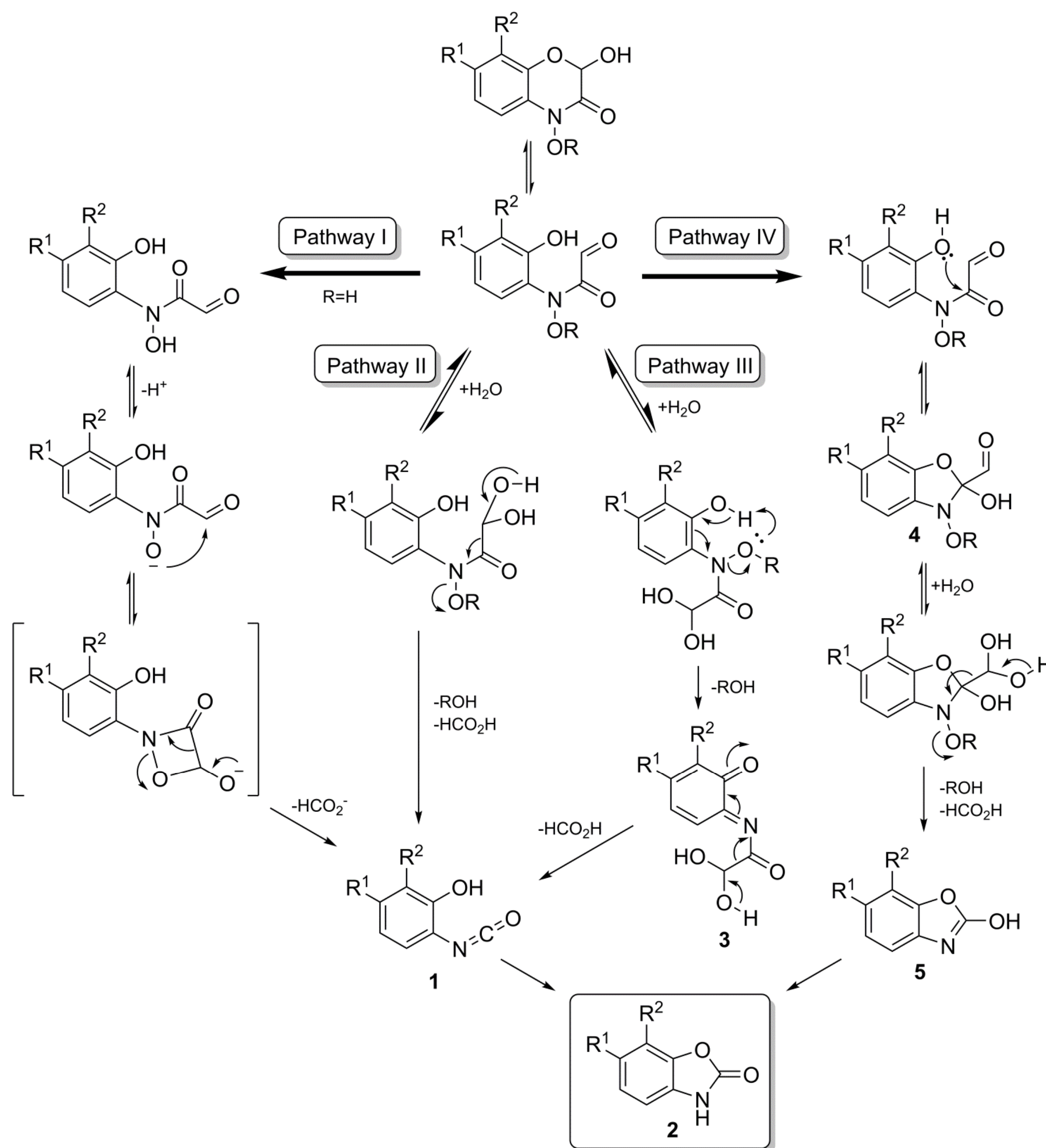
2.1. Degradation to benzoxazolinones

Due to harsh extraction methods, the first BXDs isolated were the benzoxazolinones BOA from rye and MBOA from maize and wheat, followed by the discovery that these compounds originate from DIBOA and DIMBOA, respectively.[44,45] Since then, the degradation of benzoxazinones to benzoxazolinones has been studied and deemed relevant to biological activities of BXDs. Several pathways for this degradation have been suggested, and are summarized in Scheme 3.

In general, lactams (N-H compounds) are not degraded to benzoxazolinones, whereas hydroxamic acids (N-OH) degrade readily and *N-O*-methyl derivatives degrade even faster. The half-lives of HDMBOA and DIMBOA aglucones are 1.8 h and 25 h, respectively, in buffered D_2O at pH 5.5 and 24°C.[46] This is caused by the different leaving group character of the *N*-substituent groups in these molecules, even though other structural features may influence degradation rates. Under pH 8.5 and 48°C, DIMBOA degrades faster than DIBOA, which is attributed to the strong electron donating effect from the methoxy group at position 7.[27] As will be discussed in more detail below, an electron rich aromatic ring stabilizes the positive charge on nitrogen during the transition states and accelerates degradation to benzoxazolinones. On the other hand, DIM_2BOA, with methoxy groups on positions 7 and 8, degrades more slowly than DIMBOA.[27]

Pathway I (Scheme 3) was proposed by Bredenberg *et al.*,[47] after observing by ^{14}C labelling that the carbon atom in position 2 is eliminated as formic acid during benzoxazinone degradation.[48] The authors suggested a mechanism in which the concentration of hydroxamic acid monoanion, dictated by the pH of the medium, is critical to the reaction rate. This species would act as an internal nucleophile by attacking the aldehyde carbon, forming the isocyanate **1**, presumably via a four membered ring, and ultimately leading to the benzoxazolinone **2**. This pathway is supported by the fact that DIMBOA degradation is faster in organic solvents with high donor numbers (and thus better Lewis bases), which form stronger hydrogen bonds and render the hydroxy group more nucleophilic.[49,50]

Pathway II was suggested by Grambow *et al.*[51] as a way to complement the previous pathway and explain why *N-O*-methylated derivatives also degrade to benzoxazolinones. HDMBOA would not react via pathway I, because of the poorly nucleophilic methoxy group bound to the nitrogen atom. Thus, another mechanism was suggested in which the aldehyde hydration of the open form would undergo a Grob-like heterolytic cleavage, releasing formic acid and the *N*-substituent group, and generating the isocyanate **1**. This mechanism explains why HDMBOA (N-O-methylated derivative) degrades much faster than DIMBOA (hydroxamic acid), while HMBOA (lactam) does not degrade at all in such conditions. Degradation rates by this mechanism strongly depend on the leaving group character of the *N*-substituent group: OMe > OH >> H.



Scheme 3. Proposed mechanisms for the degradation of hydroxamic acids (R = H) and *N*-*O*-methyl derivatives (R = Me) to benzoxazolinones (**2**).

Pathway III was introduced by Maresh *et al.*[46] during experiments on HDMBOA degradation. In this route, the open form phenol assists the elimination of the *N*-substituent group, forming an *o*-imidoquinone intermediate **3**, which was characterized by the authors. This intermediate can then undergo a fragmentation equal to that observed in pathway II, yielding the isocyanate **1** and the benzoxazolinone **2.** In fact, the kinetic data for HDMBOA degradation, as followed by NMR, do not fit pathway III as a unique and linear mechanism. The authors considered both pathways II and III to be

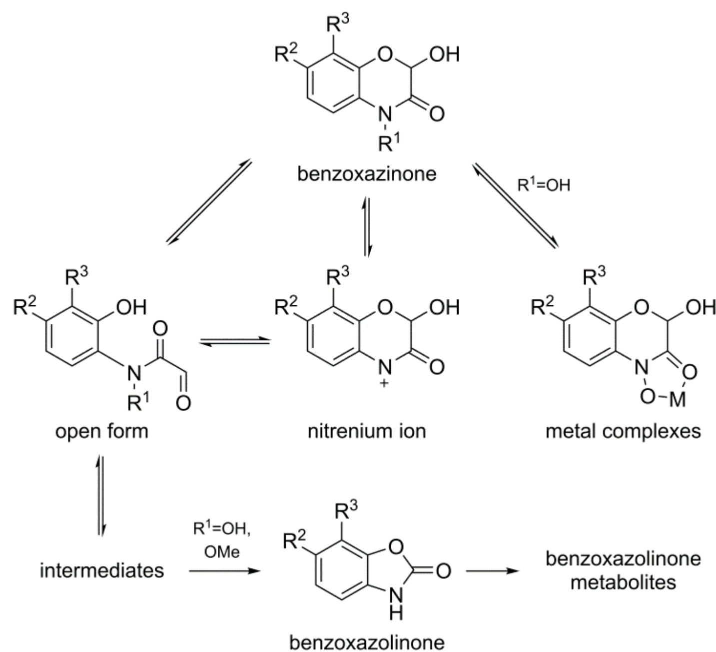
competing during degradation of HDMBOA, and included side reactions of the open form hydrate and *o*-imidoquinone into their kinetic model.

Pathway IV, proposed by Smissman *et al.*,[52] was the first to consider an active role of the phenol function in the degradation reaction. The authors suggested this possibility as an alternative to pathway I since open chain analogs of DIMBOA with the phenol either absent or ether-protected were not observed to degrade to benzoxazolinones. Therefore, the hydroxamic acid and phenol moieties may be required for degradation to occur. In this pathway, the phenol in the open form acts as a nucleophile by attacking the amide carbonyl, producing a 5-membered closed form **4** that is in equilibrium with the other forms. Similarly to pathways II and III, after aldehyde hydration a fragmentation takes place eliminating formic acid and the *N*-substituted group. In this mechanism, however, no isocyanate is formed, but rather the less stable tautomer **5** of the benzoxazolinone, which rapidly rearranges yielding the final benzoxazolinone **2**.

The proposed mechanisms all rely on the basicity and nucleophilicity of the phenol and *N*-substituent groups, and the leaving group character of the latter. Since such parameters can vary according to substitution patterns, stereoelectronic and solvent effects, and pH, any of these pathways can in theory be favored depending on the benzoxazinone structure and medium conditions. The decomposition rates of DIMBOA over different pH values show an asymmetric bell-shaped curve with a maximum around pH 9.0, which is consistent with DIMBOA acting as a diprotic acid in water (pK_a1 = 6.9, pK_a2 = 10.9) and each species (DIMBOA and its two conjugated bases) following a different degradation route.[49] Furthermore, MBOA yield from DIMBOA is not quantitative,[53] suggesting that other reactions and products might be involved in benzoxazinone degradation, with other potentially reactive and biologically relevant intermediates. Further mechanistic studies relying on modern analytical techniques and comparisons between synthetic BXD analogs could help understand the complexity of benzoxazinone degradation reactions and lead to the discovery of novel pathways, other intermediates and end-products with biological activities.

3. Possible modes of action

Due to their unique structural features, BXDs may undergo a wide range of reactions with biological relevance. Although the specific modes of action are not yet clear, many studies point to important characteristics of these compounds that might be associated with their described biological activities: (i) electrophilicity of the open form; (ii) electrophilicity of nitrenium ions; (iii) ability of hydroxamic acids to complex metal cations; and (iv) reactivity of benzoxazolinones and derivatives. These features are summarized in Scheme 4 and are discussed below.

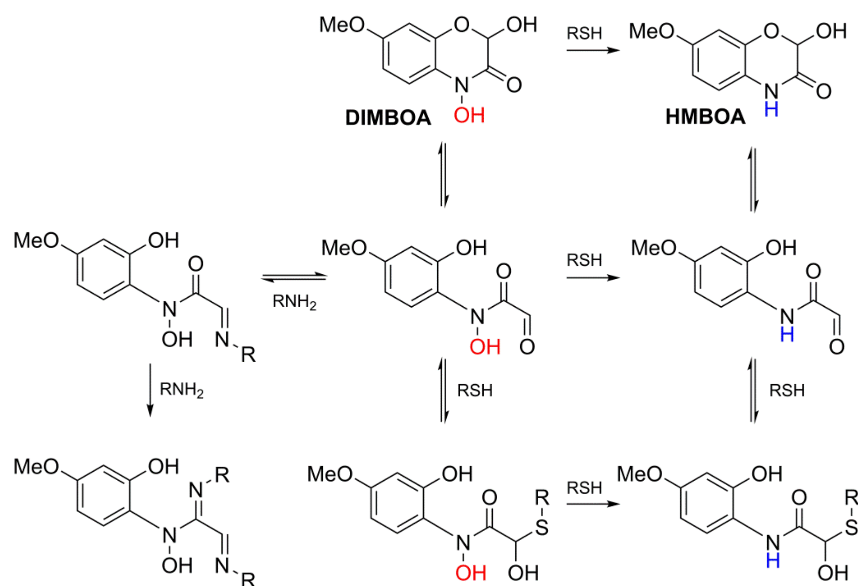


Scheme 4. Summary of possible benzoxazinoid modes of action.

3.1. Electrophilicity of the open form

The benzoxazinone open form originating from oxo-cyclo tautomerism is an α-oxo-aldehyde, which is expected to be a potent electrophile. Therefore, it has been suggested that this group might react with nucleophilic residues in proteins such as thiols and amines, causing enzymatic inhibition.

DIMBOA has been shown to react with butylamine, from which the imino products observed in Scheme 5 were identified and the reaction kinetics characterized.[54] No reaction was observed for the 2-*O*-methyl acetal derivative of DIMBOA, suggesting that ring opening is required for the formation of imino adducts. Reaction with HMBOA, a lactam, was slower than for DIMBOA, possibly due to the electron-withdrawing effect from the hydroxamic acid hydroxy group. An analogous reaction was observed with *N*-α-acetyl-lysine, a model compound for the ε-amino groups of lysine residues in enzymes.



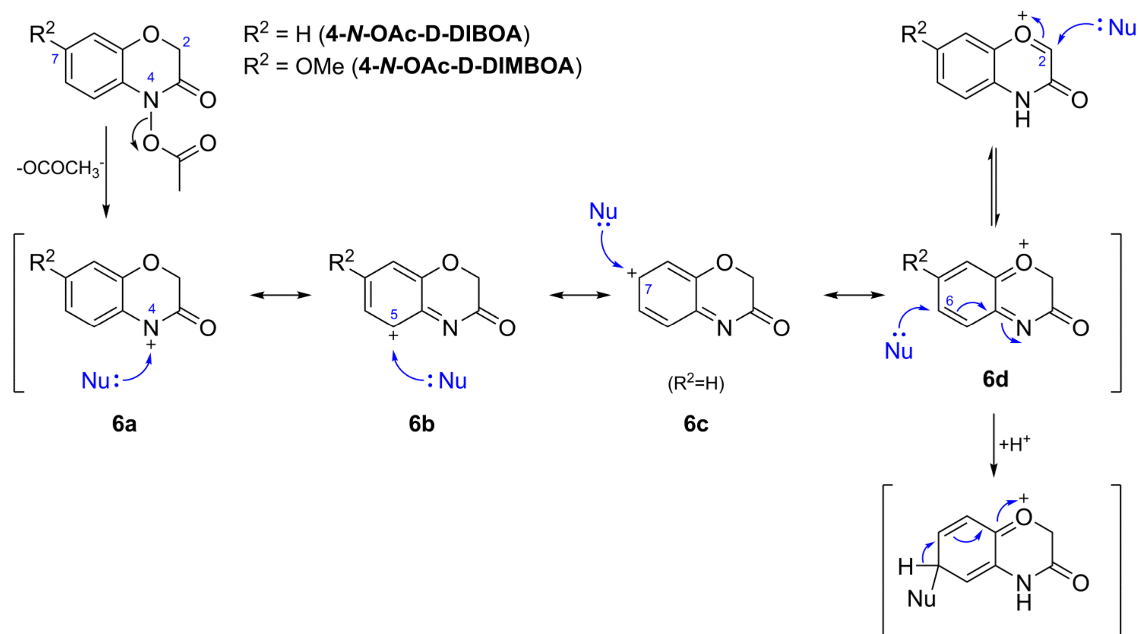
Scheme 5. Reactions of DIMBOA with amines and thiols.[27,54,55]

Thiols such as ethanethiol, cysteine, mercaptoethanol, and dithithreitol can also react with DIMBOA, forming the lactam HMBOA, and hemithioacetals originating from the open forms of DIMBOA and HMBOA, shown in Scheme 5.[55] The species to react were found to be the undissociated hydroxamic acid and the thiolate anion; thus reaction rates were correlated to thiol pK_a values. Hemithioacetals of DIMBOA and HMBOA open forms were isolated in low yields from the reaction with ethanethiol, but were shown via ^{1}H NMR to be the predominant form in solution with an excess of thiol.[27] The pattern of chemical shifts shows that the oxo-cyclo tautomerism is trapped in the open form by thiolate attack on the aldehyde carbon. The fact that the lactam is the major isolated product from this reaction is presumably due to the instability of the hemithioacetal function in basic medium. Reduction of DIMBOA to HMBOA by thiols is considered to be a consequence of the reactivity of the nitrenium ion as discussed in the next section.

3.2. Electrophilicity of the nitrenium ion

The nitrogen atom can also be an electrophilic site in the benzoxazinone skeleton, especially upon elimination of the *N*-subtituent group and formation of a nitrenium ion. A comprehensive study on this reactivity was done with 4-acetoxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (4-*N*-OAc-D-DIMBOA), referred to as AMBOA in the original article, a synthetic derivative of 2-dehydroxy DIMBOA.[41] After heterolytic *N-O* cleavage, elimination of the acetoxy group at position 4 generates a nitrenium ion, which behaves as a multi-centered electrophile as shown in Scheme 6. Reactions with

nucleophiles such as phenols, anilines, thiols, pyrroles, indoles, diazoles and pyridine gave rise to products from nucleophilic attack on positions 2, 4, 5, 6, and 7, with regioselectivities varying according to the nucleophilic atom involved (carbon, nitrogen, or sulfur). Furthermore, 4-*N*-OAc-D-DIMBOA reacts with amino acid derivatives such as protected tyrosine, histidine, and tryptophan. Adducts with guanine (but not other nucleotides) were observed after incubation of 4-*N*-OAc-D-DIMBOA with calf thymus DNA followed by enzymatic hydrolysis, and direct incubation with either 5'-deoxyguanylic or 5'-guanylic acids. This reaction accounted for as much as 4% of total guanine residues present under the experimental conditions, and occurred between the position 8 of guanine and the BXD nitrogen atom.[56]



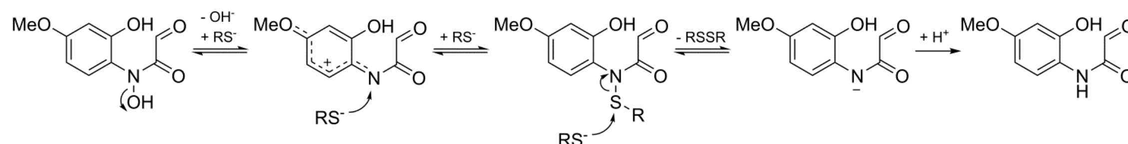
Scheme 6. Multi-centered electrophile formed from nitrenium ion (adapted from Hashimoto *et al.*[41]).

It is important to note that all reactions described for 4-*N*-OAc-D-DIMBOA are associated with the closed form of this derivative, since the absence of a hydroxy group at position 2 eliminates the oxo-cyclo tautomerism present in other benzoxazinones. However, introduction of a 2-hydroxy group enhanced the reactivity of the 4-acetoxy derivative towards nucleophiles. Moreover, 4-*N*-OAc-D-DIBOA, which lacks the 7-OMe group, showed reduced yields in reaction with phenol, suggesting that an electron donating group at position 7 can facilitate N-O heterolytic cleavage.[41] Despite being synthetic BXD derivatives, 4-*N*-OAc-D-DIMBOA and its analogs could be generated from natural BXD hydroxamic acids after activation by acyltransferases, which are important enzymes in xenobiotic metabolism. After incubation with the S-9 fraction from rat livers, the supernatant of a 9000*g* centrifugation containing high levels of enzymes of xenobiotic metabolism, D-DIMBOA (the

hydroxamic acid analog of 4-*N*-OAc-D-DIMBOA) showed increased mutagenic activity, suggesting the enhancement of its reactivity by metabolic activation.[57]

Formation of nitrenium ions is also involved in hydroxamic acid reactions in strongly acidic medium. DIBOA and DIMBOA analogs lacking the 2-hydroxy group (D-DIBOA and D-DIMBOA, respectively) react with HCl forming mainly the 7- and 5-chloro substituted products, respectively.[58] Such reactions are supposed to occur via the nitrenium ion intermediates shown in Scheme 6, but in this case the strongly acidic medium causes the protonation of the hydroxamic acid hydroxy group, making it a better leaving group and facilitating N-O heterolytic cleavage.

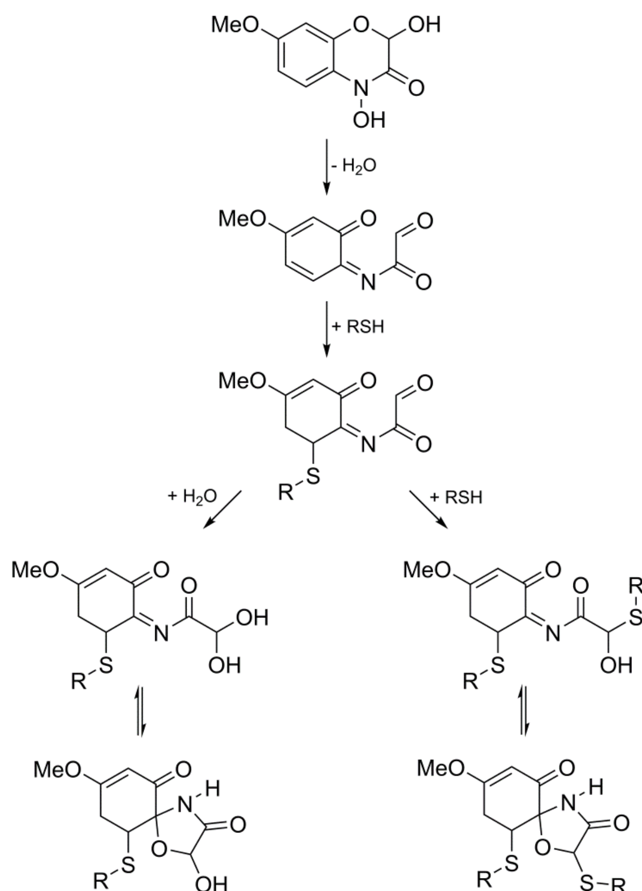
Reduction of DIMBOA to HMBOA by thiols, mentioned in the previous section, is proposed to involve the formation of nitrenium ions, as shown in Scheme 7. This mechanism is supported by the striking increase in the rate of reduction to lactam when the 7-OMe group is present, compared to other analogs.[27] Such substituents can stabilize the positive charge on the nitrogen atom by resonance, facilitating the following nucleophilic attack from the thiolate. In theory, the formation of the nitrenium ion could occur on either the closed or open forms of the hydroxamic acid, as shown in Scheme 5. However, the participation of the 7-OMe group suggests that the nitrogen atom is conjugated with the ring. This situation may be more favored in the benzoxazinone open form, since without the constraint from the heterocycle, the nitrogen lone electron pair can overlap the aromatic π-system more efficiently. Indeed, DIMBOA analogs unable to undergo oxo-cyclo tautomerism, such as 2-dehydroxy and methyl acetal derivatives and the analog lacking the heterocyclic oxygen, do not easily react with mercaptoethanol, requiring harsh conditions to provide only small yields of the lactam.[27]



Scheme 7. Proposed mechanism for the reduction of DIMBOA to HMBOA by thiols.

Reactions between DIMBOA and thiols, including mercaptoethanol and glutathione, an important thiol nucleophile in biological systems have been investigated by Dixon *et al*.[59] In this case, however, lactams were not the major observed product, but rather spirocyclic adducts with either one or two thiol equivalents were formed (Scheme 8). Such adducts had their aromatic rings remarkably altered, as shown by ^{1}H NMR. The reaction was suggested to involve an *o*-imidoquinone derivative originating from the loss of water from DIMBOA, which can be attacked by the thiol at position 5. Subsequently, the aldehyde carbon can be attacked either by water, forming a hydrate, or another thiol molecule, forming a hemithioacetal. Both compounds then undergo an internal nucleophilic

attack forming a 5-member ring spiro-fused to the original BXD 6-member ring. DIMBOA also formed an adduct irreversibly with a model enzyme (*Arabidopsis thaliana* glutathione transferase *At*GSTF8) targeting a single cysteine residue, as confirmed by tryptic digestion and MS/MS sequencing. Efficient reactions between DIMBOA and thiols suggest that BXDs can potentially cause damage to target organisms by depleting glutathione levels, but more studies are necessary to confirm such pro-oxidant activity. Although some DIMBOA reduction to HMBOA was observed, the products from these reactions with thiols were notably different from the ones obtained previously.[27,55] Formation of spirocyclic adducts with thiols could have been favored by the solvent system used (45% acetonitrile in aqueous buffer). Mixtures of water and organic solvents can modify the pH from corresponding buffers and lead to altered pK_a values of dissolved acids as well as causing solvation effects, all factors that could influence the reactivity of DIMBOA and thiols in solution.



Scheme 8. DIMBOA spiroadduct formation with thiols (adapted from Dixon *et al.*[59]).

Interestingly, the canonical structures proposed for the nitrenium ion in Scheme 6 include an *o*-imidoquinone derivative (**6d**). Reactions via the nitrenium ion are supposed to involve BXDs in a closed, positively charged form, whereas reactions via neutral imidoquinones such as **3** (Scheme 3)

should involve open form, neutral species. However, due to oxo-cyclo tautomerism, the products from both reaction pathways are indistinguishable. Indeed, imidoquinones are expected to react with nucleophiles at different sites, including the nitrogen atom, depending on conditions and the nature of the nucleophile used.[41,61] Even DIMBOA, with a poor leaving group at the nitrogen atom, can react following a nitrenium/imidoquinone pathway,[58,59] suggesting that formation of these intermediates is not only dependent on the *N*-substituent, but also the conditions used. It can be assumed that reactions via the nitrenium ion or neutral *o*-imidoquinone are similar and either can be favored under different conditions. Their competition seems to be dictated by pH conditions and structural features, such as the possibility of ring opening and stereoelectronic effects that might assist N-O heterolytic cleavage with elimination of a neutral or negatively charged fragment.

3.3. Coordination properties of hydroxamic acids

Hydroxamic acids and other compounds known collectively as siderophores are well known for their metal ion chelating properties, and are used by microorganisms, fungi, and plants in order to sequester and solubilize Fe(III) from the environment. Siderophores are also used by pathogenic fungi and bacteria to scavenge iron from their host organisms. After coordination to iron, siderophore complexes are taken up by microbial cells via specific transport systems present in the outer membrane and delivered to the cytoplasm.[62,63] Sideromycins, antibiotics covalently linked to siderophore moieties, take advantage of this recognition system and are potent naturally occurring antibiotics. The active transport of such compounds greatly reduces their minimal inhibitory concentration by enhancing delivery to cellular targets.[64]

BXDs with a hydroxamic acid function can potentially chelate metals and exert biological effects related to siderophore activity. The ability to form complexes with Fe(III) has been exploited by colorimetric methods for the detection and quantification of hydroxamic acids in BXD studies.[65] Other than iron, complexation of DIMBOA with Zn(II), Cu(II), and Mn(II) has also been observed. At the concentration levels found in plants, metal cations are complexed more by DIMBOA than by citric and malic acid, two abundant chelators from maize exudates.[66,67] As investigated by ESI-MS, lactams and methyl derivatives of hydroxamic acids do not form complexes with Fe(III).[68] Interestingly, glucosides such as DIMBOA-Glc and DIBOA-Glc have also been shown to coordinate to Fe(III),[65,69] and the similar stability constants for Fe(III) complexes with DIMBOA and DIMBOA-Glc suggest that the presence of the sugar moiety has little effect on BXD coordinating properties.[65]

Due to their ability to chelate metals, BXDs exuded by plant roots have been proposed to play a role in nutrient uptake and defense against metal toxicity. Roots of maize, wheat, and rye seedlings greatly vary in the levels of hydroxamic acids exuded in response to Fe(III) content in the growth medium.[70,71] Iron(III) complexes with DIBOA-Glc and DIMBOA-Glc are taken up by maize roots, but also by rice and oat which do not produce hydroxamic acids. Exogenous BXD glucosides absorbed

by roots of rice and oat as iron complexes can be detected in the shoots, suggesting the transport of these compounds between organs. In the leaves of iron-deficient maize, administration of Fe(III) hydroxamate complexes alleviates chlorotic symptoms and increases chlorophyll content of maize.[72] These results suggest that hydroxamic acids might be involved in iron uptake by cereals and could also be incorporated by other plants.[73]

DIMBOA might also serve as a defense against aluminum toxicity in plants. Upon Al(III) treatment, maize lines resistant to aluminum increased BXD levels in roots, especially in root tips, the region most sensitive to Al(III) toxic effects. DIMBOA also reduced callose accumulation (an indicator of cell damage that is a well described response to Al toxicity) in roots of maize lines susceptible to Al(III) that were treated with this metal. Binding of Al(III) to DIMBOA was measured by fluorescence quenching.[74]

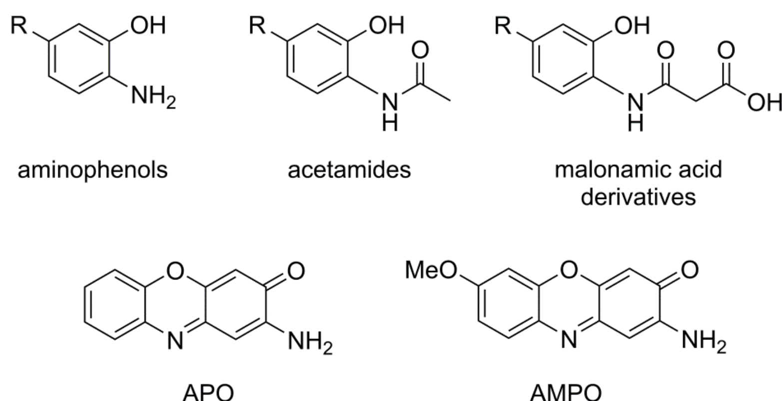
However, the importance of metal complexation by BXD hydroxamic acids in natural contexts is not yet well understood. More detailed studies are needed in order to confirm the role of BXDs in the uptake of iron and possibly other metals by plants, and distinguish the importance of their apparent siderophore behavior from their associated antimicrobial activities. It is important to note that coordination to metal cations might also be relevant to BXD glucosides, normally considered to be devoid of any toxicity or biological activity. The inhibition of enzymatic activity by BXD hydroxamic acids and derivatives could also be promoted by the chelation of such compounds to metal cofactors.[75]

3.4. Benzoxazolinones and related compounds

Even though benzoxazolinones are implicated in many biological activities, their modes of action and structure-activity relationships are even less studied than those of benzoxazinones. Structurally, benzoxazolinones resemble important signaling compounds such as melatonin, serotonin, and tryptophan. Effects on the central nervous system have been suggested to explain the stimulation of the reproductive system and appetite loss caused by MBOA in mammals.[76] In plants, benzoxazolinones are able to inhibit auxin-induced growth in roots and coleoptiles, presumably by modifying the binding affinity of auxins to receptor sites.[77-79] Even though the exact molecular mechanisms are not known, these compounds are suggested to be involved in phototropism in maize and possibly in allelopathic activities towards competing plants. The possible effects of benzoxazolinones on signaling have not been well explored in insects or microorganisms.

Benzoxazolinone metabolism by soil microorganisms has been relatively well studied within the context of the allelopathic effects of BXDs.[80] Transformation products such as aminophenols, aminophenoxazinones, acetamides, and malonamic acid derivatives have been characterized and are presented in Scheme 9. The aminophenoxazinones APO and AMPO are suggested to play a role in

allelopathy[75,81,82] and possibly interactions with other microorganisms, nematodes, and root herbivores.



Scheme 9. Transformation products of benzoxazolinones in soil

3.5. Physico-chemical properties

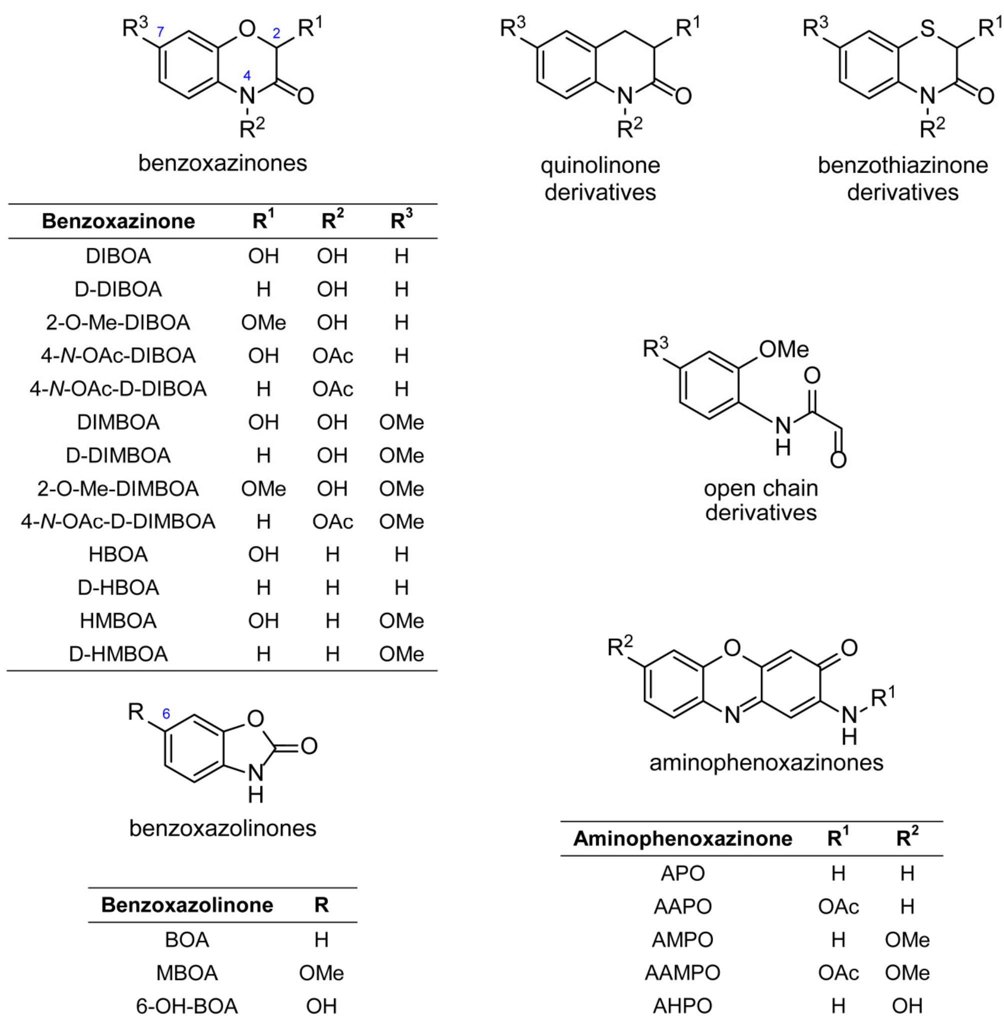
Although not directly involved in the mechanisms toxicity, some of the physico-chemical properties of BXDs are expected to influence their overall activity. For example, the diversity of ring and heteroatom substitution patterns found in BXDs modulates their lipophilicity, which can greatly influence their diffusion across cellular membranes or through soil and so affect delivery to biological targets. In soil TLC experiments, DIMBOA showed mobility intermediate between those of phenolic acids and other compounds such as vanillin and coumarin,[83] but no other BXDs were compared. BOA and MBOA showed low mobility when applied on the soil surface, being mostly recovered (71% and 97% respectively) in the top 1 cm of the soil profiles.[84]

Lipophilicity also seems to influence the biological activities of BXDs, as shown by the structure-activity relationship studies discussed on more detail on the following section. The substitution patterns of BXDs are also expected to control their acidity, which can be an important factor in the solubility, chelating properties, and diffusion under different pH conditions.

4. Structure-activity relationship studies

Extensive work has been performed in order to rationalize the influence of BXD structure on activity against a wide range of organisms. These studies have been greatly aided by the established

synthetic routes to BXDs enabling access to non-natural analogs that differ in their modes of action. Therefore, a critical evaluation of each aspect of BXD reactivity in connection to different biological activities is possible. Structure-activity relationship studies are discussed within the context of BXD biological activities in this section and interpreted according to the modes of action presented in the previous sections. Some relevant structures used for these experiments are shown in Scheme 10, including natural and non-natural BXDs analogs, as well as aminophenoxazinones, BXD soil metabolites tested for phytotoxicity.



Benzoxazinone	R^1	R^2	R^3
DIBOA	OH	OH	H
D-DIBOA	H	OH	H
2-O-Me-DIBOA	OMe	OH	H
4-*N*-OAc-DIBOA	OH	OAc	H
4-*N*-OAc-D-DIBOA	H	OAc	H
DIMBOA	OH	OH	OMe
D-DIMBOA	H	OH	OMe
2-O-Me-DIMBOA	OMe	OH	OMe
4-*N*-OAc-D-DIMBOA	H	OAc	OMe
HBOA	OH	H	H
D-HBOA	H	H	H
HMBOA	OH	H	OMe
D-HMBOA	H	H	OMe

Benzoxazolinone	R
BOA	H
MBOA	OMe
6-OH-BOA	OH

Aminophenoxazinone	R^1	R^2
APO	H	H
AAPO	OAc	H
AMPO	H	OMe
AAMPO	OAc	OMe
AHPO	H	OH

Scheme 10. Compounds used in structure-activity relationship studies. For consistency, some names have been modified from those used in the original references.

4.1. Enzymatic inhibition

Unfortunately no comprehensive *in vitro* studies have been carries out to compare the inhibition of individual enzymes by a range of BXD structures. However, the comparison of experiments with a few BXDs can give us hints about the mechanisms by which these compounds act on enzymes.

DIMBOA was shown to inhibit papain, a cysteine protease, by reacting with a cysteine residue in the active site.[85] Although D-DIMBOA, an analog lacking a lactol moiety, inhibited papain in a similar fashion, HMBOA, a lactam, did not display inhibition. Since the former compound does not undergo oxo-cyclo tautomerism and the latter does, the electrophilicity of the open form does not seem to play a role in enzyme inhibition in this case. Instead, the presence of a better leaving group as the *N*-substituent seems to be responsible for this activity, suggested to involve the reaction between the cysteine thiol group and the nitrogen atom via a nitrenium/imidoquinone derivative. Indeed, the inhibition was reversed by addition of dithiothreitol, which is consistent with the reduction of DIMBOA to HMBOA by reaction with thiols (Scheme 7). DIMBOA was also observed to inhibit α-chymotrypsin, a serine protease.[86] In this case, however, 2-*O*-Me-DIMBOA did not inhibit this enzyme, indicating that ring opening is not relevant for activity. The authors proposed that the BXD α-oxo-aldehyde reacts with the serine residue in the active site, causing the observed inhibition.

These studies indicate that even within the same enzyme class (digestive proteases), BXDs may cause inhibition by different modes of action depending on their target. The aldehyde and nitrogen electrophilic sites in the BXD structure seem to have differential reactivities depending whether the nucleophile is a thiol, hydroxyl, or amine groups, indicating that enzyme inhibition mechanisms depends on the residues at the active site as well as other conditions that modulate BXD reactivity patterns. The inhibition of digestive proteolytic enzymes is normally associated to the overall antifeedant and toxic effects of BXDs towards herbivores. Considering that insects with a alkaline gut lumen (such as most lepidopterans) rely mainly on serine-based proteinases, while insects with acidic guts (such as coleopterans) rely on thiol-based proteinases,[87] distinct chemical modes of action and molecular targets could become more relevant in different target insects, even though the physiological mode of action (inhibition of proteinases) can be considered the same.

4.2. Activities against insects

The effect of BXD analogs in inhibiting the growth of the lepidopteran chewing herbivore *Ostrinia nubilalis* was compared.[88] Among the natural BXDs tested, DIMBOA was the most toxic, followed by DIBOA and DIM_2BOA. Lactams such as HMBOA and HBOA did not significantly inhibit growth, and neither did hydroxamic acids unable to undergo ring opening (2-dehydroxy, methyl

acetals, and quinolinone derivatives). No clear correlation was found between toxicity and either degradation rates to benzoxazinones or rates of reduction to lactam by thiols. The involvement of benzoxazolinones formed by degradation during the experiment was not considered important, since MBOA required concentrations 10-20 times higher than DIMBOA to exert comparable toxic effects. However, it is important to note that the alkaline pH of the caterpillar gut (around 9 in *Spodoptera frugiperda*[89]) has an important effect on benzoxazinone stability. Analogs that degrade too quickly in such conditions might not persist long enough to manifest toxicity. Natural hydroxamic acids like DIMBOA, however, are suggested to show a balance between stability and reactivity that allows them to have considerable activity against herbivores. Although the range of compounds tested in this study was not wide enough to allow for more general interpretations, it seems that both the hydroxamic acid function and the possibility of ring opening are important for activity against caterpillars. However, either of these features alone does not seem to be sufficient, since lactams and analogs with the lactol function absent or blocked did not show activities. Since lactams can still become reactive aldehydes by ring opening, and compounds without a C-2 lactol function can still form metal complexes, these two modes of action do not seem to be critical for the observed activity. On the other hand, the simultaneous presence of a better leaving group on the nitrogen atom and the possibility of ring opening could facilitate the formation of reactive intermediates like nitrenium ion/imidoquinone derivatives. In fact, the *N*-*O*-methylated derivative HDMBOA is considered to be even more toxic to caterpillars than DIMBOA,[12] which supports that this mechanism might play an important role on inhibiting insect growth.

BXDs display both antifeedant and insecticidal activities towards a wide range of aphid species.[1] Escobar *et al.*[90] compared the antifeedant and insecticidal activities of various BXD analogs towards the aphid *Sitobion avenae*. In pairwise comparisons of benzoxazinones, the presence of a 7-OMe electron-donating group, the presence of a sulfur instead of oxygen as the heteroatom (benzothiazinone derivatives), and the possibility of ring opening were shown to increase antifeedant and mortality indices. Such a clear trend was not observed for comparisons between lactams and hydroxamic acids. Interestingly, antifeedant and insecticidal activities do not seem to be strictly correlated, suggesting that the underlying mechanisms for the two activitites do not completely overlap. For example, among the tested benzoxazolinones MBOA showed high deterrence levels but low toxicity, while 6-OH-BOA showed comparatively higher mortality but lower deterrence. HMBOA and its open chain analog with a protected phenol gave similar mortality indices towards aphids, suggesting that the electrophilicity of the open form might be important for toxicity, but the antifeedant activity of the latter was much higher. In general, benzothiazinones showed enhanced antifeedant and mortality indices, even for structures with a protected or absent lactol group. For other BXD analogs, however, toxicity seems to be enhanced by the possibility of ring opening and presence of a hydroxamic acid function, similarly to the activity observed for *O. nubilalis*. As for activity against caterpillars, the benzoxazolinones tended also to be less toxic to this aphid than benzoxazinones.

In a comparison of the antifeedant activity of BXD analogs towards a second aphid species, *Rhopalosiphum padi*,[91] the possibility of ring opening seemed to contribute to activity, and hydroxamic

acids were more active than lactams. However, more lipophilic derivatives had lower activities, suggesting that lipophilicity may reduce diffusion of compounds into the phloem fed on by aphids or subsequently into insect tissues. In this experiment, however, DIBOA was more deterrent than DIMBOA, in contrast to the experiment with *S. avenae*.[90] It is important to point out that the two aphid experiments differ in their protocols. The *S. avenae* experiment used BXDs in artificial diets (sucrose solution in sachets), whereas the *R. padi* experiment involved BXDs sprayed on barley leaves. It is not clear how efficiently BXDs sprayed on the leaf surface diffuse into cells, whether they are metabolized by the plant, and whether their tissue distribution actually mimics what an aphid would find in a BXD containing plant. On the other hand, the behavior of aphids in relation to BXDs might be altered by feeding on artificial diets.

Due to the compartmentalization of BXDs in plants as glucosides and their activation by β-glucosidases, the feeding behavior of insects likely influences the extent to which they are exposed to such compounds. Piercing and sucking insects such as phloem-feeding aphids could potentially avoid BXD activation by avoiding contact with plant glucosidases. However, since aphids are highly dependent on microbial symbionts, any antimicrobial effect of BXDs or their glucosides could also contribute to their activities on these insects. These make the study of BXD effects and mode(s) of action in sucking insects more challenging. In addition, the glucosides DIMBOA-Glc and HDMBOA-Glc showed antifeedant and insecticidal activities towards aphids,[92] suggesting that these activities derived from the BXD glucosides themselves or that the aphids are able to hydrolyze BXD glucosides with their own enzymes.

4.3. Activity against microorganisms

The antimicrobial activities of BXD hydroxamic acids were assessed against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*.[93] for A higher electron-donating character of the 7-substituent increased activity, whereas the possibility of ring opening did not seem to have a consistent influence on activity. This indicates that the electrophilicity of the open form and the degradation to benzoxazolinones are not important for antimicrobial activity. In another study investigating the activity of different BXD analogs towards *C. albicans*,[94] hydroxamic acids showed higher antifungal activity than lactams, while glucosides had no activity. Surprisingly, 4-*N*-OAc-DIBOA displayed similar antifungal activity to DIBOA, suggesting that the electrophilicity of the nitrenium ion does not contribute significantly to this activity. When evaluated with *C. albicans*, the 2-dehydro and methyl acetal derivatives showed higher activities than the corresponding lactols. Therefore, antifungal (and possibly antibacterial) activities seem to be highly dependent on the presence of a hydroxamic acid function. This suggests a mechanism in which these compounds exert toxicity due to chelation properties and/or are actively incorporated by microbial cells due to their siderophore-like

nature. However, as DIBOA-Glc and DIMBOA-Glc had no activity, possibly due to their high hydrophilicity, passive diffusion of BXDs through microbial membranes should also be considered.

Analogs of the benzoxazolinone BOA with different 6-substituents were evaluated against *S. aureus*, *E. coli*, and *C. albicans*.[95] In general, the fungus *C. albicans* was more sensitive to structural changes compared to the bacteria, and showed inhibitory concentrations on the same range as those observed for BXD hydroxamic acids. Antifungal activity increased with higher lipophilicity and was dependent on electronic effects from substituents in the aromatic ring. Activity increased with polarization of the N-H bond, which is promoted by both electron-donor (such as methoxy) and electron-acceptor (such as nitro) groups at position 6.

4.4. Activity against plants

The antialgal activity of BXD analogs was investigated against *Chlorella xantella*.[94] Due to its similarity with higher plants, this alga has been recommended as a model for phytotoxicity evaluation. The blocking of ring opening did not consistently affect antialgal activity, which was similar for 2-dehydro derivatives and lactols. This suggests that the electrophilicity of the open form is not essential to the observed antialgal activities. Glucosides such as DIBOA-Glc and DIMBOA-Glc had no activity, probably due to their high hydrophilicity. Among aglucone analogs, activity increased with the increase of lipophilicity, but was not clearly correlated with the electronic effects of aromatic ring substituents. The presence of a 4-*N*-acetoxy group decreased activity, suggesting that antialgal activity does not depend on nitrenium ion electrophilicity.

Phytotoxicity was evaluated for a range of concentrations of natural and synthetic benzoxazinones, benzoxazolinones, and their soil degradation products against wheat, onion, cress, lettuce and tomato.[96] The growth of all species was strongly inhibited by some of the tested BXDs, except for wheat, which was only moderately inhibited, suggesting that wheat is not affected by allelopathic interactions involving BXDs. In general, root length was more affected than shoot length. The benzoxazolinones BOA and MBOA were only slightly inhibitory even at the highest tested concentrations, and even promoted root growth in wheat. Thus if DIBOA and DIMBOA degraded to the corresponding benzoxazolinones during the experiment, their toxicity may have been underestimated. For most species, DIBOA and DIBOA-Glc gave similar root growth inhibition profiles, suggesting that seedlings are able to incorporate both and possibly hydrolyze glucosides with their own glucosidases. Among natural BXDs, the hydroxamic acids DIBOA and DIMBOA were toxic and the lactams HBOA and HMBOA were not. However, the 2-dehydro derivatives of both hydroxamic acids and lactams showed higher phytotoxicity, meaning that ring opening is not involved in such activity, and the presence of a hydroxamic acid function might contribute, but is not essential to activity. The acetyl derivative 4-*N*-OAc-D-DIBOA was one of the most active compounds, with considerable toxicity even in lower concentrations. On the other hand, lower toxicity was observed for

4-*N*-OAc-D-DIMBOA when compared to D-DIMBOA (2-dehydro-DIMBOA). It seems that the influence of 4-*N*-substituents may not be strictly correlated to their stereoelectronic effects, but also to other substituents and the overall lipophilic character of the molecule. Among the degradation products, only the aminophenoxazinone APO showed high activity. The inhibitory effects of aminophenoxazinones seem to increase as the lipophilicity of the compound decreases. The same compounds were tested and similar results were obtained for the weeds *Avena fatua* (wild oat) and *Lolium rigidum* (rigid ryegrass).[97] Both species belong to the family Poaceae, suggesting that the resistance to BXDs observed in wheat is not widespread in grasses. In another study on *L. sativum*, similar results were observed for root growth and α-amylase inhibition, which were correlated.[82]

The phytotoxicity of D-DIBOA has been further optimized by testing synthetic analogs with different substitution patterns at positions 2, 4, 6, 7, and 8. Quantitative structure-activity relationship (QSAR) analysis revealed optimal ranges for parameters such as lipophilicity (logP), molecular volume, dipole moment, and polarizability, as in studies for design of drugs and agrochemicals.[98-100] The influence of such parameters on phytotoxicity indicates that, beside reactivity, transport phenomena and the ability to reach the target site have critical influences on the overall biological activity observed for BXDs.

5. Conclusions

Benzoxazinoids are versatile plant chemical defenses showing activities towards a wide range of target organisms. The unique structural features of this class of compounds give rise to many possible modes of action that could be responsible for their well-documented activities against insects, microorganisms, and competing plants. In addition, the mixtures of BXD structures present in individual plants and the changing pattern in response to enemies and competitors may also contribute to the defensive roles of these secondary metabolites.

The variety of chemical properties and reactivities of BXDs make these compounds extremely interesting from the evolutionary point of view but makes the study of their modes of action quite challenging. The stability and reactivity of BXDs are highly dependent on their structures and on conditions such as pH and temperature. However, previous literature has commonly generalized the biological activities of BXDs attributing the same potential underlying mechanisms to all of them regardless of the target organism and observed effect. In light of the structure-activity relationship studies discussed, the modes of action seem to differ among the biological activities tested. Each activity is not necessarily a consequence of one chemical aspect alone, and each target organism is not necessarily susceptible to one unique mode of action.[87] Further advances in BXD research would benefit from taking into account the complexity of such relationships.

Beside the target organism, evaluation of BXD activities must also account for their stability under the bioassay conditions and the possible interference of degradation products in the results. For example, it is possible for unstable BXDs to react with components of artificial diets during insect feeding bioassays producing misleading results. Similarly, ecological experiments should critically interpret bioassay results by comparison with natural conditions. The BXD defense system in plants is highly compartmentalized and relies on temporally and spatially resolved activation by hydrolysis. It is challenging to reproduce the tissue specific distribution of BXDs that herbivores and pathogens would encounter in a plant leaf, or a gradient of BXDs and their metabolites in soil as perceived by other plants, soil microorganisms, nematodes, and root herbivores. Conclusions drawn from laboratory bioassays about the ecological role of BXDs should carefully consider such aspects.

While we have tried to summarize the general reactivities described for BXDs in the literature, the subject is still not completely clear. As the benzoxazinone degradation to benzoxazolinones is not quantitative,[53] it is possible that side reactions from intermediates shown in Scheme 3 or others are involved in biologically relevant activities. As with the reactive imidoquinone intermediate,[46] other unknown reactive species could be generated according to medium conditions and BXD structural features. Moreover, the enzymatic activation of BXDs upon metabolism yielding reactive species, as the proposed formation of 4-*N*-*O*-acetyl derivatives,[41] still needs rigorous investigation.

Studies evaluating the influence of BXD structural features on biological activitiy are especially important to clarify the mechanisms underlying their biological effects. Thanks to the development of several synthetic routes for BXDs and their non-natural analogs, it is relatively easy to access various structures and continue the work of elucidating BXD modes of action. Future experiments on exploring modes of action for BXDs would benefit from actively searching and chemically characterizing intermediates and end-products that could help differentiate between mechanisms. Advances in analytical chemistry and the wealth of literature concerning BXD analyses should make this process relatively straightforward for the modes of action discussed in this review. Experimental designs that include comparisons between structural analogs with different chemical features also have the potential to more clearly assign specific modes of action to the various biological activities.

To answer the remaining questions regarding the mode of action of BXDs will require integrated research involving ecology, evolution, biochemistry, analytical chemistry, organic synthesis, and other areas. The results will be valuable from many perspectives, from increasing our basic understanding of the interactions between plants and other organisms, to the development of BXD-inspired pesticides and new plant breeding strategies to increase protection by naturally-occurring BXDs.

6. Acknowledgments

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7. References

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Manuscript II

Biological activities and metabolism of plant defensive benzoxazinoids on insect herbivores

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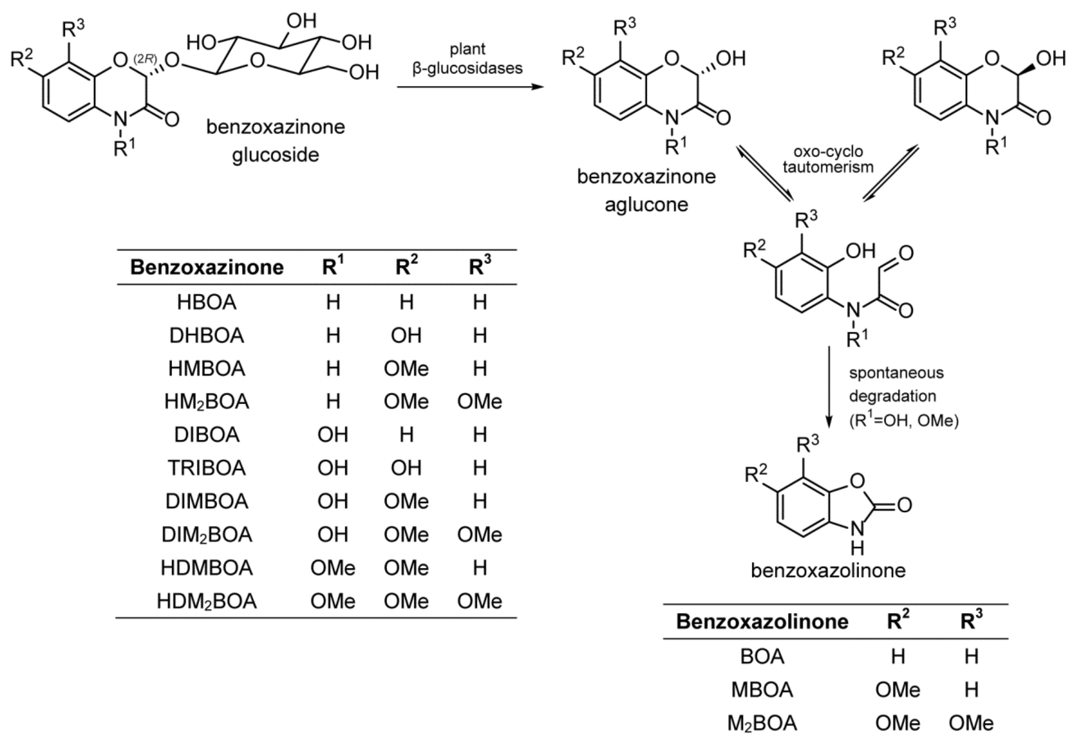
Abstract

Benzoxazinoids are a class of indole-derived plant chemical defenses, comprising compounds with a 2-hydroxy-*2H*-1,4-benzoxazin-3(*4H*)-one skeleton and their derivatives. These secondary metabolites are widespread in grasses, including important cereal crops such as maize, wheat and rye, as well as a few dicot species, and display a wide range of antifeedant, insecticidal, antimicrobial, and allelopathic activities. They are stored in plant cells as stable glucosides that yield reactive aglucones upon hydrolysis by β-glucosidases, thus forming a two-component plant defense system. Although the role of benzoxazinoids in plant defense against insects is well reported, how their specific distribution and induction patterns within plants differentially affect insect herbivores is poorly studied. Additionally, little is known about how resistant insect species metabolize these chemicals. In this context, the present review aims to summarize and critically discuss the biological activities of benzoxazinoids on chewing, piercing-sucking, and root insect herbivores, considering how their feeding behavior modulates their exposure to these plant defenses. Data on the influence of MBOA on *Spodoptera frugiperda* food consumption and utilization are presented and analyzed, as well as the efficiency of MBOA detoxification via glucosylation in this insect. Finally, advances on the metabolism of benzoxazinoids in insects are also addressed, and directions for future research are considered.

Keywords: chemical ecology, detoxification, nutritional indices, Poaceae, toxicity

Introduction

Plants have evolved a diverse repertoire of specialized or "secondary" metabolites in order to alleviate biotic and abiotic stresses. Among these, benzoxazinoids are a group of important defense chemicals widespread in grasses (Poaceae), including economically important crops such as maize, wheat, and rye (but not rice, oat, sorghum, and cultivated barley) (Niemeyer, 2009). This class of compounds is also produced by individual species within the dicot families Acanthaceae, Ranunculaceae, Plantaginaceae, and Lamiaceae (Frey *et al.*, 2009; Makowska *et al.*, 2015). These indole-derived compounds are regarded as general defense metabolites in plants, being associated with a wide spectrum of antifeedant, insecticidal, antimicrobial, and allelopathic activities (Niemeyer, 2009). Due to their structural diversity, the term benzoxazinoids (BXDs) will be used in this article to refer to both benzoxazinones (glucosides and corresponding aglucones containing a 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one skeleton) and their degradation products, benzoxazolinones. The most common naturally occurring BXD structures, simplified activation and degradation routes, and commonly used acronyms are presented in Fig. 1.



Benzoxazinone	R^1	R^2	R^3
HBOA	H	H	H
DHBOA	H	OH	H
HMBOA	H	OMe	H
HM_2BOA	H	OMe	OMe
DIBOA	OH	H	H
TRIBOA	OH	OH	H
DIMBOA	OH	OMe	H
DIM_2BOA	OH	OMe	OMe
HDMBOA	OMe	OMe	H
HDM_2BOA	OMe	OMe	OMe

Benzoxazolinone	R^2	R^3
BOA	H	H
MBOA	OMe	H
M_2BOA	OMe	OMe

Fig. 1 Glucoside hydrolysis of naturally occurring benzoxazinones and degradation to benzoxazolinones via oxo-cyclo tautomerism.

Benzoxazinones are mainly stored as glucosides in vacuoles of undamaged plant cells, and activation by glucosidases increases their reactivity and toxicity (Frey *et al.*, 2009; Niemeyer, 2009). Plant BXD β-glucosidases can be found in plastids, cytoplasm, and cell walls (i.e. spatially separated from their glucoside substrates) (Nikus et al., 2001) which, upon damage to the plant cell and loss of tissue and cell integrity, promote the hydrolysis of benzoxazinone glucosides. The resulting unstable benzoxazinone aglucones and their benzoxazolinone degradation products are regarded as the active compounds involved in most observed BXD biological activities (Niemeyer, 2009; Sicker and Schulz, 2002). This compartmentalized system consisting of a stable compound and an activating enzyme resembles other activated two-component defense systems in plants such as glucosinolates and cyanogenic and iridoid glycosides (Morant *et al.*, 2008; Pentzold *et al.*, 2014b).

The stability and reactivity, and therefore the biological activities of BXDs are known to vary according to their chemical structures and with conditions such as pH and temperature. However, despite numerous reports of BXD effects on survival, growth and feeding behavior of a range of target organisms, the underlying molecular modes of action and physiological mechanisms responsible for these effects are not completely understood. Similarly, the importance of these chemical defenses in a natural context and their modulation by specific characteristics, such as physiology of the target organism and its feeding behavior, have not always been critically evaluated. Given the tissue-specific distribution of BXDs in plants and their activation by glucosidases, the feeding behavior of insect herbivores dictates the amount and the nature of BXDs that they will encounter, based on the attacked tissue and the extent of plant cell damage. However, the metabolism of BXDs by the target organisms, as well as other strategies possibly employed to avoid toxicity (e.g. rapid excretion), are not well explored.

Due to the economic and ecological importance of BXDs, many aspects of these compounds have been reviewed, including their biological activities (Macías *et al.*, 2009; Niemeyer, 2009), strategies for organic synthesis (Macías *et al.*, 2006; Sicker and Schulz, 2002), chemical reactivities (Hashimoto and Shudo, 1996), and biosynthetic evolution and genetics (Frey *et al.*, 2009; Makowska *et al.*, 2015). The present review aims to update and categorize the spectrum of biological activities observed for BXDs towards and insect herbivores with different feeding behaviors: chewing, piercing-sucking, and root herbivores, as well as towards purified enzymes. These effects are discussed in the ecological context of each interaction. The toxic, digestibility-reducing and antifeedant activities of BXDs are evaluated and their contribution to the overall effects observed on insect herbivores is addressed. Additionally, the current knowledge about the metabolism of BXDs in various organisms is summarized.

Biosynthesis and distribution of BXDs in plants

The BXD biosynthetic pathway has been mostly established in maize (Frey *et al.*, 2009; Meihls *et al.*, 2013), but other BXD-producing plants have also been investigated (Dick *et al.*, 2012; Schullehner *et al.*, 2008). The general reactions and compartmentalization of BXD biosynthesis are shown in Fig. 2. The formation of BXDs, as well as genetic aspects and their evolution in plants have been comprehensively reviewed (Frey *et al.*, 2009; Gierl and Frey, 2001; Makowska *et al.*, 2015).

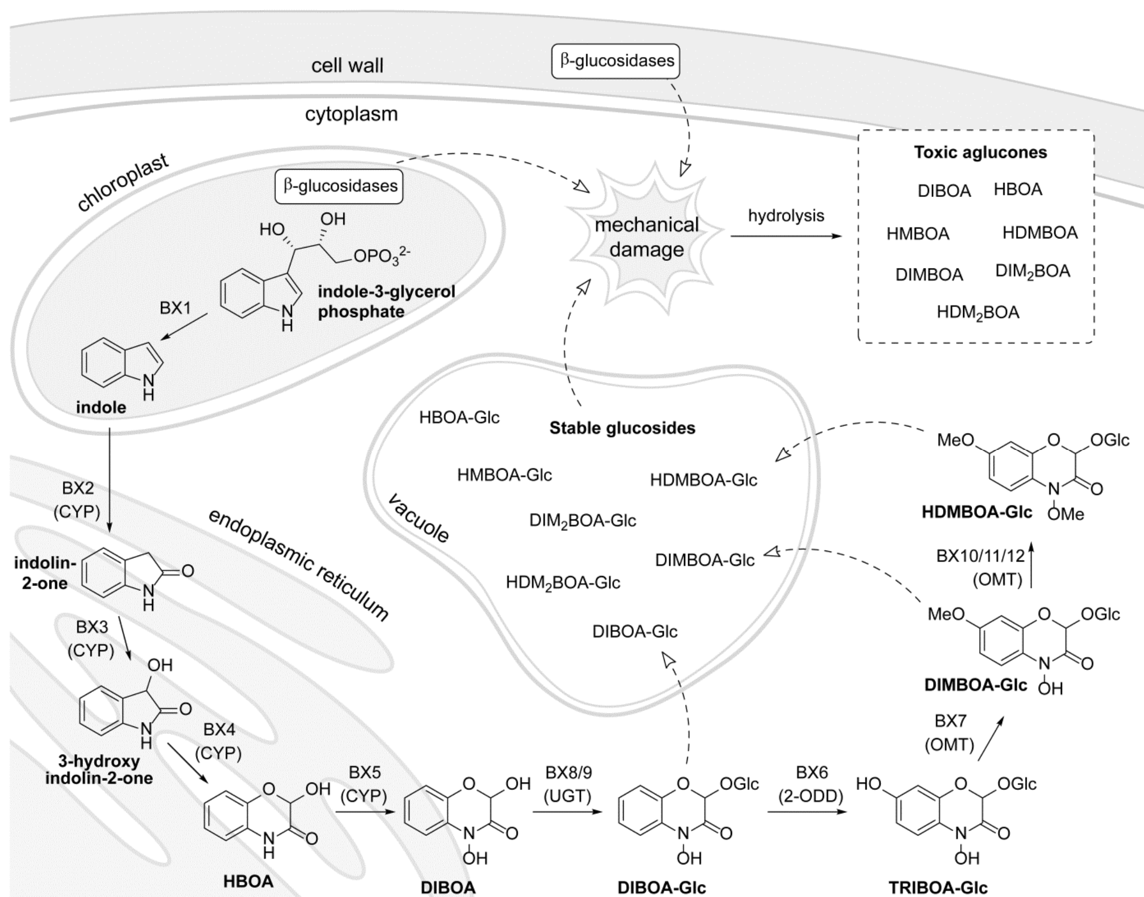


Fig. 2 BXD biosynthesis and compartmentalization in plant cell.

The first committed step of the pathway is catalyzed by BX1, which converts indole-3-glycerol phosphate into indole in the chloroplasts. This enzyme is a homolog of the α-subunit of tryptophan synthase (TSA). In tryptophan synthase, the resulting indole is not released, but rather channeled to the active site of a β subunit (TSB), where it reacts with serine, yielding tryptophan and water. Most likely, the gene encoding *bx1* originated from *TSA* by duplication and modification of both function and

expression patterns and giving rise to both *bx1* and *Igl* (indole-3-glycerol phosphate lyase), which mostly produces the free indole released by the plant as a volatile (Gierl and Frey, 2001). Even though free indole produced by IGL is normally not used by the BXD pathway, this enzyme can supply indole in *bx1* mutant maize lines (Kohler *et al.*, 2015). After this first step, free indole is converted to DIBOA by incorporation of four oxygen atoms. These oxidations are carried out by four cytochrome P450-dependent monooxygenases, BX2-BX5, which are located in the endoplasmic reticulum and are substrate-specific and regioselective for the sequential introduction of oxygen atoms. The DIBOA aglucone thus produced is the first toxic intermediate in the pathway, and is rendered less reactive in the cytoplasm via glucosylation by the action of the UDP-glucosyltransferases (UGTs) BX8 and BX9, probably to avoid autotoxicity and to provide a stable intermediate for further modifications. DIBOA-Glc can be then hydroxylated by the 2-oxoglutarate-dependent dioxygenase BX6, and further *O*-methylated by the *O*-methyltransferase BX7, forming DIMBOA-Glc. Recently, another *O*-methylation step catalyzed by a group of three homologous *O*-methyltransferases, BX10, BX11, and BX12, was elucidated and implicated in the production of HDMBOA-Glc from DIMBOA-Glc (Meihls *et al.*, 2013). The *O*-methylation steps leading to DIM_2BOA-Glc and HDM_2BOA-Glc are also known. (Vinzenz Handrick, submitted)

The stable BXD glucosides are considered to be transported and stored in the vacuole, while the β-glucosidases are thought to be present in plastids (Frey *et al.*, 2009; Niemeyer, 2009; Sicker and Schulz, 2002), but this distribution is not well established and might vary with plant species, tissue and age. Some direct evidence of BXD glucoside distribution was provided by MALDI-MS imaging of metabolites in a maize leaf cross-section, which revealed that DIMBOA-Glc and HMBOA-Glc were localized in cell vacuoles (Korte *et al.*, 2015). The use of antibodies suggests that the subcellular distribution of β-glucosidases varies among plant species and tissues (Nikus *et al.*, 2001). Wheat and rye β-glucosidases are mainly localized in cell walls and cytoplasm, while in maize they are mostly found in plastids and proplastids. However, deviations from these trends have been noted. For instance, Massardo *et al.* (1994) observed, through cell fractionation, the presence of BXD β-glucosidases in the vacuole and of DIMBOA-Glc in the extravacuolar space of maize parenchyma cells.

Upon destruction of the tissue and cellular organization, caused for example by herbivore damage or pathogen attack, the stable glucosides come into contact with β-glucosidases and are hydrolyzed to reactive aglucones, which are then implicated in BXD toxicity (Cambier *et al.*, 1999). But, in some cases aglucones accumulate. For example, the apoplast of maize leaves contains DIMBOA as well a DIMBOA-Glc, and HDMBOA-Glc (Ahmad *et al.*, 2011), while waxes on the surface of maize whorls contain considerable amounts of HDMBOA aglucone (which seems to be stable in the waxy layer), together with DIMBOA and MBOA (Hedin, 1993). In roots, BXD aglucones are considered to be actively exuded from the plant tissue and diffuse into the soil where they exert their effects on soil microorganisms, root herbivores, and other plants (Belz and Hurle, 2005; Pérez and Ormeño-Nuñez, 1991). The identity of BXDs exuded by roots seems to vary according to growing conditions, sampling, and analytical methods. The aglucones DIBOA and DIMBOA were the main

BXDs exuded from roots of hydroponically grown *Secale cereale* and three *Triticum* species: *T. aestivum*, *T. durum*, and *T. spelta* (Belz and Hurle, 2005). Maize root exudates collected by a trapping system contained the hydroxamic acids DIMBOA and DIBOA, the lactam HMBOA, and the benzoxazolinones MBOA and BOA. In maize hydroponic cultures, however, the glucoside DIMBOA-Glc was additionally detected (Friebe *et al.*, 1998). Exudate extracts obtained by dipping maize roots in dichloromethane contained mainly the unstable derivative HDMBOA (Zhang *et al.*, 2000). However, a method based on liquid extraction surface analysis detected glucosides such as HDMBOA-Glc and DIMBOA-Glc in maize root exudates (Robert *et al.*, 2012). Since highly polar compounds are readily adsorbed onto the soil matrix and do not easily diffuse (Li *et al.*, 2013), these data suggest that BXD glucosides are restricted to the root surface and immediate surroundings, while benzoxazinone aglucones and degradation products such as benzoxazolinones show higher mobility through the soil.

The abundance of BXDs and their proportions vary between plant species and varieties, and also among tissues and developmental stages within plants. For example, the main BXD in rye is DIBOA-Glc (Oikawa *et al.*, 2002), whereas DIMBOA-Glc is the major BXD in aerial parts of wheat and maize (Cambier *et al.*, 2000; Oikawa *et al.*, 2002). In contrast, HDMBOA-Glc is dominant in maize roots, with BXDs being more concentrated in crown roots than in primary and secondary roots (Robert *et al.*, 2012). In maize, BXDs reach the highest concentrations in seedlings up to 10 days old and decrease as the plant grows (Cambier *et al.*, 2000). Moreover, BXDs are differently allocated in leaves according to their age: DIMBOA-Glc was the predominant BXD in young maize leaves from growth stages L2 to L7, but in older leaves DIMBOA-Glc was found at initial growth stages, while DIBOA-Glc and HMBOA-Glc became the most abundant BXDs in old leaves after stage L5 (Kohler *et al.*, 2015).

The total concentrations of BXDs in different plants can vary with age and biotic stresses, and can reach >0.1% of maize leaf fresh weight after caterpillar attack (Dafoe *et al.*, 2011; Glauser *et al.*, 2011). HDMBOA-Glc is highly induced in maize after herbivory (Dafoe *et al.*, 2011; Glauser *et al.*, 2011), fungal attack (Oikawa *et al.*, 2004), and in both maize and wheat upon jasmonic acid treatment (Oikawa *et al.*, 2001; Oikawa *et al.*, 2002). Moreover, young maize leaves display higher inducibility of HDMBOA-Glc and HDM_2BOA-Glc upon herbivory than old leaves (Kohler *et al.*, 2015).

Chemical properties and reactivity of BXDs

Benzoxazinones (Fig. 1) can be divided according to their substituent group R^1 as lactams (R^1=H), hydroxamic acids (R^1=OH), and *N*-*O*-methylated derivatives (R^1=OMe). These functional groups and other substituents modulate the stability and reactivity of each compound and therefore their biological activities.

Benzoxazinone glucosides are remarkably stable (Hietala *et al.*, 1960) and require the action of glucosidases for their hydrolysis. The resulting aglucones are cyclic hemiacetals that undergo oxo-cyclo tautomerism via a fast, reversible ring opening reaction (Copaja *et al.*, 1986), and therefore occur as racemic mixtures in solution. Interestingly, all known benzoxazinone glucosides produced by plants are (2*R*)-2-β-D-glucosides (Hartenstein *et al.*, 1993; Hartenstein and Sicker, 1994; Kluge *et al.*, 1997a; Nagao *et al.*, 1985) that, upon glucosidase activity, originate racemic mixtures of (2*R*) and (2*S*) aglucones.

The high activity of BXD β-glucosidases and the instability of aglucones represent a challenge in their extraction and quantitative analysis from natural samples. Once plant material is mechanically disrupted, plant β-glucosidases quickly hydrolyze benzoxazinone glucosides to aglucones, which spontaneously degrade to benzoxazolinones. The extraction and analytical methods have been thoroughly evaluated and compared (Villagrasa *et al.*, 2009), and modern LC-MS protocols are sensitive and accurate. It is important to note, however, that earlier experiments sometimes used colorimetric methods to determine total hydroxamic acids, with no distinction between different structures or even between glucosides and aglucones. Other methods have included calculations of DIMBOA content based on degradation to MBOA and quantification, which can be inaccurate due to the non-quantitative nature of this transformation (Woodward *et al.*, 1978) and the fact that HDMBOA also degrades to MBOA. In fact, probably due to its instability, HDMBOA was often missed and only started to be considered as a major benzoxazinoid in the 1990s (Hedin, 1993), although the glucoside was already identified in the 1970s (Hofman *et al.*, 1970). Therefore, when interpreting results from the literature, one must consider possible quantification errors and the presence of BXDs that were not detected.

Large scale isolation of DIMBOA(-Glc) and DIBOA(-Glc) can be achieved from maize or rye seedlings (Larsen and Christensen, 2000; Macías *et al.*, 2006), and semi-preparative LC of maize extracts can yield other abundant BXDs (Glauser *et al.*, 2011). BXDs have also been obtained synthetically, both as natural structures and analogs with various substitution patterns and functional groups (Atkinson *et al.*, 1991; Hashimoto *et al.*, 1991; Kluge *et al.*, 1997; Kluge and Sicker, 1996; Sicker *et al.*, 1994). Synthetic approaches to BXDs have been summarized elsewhere (Macías *et al.*, 2006; Sicker and Schulz, 2002).

In general, lactams (N-H compounds) are not degraded to benzoxazolinones, whereas hydroxamic acids (N-OH) degrade readily and *N-O*-methyl derivatives degrade even faster. For example, the half-lives of HDMBOA and DIMBOA aglucones in buffered D_2O at pH 5.5 and 24°C are 1.8 h and 25 h, respectively (Maresh *et al.*, 2006). The degradation rates depend on conditions such as pH, temperature and solvent, and structural features such as nature of the *N*-substituent group and other substituents at the aromatic ring (Atkinson *et al.*, 1991). Several pathways have been proposed to explain this degradation mechanism (Bredenberg *et al.*, 1962; Grambow *et al.*, 1986; Maresh *et al.*, 2006; Niemeyer *et al.*, 1982a; Smissman *et al.*, 1972).

Due to their unique structural features, BXDs present a wide range of possible reactions and properties of biological relevance. Upon ring opening, benzoxazolinone aglucones become α-oxo-aldehydes, which are potent electrophiles capable of reacting with nucleophilic residues in proteins such as thiols and amines, and causing enzymatic inhibition (Atkinson *et al.*, 1991; Niemeyer *et al.*, 1982b; Pérez and Niemeyer, 1989a). The nitrogen atom can also be an electrophilic site upon elimination of the *N*-substituent group and formation of a nitrenium ion or a reactive *o*-imidoquinone intermediate (Atkinson *et al.*, 1991; Dixon *et al.*, 2012; Hashimoto *et al.*, 1991; Maresh *et al.*, 2006; Quiroz and Niemeyer, 1991). BXD hydroxamic acids possess metal ion chelating properties (Tipton and Buell, 1970) that can play a role in Fe(III) uptake (Pethő, 1992a, b, 1993, 2002) and Al(III) resistance by roots (Poschenrieder *et al.*, 2005). Furthermore, benzoxazolinones can interfere with auxin binding in plants (Hasegawa *et al.*, 1992; Hoshisakoda *et al.*, 1994; Venis and Watson, 1978), and the products from their metabolism by soil microorganisms are suggested to play a role in allelopathy (Kato-Noguchi *et al.*, 2010; Schulz *et al.*, 2013; Venturelli *et al.*, 2015).

Biological effects of BXDs

Since their discovery, BXDs have been considered to function as resistance factors against herbivores, pathogens, and other plants. Many *in vitro* studies and bioassays demonstrate that these compounds have inhibitory and toxic effects towards a wide range of target enzymes and organisms, particularly insect herbivores. Most reports focus on important pest species of cereal crops, covering different ecological guilds: caterpillars (chewing herbivores), aphids (piercing-sucking herbivores), and rootworms (root herbivores). These studies are summarized and discussed in the following sections in the ecological context of the insect feeding behavior. Moreover, the influence of toxic, digestibility-reducing, and antifeedant activities of BXDs on the insect physiology is addressed. Allelopathic and antimicrobial activities of BXDs will not be covered in this section, as they have already been comprehensively discussed in recent reviews (Macías *et al.*, 2009; Niemeyer, 2009; Schulz *et al.*, 2013).

BXDs have been shown to be present in whole grain cereals, flours, sprouts, bread and beverages based on rye, wheat, and maize, and might influence human health or be used for therapeutic purposes. Anti-inflammatory, anticancer, and antimicrobial activities, as well as stimulatory effects on the central nervous and reproductive systems have been reported for BXDs, but mostly using *in vitro* studies. This human therapeutic potential of BXDs has also been recently reviewed (Adhikari *et al.*, 2015).

Effects on purified enzymes

DIMBOA has been shown to inhibit the cysteine proteinase papain, and this inhibition was reversed by addition of dithiothreitol, supporting a model where DIMBOA reacts directly with the enzyme's catalytic cysteine residue (Pérez and Niemeyer, 1989). Since inhibition was also observed for 2-dehydro-DIMBOA, but not for HMBOA, the enzyme thiol group is proposed to react with the BXD nitrogen group, and not with the α-oxo carbonyl in the open form. DIMBOA also inhibited the proteinase α-chymotrypsin by reacting with the serine residue in the active site (Cuevas *et al.*, 1990). In contrast to assays with papain, the 2-methyl acetal derivative of DIMBOA did not inhibit this serine proteinase, suggesting that the open form is involved in the latter inhibitory activity. These studies indicate that the overall effects of BXDs in herbivores can result in part from the inhibition of digestive enzymes.

BXDs also inhibit reactions of energy metabolism *in vitro* in both plant and animal systems. DIMBOA inhibits ATP synthesis and its coupled electron transport in chloroplasts with an IC_{50} of about 1 mM. DIMBOA-Glc caused weaker inhibition, with an IC_{50} ~4 mM (Queirolo *et al.*, 1981). Such inhibition was reversible and uncompetitive, and proposed to occur partially via reaction with sulfhydryl groups in enzymes (Queirolo *et al.*, 1983). In bovine submitochondrial cell fractions, DIMBOA inhibits ATP synthesis, the P_i-ATP exchange reaction, ATPase activity, and electron transport (Niemeyer *et al.*, 1986). Similar effects were observed for the benzoxazolinone BOA (Niemeyer *et al.*, 1987). In general, however, chloroplasts seem to be more sensitive than animal mitochondria regarding inhibition of energy metabolism. In maize, DIMBOA was shown to reduce electron transport in both mitochondria and chloroplasts with IC_{50} values of 1.8 mM and 1.2 mM, respectively, while DIMBOA-Glc showed no activity. These concentrations are below the levels of BXDs in the plant cell, suggesting that glucosylation and compartmentalization of these defenses indeed contribute to minimizing autotoxicity (Massardo *et al.*, 1994).

In the plasma membrane of *Avena sativa* and *Vicia faba* root cells, H^+-ATPase was considerably inhibited by DIMBOA in concentrations as low as 0.25 mM (Friebe *et al.*, 1997). DIBOA, BOA, and MBOA were weaker inhibitors. The effects of DIBOA and BOA on ATPase correlated well with the inhibition of root elongation in *A. sativa*, suggesting that this molecular mechanism might be involved in the observed allelopathic effects promoted by BXDs.

NADH oxidation catalyzed by horseradish peroxidase was shown to be stimulated by DIMBOA and, to a lesser extent, by DIBOA, while BOA and MBOA did not have an effect (Rojas *et al.*, 1997). Peroxidases play a role in cell growth by catalyzing oxidative cross linking of cell wall polysaccharides and proteins. A mechanism for DIMBOA-induced inhibition of plant growth could involve peroxidase stimulation and consequent accumulation of H_2O_2 in cell walls leading to higher rigidity.

The aminophenoxazinones APO and AMPO, degradation products from DIBOA and DIMBOA in soil, inhibited histone acetyltransferases and deacetylases (HDAC) from *Arabidopsis thaliana* nuclear extracts in a dose-dependent fashion (Venturelli *et al.*, 2015). Docking simulations showed that these molecules coordinate to the Zn cofactor of model HDACs as a bidentate ligand. This activity was proposed as a mechanism for BXD allelopathy, in which aminophenoxazinones inhibit HDAC thus interfering in gene regulation within the target plant and leading to inhibition of root growth, as observed for *A. thaliana* seedlings upon treatment with APO and AMPO.

Effects on chewing herbivores

Due to their feeding behavior, chewing herbivores such as lepidopteran larvae disrupt the compartmentalization of BXDs in plant cells during ingestion, and are therefore directly exposed to high amounts of BXD aglucones, especially when feeding on leaves from young seedlings where BXD concentrations are highest. Indeed, DIMBOA and BXDs in general have been long known for their toxic and antifeedant activities on caterpillars and for being the primary factors protecting resistant maize lines from herbivore attack (Klun *et al.*, 1967; Reed *et al.*, 1972; Robinson *et al.*, 1978). When evaluating the effects of BXDs on herbivores, it is essential to take into account that most lepidopterans possess an alkaline gut environment (Berenbaum, 1980) that facilitates the degradation of hydroxamic acids and *N*-*O*-methylated hydroxamic acids into benzoxazolinones, thus leading to biological activities that can be specific to these insects. Furthermore, the differential induction of plant BXDs caused by herbivore feeding is also important when considering the natural context of such interactions.

When investigating the effects and modes of action of plant toxins on insect herbivores, it is also important to distinguish how different aspects of their nutrition are affected. Following the definitions of Duffey and Stout (1996), a compound is considered toxic if it has an overall detrimental effect on the insect itself, i.e. its tissues or internal processes. On the other hand, a compound is considered a digestibility reducer if it affects processes external to the insect proper, such as digestion. This activity can be further divided into antidigestive, limiting the rate of enzymatic conversion of ingested food (e.g. via inhibition of digestive enzymes); and antinutritive, limiting the utilization of food by altering its physical availability or chemical identity (e.g. via precipitation or modification of food proteins that prevent their breakdown by digestive proteases). Additionally, compounds can modify diet palatability and elicit behavioral responses that decrease ingestion by the insect, being therefore classified as antifeedants (Koul, 2008). For example, DIMBOA has been shown to interfere with gustatory chemoreceptors in the lateral sensillum styloconicum of *Mythimna separata* larvae, inducing impulse discharges in the deterrent cell and reducing responsiveness of the sugar cell to sucrose solution (Yang *et al.*, 1997). However, the underlying effects on insect gustatory and olfactory systems are usually not well understood mechanistically.

The nutritional indices proposed by Waldbauer (1982) constitute a method for evaluating the effects of a compound on insect nutrition, distinguishing between antifeedant, digestibility-reducing, and toxic activities. These parameters include relative growth rate (RGR), consumption index (CI), approximate digestibility (AD), efficiency of conversion of ingested food into biomass (ECI), and efficiency of conversion of digested food into biomass (ECD). The comparison of these indices among treatments allows a better assessment of how a certain compound affects food consumption and utilization. However, it is important to note that such indices interact in a complex manner, partly due to homeostatic compensations by the insect (Scriber and Slansky, 1981). For example, the effect of a toxic compound that reduces ECI and ECD could be compensated by increased CI, keeping a high RGR and overall performance. Furthermore, the activity of chemicals might be modulated by food quality and co-occurring chemicals and enzymes in the diet (Duffey and Stout, 1996).

Genus *Ostrinia*: The toxicity and deterrence of BXDs towards the European corn borer (ECB, *Ostrinia nubilalis*) have been extensively investigated due to its economic importance as a pest of maize crops. Two comprehensive studies of the toxicokinetics of DIMBOA and MBOA in *O. nubilalis* have been reported (Campos *et al.*, 1988, 1989). DIMBOA concentrations between 0.05 and 0.5 mg/g (~0.24 and 2.37 mM, respectively) in artificial diets did not affect total weight gain in larvae up to the fifth instar. The same was observed for MBOA at concentrations of 0.5 – 4.0 mg/g (~3 – 24 mM, respectively). However, both BXDs increased mortality and developmental times to pupation in a dose-dependent way. DIMBOA reduced pupal and adult weights in all tested concentrations, and the highest concentration also increased pupal mortality and delayed the emergence of adults. Despite not influencing pupal and adult weights, and not consistently leading to pupal mortality, MBOA also delayed adult emergence, although not as dramatically as DIMBOA. Female to male sex ratio was not modified by DIMBOA, but was decreased by MBOA. This might suggest that females are more susceptible to MBOA, although the effect was not proportional to its concentration in diet. Both DIMBOA and MBOA reduced the number of eggs produced by females. In general, the effects caused by MBOA were similar to those caused by DIMBOA but required higher concentrations, agreeing with the trend that benzoxazolinones are less toxic than benzoxazinones.

These studies also tracked BXD distribution and excretion dynamics in *O. nubilalis* using radioactive ^{3}H-labeled DIMBOA and MBOA. In both cases, the radioactivity was mainly excreted by larvae in the frass and transferred to the pupal case after emergence, suggesting that the adult insect avoids accumulation of BXDs. However, the body burden (ratio of radioactivity between body and frass) was constant for all tested concentrations, implying that larvae are not able to increase excretion rate when faced with higher levels of BXDs. The highest concentration of DIMBOA (0.4 mg/g, 1.9 mM), caused a higher accumulation of ^{3}H in body and frass than the other concentrations (yet with the same burden). This is probably consequence of a higher diet consumption, which is consistent with a digestibility-reducing activity of DIMBOA at this concentration. On the other hand, MBOA did not alter diet consumption, thus its effects are due to toxicity and not to an antifeedant activity. No long-term accumulation of ^{3}H labeled DIMBOA or MBOA in specific tissues was observed,

indicating that *O. nubilalis* does not sequester BXDs. In fact, a short-term higher level of radioactivity in hemolymph compared to other tissues suggests that these compounds are rapidly transported and excreted. Furthermore, topical applications of DIMBOA and MBOA show that these compounds are rapidly absorbed through the skin and excreted. In both cases, the accumulation of label in hemolymph reached a peak at 2h, and most of the label was excreted in frass (88% after 48h for DIMBOA, and 82% after 24h for MBOA).

Further experiments evaluating the effects of DIMBOA and MBOA on food consumption and utilization in *O. nubilalis* suggest that these compounds have distinct modes of action on the insect physiology (Houseman *et al.*, 1992). In 4-day artificial diet feeding assays, both DIMBOA (0.2 mg/g, ~1 mM) and MBOA (3.0 mg/g, ~18 mM) reduced weight gain in *O. nubilalis*. This was not caused by deterrence effects, since CI increased for DIMBOA and remained the same for MBOA treatments. DIMBOA decreased both AD and ECI, but did not change ECD, indicating that DIMBOA affects digestive processes (e.g. inhibiting digestive enzymes), but does not modify the utilization of nutrients after digestion. Such reduction in digestibility could lead to the observed increase in diet consumption, as an attempt by the larvae to compensate for the decrease in food quality. On the other hand, MBOA ingestion did not change AD or ECI, but decreased ECD, suggesting that this compound has an effect on processes occurring after digestion. In agreement with these results, both *in vivo* and *in vitro* assays showed that DIMBOA inhibits trypsin and chymotrypsin, while MBOA inhibited just trypsin.

In a structure-activity relationship study, the effect of various BXDs on *O. nubilalis* growth was evaluated using artificial diets containing 0.5 mM of individual BXDs (Atkinson *et al.*, 1992). Among the natural BXD structures, DIMBOA and DIBOA showed higher toxicity, followed by DIM_2BOA. The authors suggested that the resulting toxicities are linked to the degradation rates of these hydroxamic acids to benzoxazolinones. Indeed, the lactams HMBOA and HBOA, which do not form benzoxazolinones upon degradation, did not inhibit larval growth.

The activities of several detoxification enzymes in *O. nubilalis* midgut (cytochrome *b*5, NADPH cytochrome *c* reductase, NADPH oxidase, and *O*-demethylase) increased when larvae fed on maize leaves, compared to meridic diets based on wheat germ (Feng *et al.*, 1992a). The levels of induction of these enzymes were positively correlated to the resistance of maize lines towards leaf damage and their DIMBOA concentrations. Some of these results were also observed *in vitro* by adding DIMBOA to enzyme assays, and *in vivo* by feeding on artificial diets with different levels of DIMBOA or MBOA (Feng *et al.*, 1992); however, it is not clear whether some of these effects might be mediated by other secondary metabolites in the plant.

The effects of *O. nubilalis* herbivory on maize metabolism are also relevant for the ecology of this interaction. Larval feeding induced the production of HDMBOA-Glc in stems, with a consequent decrease in DIMBOA-Glc (Dafoe *et al.*, 2011). Even though HDMBOA is considered more toxic than DIMBOA, larvae feeding on previously induced stem sections grew more and consumed more plant tissue than on uninduced stems. Feeding on induced stems decreased CI and AD, but increased ECI and ECD, suggesting that, despite being better defended, such tissues are more nutritive than non-

induced stems. Indeed, induction by *O. nubilalis* herbivory increased protein, sucrose, and linoleic acid levels in stems. The authors suggested that high amounts of the auxin 3-indole-acetic acid in *O. nubilalis* oral secretion and frass could affect plant metabolism and promote the increase in nutritional value of attacked tissue.

It is important to note, however, that the amounts of HDMBOA-Glc and other BXDs encountered by *O. nubilalis* in maize stems at the L11-L13 stage (3.6 µg/g after 48h induction) are much lower than those found in leaves of young seedlings. For comparison, *S. frugiperda* larvae feeding on maize at the L4 stage preferred the youngest leaves with more highly inducible HDMBOA-Glc concentrations (around 30 µg/g constitutively, and 300 µg/g after herbivore induction) (Kohler *et al.*, 2015). Screening of maize lines for resistance towards *O. nubilalis* indicates that, in general, DIMBOA concentrations of more than 100 µg/g (0.47 mM) in whorl tissues are associated with lower leaf consumption upon infestation (Barry *et al.*, 1994). Therefore, it has been proposed that *O. nubilalis* restricts its diet to tissues and plant growth stages that present lower BXD concentrations in order to avoid their toxicity (Maag *et al.*, 2014). This is consistent with their apparently low capability of biochemically detoxifying BXDs, as will be discussed in more detail below. Alternatively, the low BXD levels normally encountered by this species might not have evolutionarily selected for strong detoxification mechanisms against these compounds.

In natural situations, the amount of DIMBOA is not the only factor guiding *O. nubilalis* feeding behavior. In maize plants, *O. nubilalis* larvae prefer to feed on immature whorl tissues, despite their higher BXD levels compared to mature tissues (Bergvinson *et al.*, 1995a; Bergvinson *et al.*, 1995b). Such preference was suggested to be a consequence of higher fiber content and cell wall phenolics in mature tissues, which increase leaf toughness and constitute a mechanical defense towards herbivore feeding. Therefore, it was proposed that neonate larvae feed on the younger, tenderer maize whorl leaves which contain higher amounts of protein, in spite of their higher DIMBOA concentrations. As they grow and develop stronger mandibles better able to chew tougher tissues, the larvae move to more mature parts of the plant with lower levels of BXDs. Another study had observed a similar trend by comparing maize lines with varying DIMBOA concentrations grown under different light conditions. Even though DIMBOA seems to be responsible for high resistance in some genotypes, neonate leaf consumption and survival correlate better with nitrogen content and nutritional value of plants when comparing different light regimes (Manuwoto and Scriber, 1985b).

DIMBOA was shown to induce cytochrome P450 and glutathione *S*-transferase activities in the Asian corn borer (*Ostrinia furnacalis*) (Yan *et al.*, 1995). Acetylcholinesterase was inhibited, while inhibition or induction of general esterases depended on the tissue analyzed. These results indicate that BXDs modulate general detoxification activities and nervous system enzymes in caterpillars. However, treatments were administered by cabbage leaf disks briefly dipped into DIMBOA solutions (400 ppm, 1.89 mM) in acetone, which makes it difficult to calculate actual DIMBOA intake by larvae, especially because the stability of DIMBOA throughout the experiment is not known. Further choice assays revealed that DIMBOA applied to cabbage leaves shows increasing antifeedant effect on *O. furnacalis* up to 0.8 mg/g (3.79 mM) (Yan *et al.*, 1999). Moreover, artificial diets with 1 mg/g DIMBOA

(4.73 mM) inhibited growth and extended larval developmental time in this species. In a comparative study, *O. furnacalis* was less affected than *O. scapulalis* when feeding on artificial diet containing a 2:1 mixture of DIMBOA and DIM_2BOA (Kojima *et al.*, 2010). Growth rates and pupal weights decreased for both species at 0.3 mg/g and 0.7 mg/g treatments, compared to controls. Survival and duration of larval development of *O. furnacalis* did not differ at the 0.3 mg/g treatment, but were strongly affected by 0.7 mg/g BXD concentration. Although *O. furnacalis* suffered adverse effects from feeding on BXDs, it was less strongly affected than *O. scapulalis*. Another experiment comparing *O. furnacalis*, *O. scapulalis*, and their hybrids gave similar results (Phuong *et al.*, 2015). At a DIMBOA level of 0.3 mg/g, *O. furnacalis* was not greatly affected, but stronger detrimental effects were observed at 0.7 mg/g. The hybrids responded to DIMBOA similarly to *O. furnacalis*, indicating tolerance to low levels of BXDs.

Genus *Spodoptera*: The susceptibility of *S. littoralis* and *S. frugiperda* towards BXDs has been compared using artificial diets (Glauser *et al.*, 2011). *S. littoralis* larvae feeding for 7 days on 40 µg/g DIMBOA (0.19 mM, the level encountered by larvae in non-induced maize plants) did not grow differently than when feeding on a control diet. In contrast, *S. frugiperda* grew more quickly on the DIMBOA-containing diet. When feeding on DIMBOA at 200 µg/g (0.95 mM, the level found in herbivore-induced maize plants), *S. littoralis* grew significantly less, whereas *S. frugiperda* growth was not affected. However, while both species showed no differences in preference or short-term consumption between artificial diets containing 50 and 500 µg/g HDMBOA-Glc, the addition of maize plant extract to such diets promoted deterrence and lower food intake in the high-HDMBOA-Glc treatment for both species. This was presumably a consequence of the β-glucosidase activity present in the plant extract, causing hydrolysis of HDMBOA-Glc and releasing the highly toxic HDMBOA aglucone, which persists in the diet for around 30 min. These experiments indicate that *S. frugiperda* is more resistant towards the toxic effects of DIMBOA than *S. littoralis*, and may even benefit from its presence in the diet, while HDMBOA seems to be similarly toxic to both species (Glauser *et al.*, 2011). On the other hand, artificial diet containing MBOA at 330 µg/g (2 mM) did not impair *S. frugiperda* and *S. littoralis* growth, but decreased *O. nubilalis* growth (Maag *et al.*, 2014).

We investigated the effects of MBOA on food consumption and utilization by *S. frugiperda* feeding on bean-based artificial diets by measuring the weights of larvae, diet consumed and frass excreted. MBOA at concentrations of 50 and 1000 µg/g (0.3 and 6.06 mM respectively) did not significantly affect larval growth curves (Fig. 3) or RGR after 12 days of feeding (Table 1), compared to feeding on a control diet lacking MBOA. In the high-MBOA treatment, however, we observed a significant decrease in CI and AD. This seems to be compensated by an increase in ECI and, especially, on ECD. Taken together, these data suggest *S. frugiperda* is not affected by MBOA toxicity even at the higher concentration tested, which is higher than the physiological levels encountered in maize seedlings (approximately 2 mM) (Maag *et al.*, 2014). Two hypotheses could be proposed from these results: (i) MBOA exhibits antifeedant activity and decreases CI, and may be used by the insect as a cue for (degraded) BXDs, but the insect adapts by utilizing the ingested and digested food more

efficiently; or (ii) MBOA serves as a nutrient for the insect and raises ECD, possibly by increasing nitrogen availability and uptake, with the insect decreasing consumption as a response to a richer diet. In any case, MBOA seems to act as a digestibility reducer, either by inhibiting digestive enzymes or interacting with nutrients in the diet and preventing them to be digested. The remarkably high effect on ECD indicates that MBOA acts more critically after the digestion process, suggesting a nutrient role for it. However, *S. frugiperda* has been shown to metabolize MBOA via *N*-glucosylation and to excrete a considerable fraction of it in the frass (Maag *et al.*, 2014). This supports the opposite conclusion, that *S. frugiperda* would recruit detoxification enzymes and expend energy (as UDP-glucose) to facilitate MBOA excretion, thus being subject to a higher metabolic cost that should be reflected in a lower ECD. The quantification of MBOA metabolism and excretion is underway, and should provide more insight about its possible role as a nutrient. It is important to note that bioassays performed with minimal diets might overestimate the nutritional value of nitrogen-containing compounds and not necessarily reflect the importance of MBOA in the natural context. Nevertheless, *S. frugiperda* seems to have the capacity of feeding on high MBOA diets without negative effects on growth, and the resulting physiological effects are different than those reported for *O. nubilalis* (Houseman *et al.*, 1992).

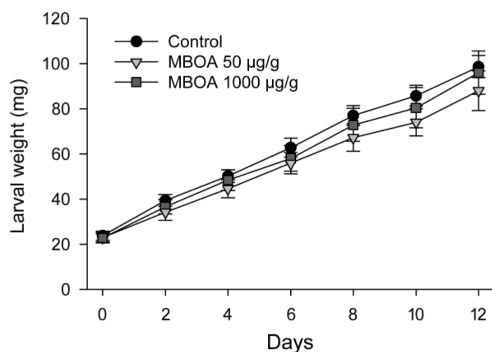


Fig. 3 Growth curves (±SEM) for *S. frugiperda* larvae feeding on artificial diets containing MBOA

Table 1. Nutritional indices (±SEM) for *S. frugiperda* larvae feeding on artificial diets containing MBOA (*P<0.05, **P<0.01, Tukey's test for unequal sample sizes)

Treatment	N	RGR (mg/mg per day)	CI (mg/mg per day)	AD (%)	ECI (%)	ECD (%)
Control	13	0.1055 ± 0.0080	0.9904 ± 0.0530	43.03 ± 3.11	10.83 ± 0.83	27.14 ± 2.88
MBOA 50 µg/g	15	0.0941 ±0.0055	0.8337 ± 0.0505	39.63 ± 3.41	12.21 ± 1.27	34.98 ± 5.26
MBOA 1000 µg/g	14	0.0989 ± 0.0042	0.6739 ± 0.0387 **	27.67 ± 2.83 **	15.15 ± 0.85 *	66.54 ± 10.02 **

The feeding preferences of *S. frugiperda* and *S. littoralis* on maize plants at different growth stages have been investigated (Kohler *et al.*, 2015). While *S. frugiperda* preferred to feed on younger leaves in spite of higher BXD induction, *S. littoralis* feeding was more distributed over different leaves, but especially concentrated on older ones. This indicates that *S. littoralis* moved more during foraging, possibly to avoid induced BXDs. After maize stage L6, however, *S. littoralis* switched to young leaves, presumably due to the overall decrease in both BXD levels and inducibility as the plant grows older. Moreover, in mutants containing lower BXD levels, the preference of *S. frugiperda* for young leaves disappeared, and larvae grew less than on high-BXD WT plants. This suggests that BXDs differentially influence feeding patterns in both *S. frugiperda* (serving as feeding stimulants or nutrients) and *S. littoralis* (acting as deterrents and toxins).

Food consumption and utilization by the southern armyworm (*Spodoptera eridania*) was compared among maize lines with different DIMBOA levels, which had been bred for resistance towards *O. nubilalis* (Manuwoto and Scriber, 1982). Penultimate instar *S. eridania* grew less when feeding on high-DIMBOA plants, which resulted in lower ECD and ECI. These larvae displayed higher CI and AD, even though this was not enough to compensate for the high metabolic costs of feeding on tissue with high DIMBOA levels. However, last instar larvae grew more on lines with more DIMBOA, possibly because of the induction of detoxification pathways or more efficient food processing caused by previous contact with BXDs. In another study, these authors analyzed the same parameters for *S. eridania* comparing two maize genotypes grown under iron and nitrogen deficient conditions, which affects water content, and nitrogen and DIMBOA levels (Manuwoto and Scriber, 1985). Overall, Fe-deficient plants showed higher DIMBOA levels, whereas N-deficient plants had lower DIMBOA levels. Surprisingly, larvae feeding on genotype CI31A, with high DIMBOA, grew more than the group feeding on the “ECB-susceptible” genotype WF9, with low levels of DIMBOA. Fifth-instar larvae feeding on Fe-deficient plants displayed lower CI and AD, but these were compensated by higher ECD and ECI, resulting in no differences in growth. Nitrogen-deficient plants, however, did not support the development of *S. eridania*, resulting in lower growth, CI, ECD, and ECI, together with high mortality rates, even though these plants contained lower DIMBOA levels compared to ones grown with complete nutrient medium. These results suggest that, even though DIMBOA is important in plant resistance to herbivore attack, other factors such as nitrogen and water content also play a role in determining consumption and utilization of food by insects. Deprivation of nutrients might also affect many other primary and secondary metabolites in plants, which have an impact on its nutritional value.

In choice assays, beet armyworm (*Spodoptera exigua*) larvae were deterred by barley leaves treated with DIMBOA, while this treatment stimulated feeding for *S. frugiperda* (Rostás, 2006). Feeding on artificial diets containing 500 µg/g DIMBOA (2.37 mM) increased mortality and reduced growth in *S. exigua* in short term experiments. In long term assays, mortality and pupal weight were not affected, but developmental time increased for *S. exigua* on DIMBOA treatment, while *S. frugiperda* was not affected. Longer developmental times can affect insect survival in nature by prolonging the larval stages, making them more vulnerable to predators, parasitoids, and pathogens.

However, it is uncertain whether the overall negative effects observed on *S. exigua* are consequence of DIMBOA toxicity or antifeedant effects.

In summary, benzoxazinones such as DIMBOA seem to be more toxic to caterpillars than benzoxazolinones such as MBOA. The *N*-*O*-methyl derivative HDMBOA is suggested to be even more toxic, including towards BXD-resistant species. Experiments with lepidopteran herbivores indicate that *Spodoptera* spp. are generally more adapted to BXDs than *Ostrinia nubilalis*. And within the genus *Spodoptera*, *S. frugiperda* seems to be more resistant to BXD toxic effects than *S. littoralis*, *S. eridania*, and *S. exigua*, possibly even benefiting from the presence of BXDs in the diet. Such gradients in BXD resistance are partly explained by the detoxification and metabolic capabilities of each species, as will be discussed in more detail in the following sections. However, insect adaptation to BXD-containing plants might also be influenced by larval feeding behavior and trade-offs between nutritional content and both concentration and induction of chemical defenses (not exclusively BXDs) in plant tissues.

Effects on Aphids

Piercing-sucking herbivores from the Order Hemiptera, such as aphids, possess modified mouthparts called stylets that are used to pierce through the plant cuticle, epidermis, and mesophyll cells, and feed on the highly nutritious phloem sap (Douglas, 2003). Because of this particular feeding behavior, aphids are considered to minimize tissue disruption and consequent activation of glucosylated defenses (Pentzold *et al.*, 2014b). However, the dynamic allocation patterns of BXDs still make them effective defensive compounds towards aphids.

BXDs have been considered resistance factors of cereals towards several aphid species. Hydroxamic acid levels in cereals were correlated with their resistance towards *Metopolophium dirhodium* (Argandoña *et al.*, 1980), *Schizaphis graminum* (Corcuera *et al.*, 1982), and *Sitobion avenae* (Bohidar *et al.*, 1986). Moreover, *Rhopalosiphum padi* displayed higher weight gain and survival when feeding on mutant maize plants with reduced BXD levels compared to wild-type plants (Ahmad *et al.*, 2011). Notably, however, BXD levels in maize were not correlated with resistance towards *R. maidis* (Bing *et al.*, 1990).

The detrimental effects of BXDs on many aphid species have been explored using both artificial diets and plant cultivars with different levels of BXDs. In artificial diets, DIMBOA and MBOA increased mortality in *M. dirhodium* (Argandoña *et al.*, 1980). In *R. padi*, MBOA increased reproduction rate in concentrations up to 0.1 mM, but had the opposite effect above this threshold (Hansen, 2006). DIBOA increased *R. padi* mortality in artificial diets, and this was substantiated in a comparison among wild *Hordeum* species containing different DIBOA levels (Barria *et al.*, 1992). DIMBOA also increased *S. graminum* mortality in concentrations as low as 1 mM (Argandoña *et al.*,

1983; Argandoña *et al.*, 1981) and decreased its reproduction rate in sub-lethal concentrations (0.1 mM) (Corcuera *et al.*, 1982), with DIBOA increasing mortality as effectively as DIMBOA (Zuñiga *et al.*, 1983). In choice assays, DIMBOA had antifeedant activity towards *S. graminum* in artificial diets (Argandoña *et al.*, 1983), whereas *R. padi* avoided wheat leaves from high-DIMBOA cultivars (Givovich and Niemeyer, 1991) and barley leaves treated with DIBOA (Copaja *et al.*, 2006). Additionally, aphid species differ in their susceptibility to BXDs. While DIMBOA up to 2 mM increased the mortality of *S. graminum* and *M. dirhodium*, it did not affect *R. maidis* (Corcuera *et al.*, 1982).

Structure-activity relationships comparing the toxicity and antifeedant activity of several BXD aglucones and analogues towards *S. avenae* were determined in artificial diets (Escobar *et al.*, 1999). Among the natural compounds tested, DIMBOA and DIBOA elicited remarkably higher mortality (>50% after 89h) than the lactams HMBOA and HBOA (<20%), and benzoxazolinones (<10%). Antifeedant activity and toxicity did not follow the same patterns among the tested compounds. For example, MBOA caused low mortality, but was one of the most deterrent compounds. Such discrepancies indicate that the antifeedant and toxic activities of BXDs do not necessarily arise from the same structural features. In another study, DIBOA and DIMBOA were significantly more repellent to *R. padi* than HBOA and HMBOA (Bravo *et al.*, 2004). However, BXDs were sprayed on barley leaves, and their allocation and stability during the experiment are not known.

Interestingly, BXD glucosides are also active towards aphids. In *S. graminum*, DIMBOA and DIMBOA-Glc increased mortality with LD_{50} values (24 h feeding) of 1.2 mM and 4 mM respectively. Both compounds also decreased reproduction rates at concentrations as low as 0.25 mM and caused appreciable feeding deterrence at 0.5 mM (Corcuera *et al.*, 1985). The glucosides DIMBOA-Glc and HDMBOA-Glc increased mortality of *M. dirhodium* with LD_{50} values (3 days feeding) of 5.3 mM and 1 mM, respectively, while also decreasing fecundity. Mortality curves similar to fully unfed treatments suggest that these glucosides are also antifeedant in high concentrations (Cambier *et al.*, 2001). However, it is not clear if BXD glucosides display inherent biological activity towards aphids or are activated (hydrolyzed or otherwise metabolized) by their own enzymes after ingestion.

The lower performance of aphids of high-BXD containing plants derives from a combination of toxic and antifeedant effects. Mortality curves for *S. graminum* feeding on artificial diets with 8 mM DIMBOA were similar to a non-fed treatment, suggesting that aphids died due to starvation caused by the antifeedant activity of high DIMBOA concentrations rather than toxicity. In order to separate these two effects, aphids were first exposed to diets containing DIMBOA and then transferred to diets without BXDs (Argandoña *et al.*, 1983). Mortality rates followed a biphasic distribution, being highest at intermediate DIMBOA concentrations (3-4 mM), due to the toxicity of DIMBOA ingested in the first diet. Mortality decreased at higher DIMBOA concentrations due to lower diet ingestion and DIMBOA uptake, caused by its strong antifeedant effect. A similar experiment comparing DIMBOA and DIMBOA-Glc resulted in highest mortalities at 4 mM and 6 mM respectively (Corcuera *et al.*, 1985). Upon feeding on wheat with different DIMBOA contents, the same biphasic profile was observed for DIMBOA content in bodies of *M. dirhodium* and *S. avenae* (Niemeyer *et al.*, 1989), and for both

production and DIMBOA-Glc content in honeydew of *R. padi* (Givovich et al., 1992), which are parameters reflecting food ingestion.

BXDs might increase aphid susceptibility to other plant chemical defenses and insecticides by decreasing their detoxification capabilities. In *R. padi*, DIMBOA inhibits glutathione-*S*-transferases and esterases *in vivo* at dietary concentrations as low as 1.0 and 0.5 mM, respectively (Mukanganyama *et al.*, 2003). UGT activities in *S. avenae* were lower when the aphids fed on high-DIMBOA wheat cultivars than on low-DIMBOA cultivars, and UGT inhibition by DIMBOA was confirmed *in vitro* (Leszczynski *et al.*, 1992).

The distribution and concentration of BXDs in the phloem sap and other cell structures perceived by the aphid during probing behavior are essential to their defensive role. DIMBOA-Glc was detected in honeydew of *R. padi* (Givovich *et al.*, 1992) and *S. avenae* (Leszczynski and Dixon, 1990) feeding on wheat seedlings, with low or absent DIMBOA aglucone and MBOA. Accordingly, DIMBOA-Glc was detected at concentrations around 1 mM as the only BXD hydroxamic acid in wheat phloem sap. Average DIMBOA-Glc concentrations in the phloem did not differ greatly among cultivars with different BXD levels. However, a high variability of DIMBOA-Glc levels among phloem samples from the same cultivar may reflect different biosynthesis or transport activity in particular sieve tubes, which suggests that aphids choose sieve tubes according to their BXD levels (Givovich *et al.*, 1994). BXD hydroxamic acids were also detected in vascular bundles and mesophyll cells, but not in the xylem in maize and wheat leaves (Argandoña and Corcuera, 1985; Argandoña *et al.*, 1987). The unstable aglucone HDMBOA has been detected in waxes on the surface of maize leaves (Hedin, 1993), and could be used by aphids as a chemical cue on the leaf surface to assess suitability of the host plant (Niemeyer, 1990). Furthermore, the aglucone DIMBOA and the glucosides DIMBOA-Glc and HDMBOA-Glc were present in the apoplast in maize (Ahmad *et al.*, 2011) and might also be perceived during stylet penetration.

Due to the feeding behavior of aphids, a compound present in the phloem sap can exert both antifeedant and toxic effects, while compounds located in the path followed by the aphid stylet are considered to be restricted to antifeedant effects (Cambier *et al.*, 2001). In this context, the electrical penetration graphic (EPG) technique (Tjallingii, 1985) allows the evaluation of individual parameters of the overall probing behavior and their modification by BXDs. As the hydroxamic acid content of wheat cultivars increases, *S. graminum*, *R. padi*, *S. avenae*, and *M. dirhodium* took longer to achieve phloem ingestion, and increased xylem ingestion, possibly in order to “dilute” ingested BXDs (Givovich and Niemeyer, 1991; Givovich *et al.*, 1994). On the other hand, for *R. maidis*, time to reach the phloem was not influenced by the plant BXD content, suggesting that this species is insensitive to the antifeedant effect of BXDs while searching for sieve elements. Furthermore, the duration of committed phloem ingestion did not change according to BXD levels in the plant in any of these five species, implying that BXDs are more critical before this event. Surprisingly, DIMBOA and DIMBOA-Glc offered in artificial diets decreased ingestion time for all five species, including *R. maidis*, indicating that they are all similarly responsive to the dose-dependent antifeedant activity of both compounds once they are exposed to them. The feeding strategy adopted by *R. maidis* consists in

puncturing fewer cells before reaching the phloem, as indicated by EPG comparisons to *R. padi*. Therefore, *R. maidis* minimizes exposure to BXDs in mesophyll cells and avoids their antifeedant effects, although it still suffers from them when administered via artificial diets (Givovich and Niemeyer, 1995). The influence of BXDs in the feeding behavior of *Diuraphis noxia* is similar to the sensitive species described above (Givovich and Niemeyer, 1996; Mayoral *et al.*, 1996).

Avoidance of BXDs might be learned by previous experience, as shown by *Sitobion fragariae* aphids feeding on wheat cultivars with different BXD levels (Ramirez *et al.*, 1999). In a high-BXD cultivar, the time taken by naïve aphids to reach the phloem was higher than in a low-BXD cultivar. However, on a second probing, the time to achieve phloem ingestion did not differ. A similar trend was observed for the number of mesophyll cell punctures. Such effects were also observed upon feeding on attacked and non-attacked plants, indicating that no aphid-induced effects in the plant are involved. These data suggest that aphids can adapt their feeding behavior after exposure to BXDs, avoiding them in future probes by minimizing mesophyll cell damage.

In summary, these data demonstrate that BXDs protect plants against most aphid attacks, acting in multiple instances from probing to feeding. Before probing has started, aphids can potentially already sense BXD aglucones such as HDMBOA present on the leaf surface waxes. Once the epidermis is penetrated, the stylet follows mostly through the apoplast, where BXD glucosides are also present, as well as punctures and probes mesophyll cells, possibly with mechanical disruption of organelles (Brzezina *et al.*, 1986; Hewer *et al.*, 2011). Such a scenario could lead to hydrolysis of BXD glucosides by plant β-glucosidases and expose the aphid to locally high concentrations of antifeedant BXD aglucones. Even if BXD hydrolysis does not occur, BXD glucosides appear to exert antifeedant activity as well. It is not clear whether BXD aglucones and glucosides present in the apoplast are perceived by aphid chemoreceptors and provoke antifeedant responses. However, aphid infestation induced BXD accumulation specifically in the apoplast (Ahmad *et al.*, 2011), supporting the role of BXDs as apoplastic defenses towards aphids. Once the aphid reaches a suitable sieve element and starts committed ingestion of phloem sap, it ingests considerable amounts of BXDs, mostly glucosides. DIMBOA-Glc and HDMBOA-Glc displayed antifeedant and toxic effects when present in artificial diets. However, the duration of phloem ingestion did not correlate to BXD content in plants, which suggests that BXDs in phloem sap are not concentrated enough to exert antifeedant effects or are masked by other phloem consituents.

If the antifeedant effects of BXDs represent a first line of defense against aphids, plant resistance towards aphids might not necessarily arise from the same BXDs implicated in resistance against chewing herbivores. That is, when selecting resistant cereal lines, one should be aware that BXD antifeedant and insecticidal activities are not necessarily correlated. Moreover, due to the dependence of aphids on bacterial endosymbionts, antimicrobial activities from BXDs could also contribute to their overall detrimental effects observed on aphids.

Future investigations on the effects of BXDs on aphids would benefit from taking into account their specialized feeding behavior. Studies using artificial diets offer BXDs as a homogeneous solution

and do not account for their specific allocation to leaves and possible aphid avoidance behavior during probing. Although such bioassays are useful to assess BXD toxicity, they might overestimate antifeedant effects. Alternatively, EPG studies are useful to determine what features of feeding behavior are associated with BXD avoidance, and how insects respond to them, especially insensitive species such as *R. maidis*.

Effects on root herbivores

Insect herbivores feeding on belowground plant tissues have had to adjust to the challenges posed by living in the soil, including root primary and secondary metabolites. Root herbivores are subjected to the antifeedant and toxic activities of plant chemical defenses and exploit root volatiles and exudates for host location and foraging similarly to aboveground herbivores (Erb *et al.*, 2013; Hiltpold *et al.*, 2013). However, although progress is being made in this field, the release of BXDs and other secondary metabolites by roots is still not well understood (Baetz and Martinoia, 2014; Park *et al.*, 2004).

The effect of BXDs on the Western corn rootworm (*Diabrotica virgifera virgifera*) has been widely studied due to its economic importance as a specialist maize pest. DIMBOA applied to corn roots increased mortality of *D. v. virgifera*, but is not clear how DIMBOA was absorbed and possibly metabolized by maize roots and the actual concentrations experienced by the insects. In the highest concentrations, larvae came out of the roots before dying, suggesting an antifeedant effect and death by starvation. However, larval death was also observed inside the root, indicating that toxicity also contributes to high mortality. Upon infestation experiments with *D. v. virgifera* eggs, a high-DIMBOA maize line (1300 µg/g root fresh weight, 6.16 mM) suffered less damage than a low-DIMBOA line (400 µg/g, 1.90 mM). Insect infestation in the high-DIMBOA line led to lower adult emergence rate and size when compared to the low-DIMBOA line (Xie *et al.*, 1990). In another experiment, *D. v. virgifera* feeding on maize lines with root DIMBOA levels ranging from 90-250 µg/g (0.43 – 1.18 mM) did not differ in developmental time and survival, confirming that these values are below a threshold for resistance against this herbivore (Davis *et al.*, 2000). Nevertheless, the positive correlation between root BXD contents and resistance towards *D. v. virgifera* damage was also observed in field experiments with different maize lines (Assabgui *et al.*, 1995a; Assabgui *et al.*, 1995b).

When applied to maize roots, DIMBOA, DIBOA, DIM$_2$BOA, HMBOA, and MBOA were repellent to *D. v. virgifera* larvae, as observed by choice assays (Xie *et al.*, 1992). Fewer larvae were found inside roots and more stayed on or outside roots when compared to control treatments. BXD treatment also modified host searching behavior, decreasing number of turns and increasing area searched and locomotor rate of larvae. A first bioassay-driven fractionation of maize root extracts suggested that MBOA is attractive to *D. v. virgifera* and could be used as a volatile chemical cue for location of grass hosts in a CO_2 background (Bjostad and Hibbard, 1992). However, in a second

study, the authors found CO_2 to be the only compound responsible for larval attraction, rather than other components of maize extracts (Bernklau and Bjostad, 1998). Furthermore, MBOA applied to maize roots did not show antifeedant or toxic activity towards *D. v. virgifera* (Abou-Fakhr *et al.*, 1994).

The use of BXDs by *D. v. virgifera* as chemical cues for foraging has also been investigated. Maize crown roots were shown to be more nutritious and also to present higher levels of total and exuded BXDs than primary roots. Even in a high-BXD line, *D. v. virgifera* preferred to feed on crown roots, while feeding by the generalist *D. balteata* was more distributed between crown, primary, and secondary roots. Accordingly, in no-choice assays, *D. v. virgifera* larvae gained more weight feeding on crown roots compared to primary roots in both high- and low-BXD mutant plants, reflecting their superior nutritional value. However, when given the choice between crown and primary roots, *D. v. virgifera* did not show any preference for low-BXD mutants. This suggests that *D. v. virgifera* uses BXDs as chemical cues to locate highly nutritious roots (Robert *et al.*, 2012).

The performance of *D. v. virgifera* was compared to the generalist southern corn rootworm (*Diabrotica undecimpunctata howardi*) when feeding on a BXD deficient mutant with low BXD levels and its parental line with high BXD levels (Alouw and Miller, 2015). Survival and developmental time were the same when comparing both maize lines. However, *D. v. virgifera* grew better on the high-BXD line compared to the low-BXD mutant, while no differences were observed in *D. u. howardi* growth. The better performance of the specialist *D. v. virgifera* might be related to its ability to exploit BXDs to find nutritious tissues (Robert *et al.*, 2012), while the generalist *D. u. howardi* is unable to do so.

Another specialized root herbivore, the wheat bulb fly (*Delia coarctata*), showed remarkable attraction to wheat seedling exudates (Rogers and Evans, 2013). MBOA attracted larvae in a dose-dependent fashion and might contribute to the activity of the exudate, while DIMBOA elicited a weaker response. Since MBOA is more stable than DIMBOA in the soil (Macías *et al.*, 2004), it constitutes a more reliable chemical cue for host location.

In soil, BXDs and their derivatives are present as a complex and dynamic mixture whose composition depends on many factors such as temperature, pH, and soil microbiota. Moreover, soil fungi are known to metabolize benzoxazolinones and produce a variety of aminophenols and aminophenoxazinones, as well as their malonylated and acetylated derivatives (Fomsgaard *et al.*, 2004). Such compounds could also possess biologically relevant activities on root herbivores, but this has not yet been investigated in detail. Likewise, soil nematodes and microbial pathogens are exposed to similar BXD profiles in soil environment and are subject to their biological activities (Meyer *et al.*, 2009; Zasada *et al.*, 2005). Due to the complexity of the soil matrix, it is difficult to design bioassays that reflect the BXD concentrations and allocation in the root tissue as perceived by a root herbivore in the natural context. Further studies on BXD degradation and diffusion through soil could allow better experimental setups and evaluation of BXD influence on underground natural enemies.

Metabolism of BXDs

Despite the toxicity and allelopathic and antimicrobial activities of BXDs, many animals, plants, and microorganisms have adapted to counteract these detrimental effects. Several studies have increased our understanding about how BXDs are metabolized, absorbed, and excreted by many target organisms. Such knowledge provides insight into the coevolution of these plant chemical defenses with their natural enemies and on the effects of cereal products on human health.

Metabolism in insects

Several insect species use BXD-containing plants as food, which suggests they have developed resistance strategies such as avoidance, rapid excretion, sequestration, detoxification, and target-site mutation (Després *et al.*, 2007).

Larvae of *M. separata* fed on artificial diet containing DIMBOA excreted DIMBOA-Glc, HMBOA-Glc and 1-(2-hydroxy-4-methoxyphenylamino)-1-deoxy-β-glucopyranoside-1,2-carbamate (referred here as MBOA-Glc-carbamate, Fig. 4) in the frass (Sasai *et al.*, 2009). Incubation of midgut homogenates with DIMBOA and UDP-glucose yielded DIMBOA-Glc, indicating a UGT activity. The *in vitro* DIMBOA glucosylation activity for *M. separata* was higher than for the non-adapted *B. mori*.

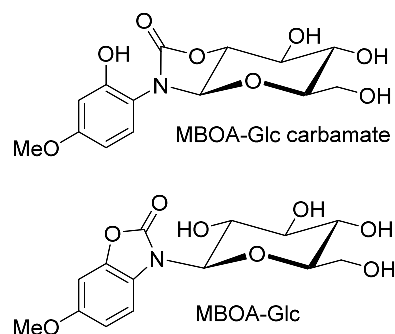


Fig. 4 Structures of MBOA metabolites detected in plants and insect frass

Metabolism of DIMBOA is observed upon incubation with *O. furnacalis* larval gut homogenates and UDP-glucose (Kojima *et al.*, 2010). This degradation is not affected by the presence of NADPH and glutathione, and is decreased if the homogenate is treated with heat or proteinases, confirming the enzymatic nature of this transformation. Assays with larvae of the less adapted species *O. scapulalis* and *O. latipennis* resulted in lower DIMBOA degradation. DIMBOA enzymatic catabolism by *O. furnacalis* larval gut homogenates had a pH optimum between 7.2 and 7.8 and was induced after feeding on DIMBOA-containing diet or maize, which was not observed for

O. scapulalis (Phuong *et al.*, 2015). However, DIMBOA-Glc was not detected and other final products were not identified.

Other well adapted species such as *S. frugiperda* have also been suggested to have resistance mechanisms towards BXDs. *S. frugiperda* and *S. littoralis* larvae feeding on DIMBOA-containing diet diminished its toxicity by glucosylation reactions, excreting DIMBOA-Glc, HMBOA-Glc, and MBOA-Glc in the frass (Glauser *et al.*, 2011). On the other hand, HDMBOA is to too unstable in the alkaline insect gut in order to be efficiently conjugated, in agreement with its potent toxic and antifeedant effect even towards the BXD-resistant *S. frugiperda*.

In a screening of many lepidopteran species, glucosylation was shown to be an important mechanism in BXD metabolism. When feeding on maize leaves, larvae of the more resistant species *S. frugiperda*, *S. littoralis*, and *S. exigua* excreted DIMBOA-Glc in the frass, while the more susceptible *Mamestra brassicae* and *Helicoverpa armigera* did not (Wouters *et al.*, 2014). *In vitro* assays confirmed that DIMBOA is glucosylated by *S. frugiperda* larval gut homogenates in the presence of UDP-glucose, suggesting the contribution of UGT enzymes. The insect-derived product, (2*S*)-DIMBOA-Glc, is an epimer of the original (2*R*)-DIMBOA-Glc produced by the plant. Such a change in stereochemistry renders the insect metabolite inert towards plant BXD β-glucosidases, which are still present and active in the gut lumen, and thus represents a detoxification mechanism that prevents further activation of this chemical defense.

The benzoxazolinone MBOA is also glucosylated by lepidopteran herbivores. Besides showing inherent toxicity, MBOA is a product of the spontaneous degradation of DIMBOA and HDMBOA (Fig. 1), two of the most abundant BXDs present in maize leaves. Furthermore, the alkaline gut of larvae of most lepidopteran species further accelerates DIMBOA and HDMBOA degradation to MBOA, suggesting that insects are exposed to high levels of this BXD. *S. frugiperda* and *S. littoralis* larvae feeding on artificial diets containing MBOA excreted considerable amounts of MBOA-Glc, while *O. nubilalis* larvae were less efficient in this conjugation reaction (Maag *et al.*, 2014). Detailed structural elucidation revealed that the observed MBOA metabolite was 3-β-D-glucopyranosyl-6-methoxy-2-benzoxazolinone (MBOA-Glc, Fig. 4) rather than the isomeric MBOA-Glc-carbamate previously characterized as a plant detoxification product (Hofmann *et al.*, 2006; Sicker *et al.*, 2001).

We further quantified the specific activity of MBOA glucosylation in gut homogenates of *S. frugiperda* and *O. nubilalis* larvae, as shown in Fig. 5. *S. frugiperda* displayed a specific activity more than 20-fold higher than *O. nubilalis*, and no significant differences were observed between *O. nubilalis* univoltine and bivoltine strains. Accordingly, the tolerance of *O. nubilalis* to BXD-containing plants was suggested not to depend on detoxification mechanisms, but rather on temporal aspects and foraging preferences (Maag *et al.*, 2014). It is not known, however, whether this UGT activity is induced upon exposure of *O. nubilalis* to BXDs. The regulation and identity of UGTs responsible for BXD detoxification in *S. frugiperda* are being currently investigated.

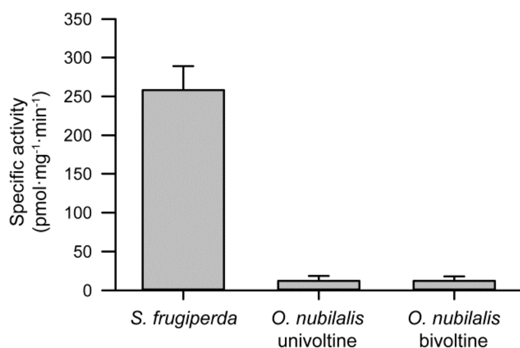


Fig. 5 Specific activity of MBOA glucosylation (±SEM) in gut homogenates of *S. frugiperda* and *O. nubilalis* (univoltine and bivoltine strains) (N=3)

A recent comparison between performance and transcriptional profiles of *S. frugiperda* and *S. littoralis* showed that total expression of UGT-encoding genes levels did not change between larvae feeding on artificial diet and on maize leaves (Roy *et al.*, 2016). This supports the hypothesis that enzymes responsible for BXD glucosylation are constitutively expressed in *Spodoptera* spp., rather than being induced upon contact with BXDs. However, basal UGT expression levels are higher on a *S. frugiperda* strain that uses maize as a main host, compared to a rice strain. Furthermore, *S. frugiperda* performance on maize was higher than *S. littoralis*, which might be due to a lower tolerance to BXDs in the latter.

Unfortunately, to the best of our knowledge there are no studies investigating metabolites of BXD detoxification in aphids. However, *S. avenae* reared on wheat over 10 generations possessed increased activities of cytochrome P450 monooxygenases, NADPH cytochrome *c* reductase, GSTs, and esterases, but not catalases (Loayza-Muro *et al.*, 2000). In general, these increases were more pronounced in the low-BXD wheat cultivar tested, presumably due to antifeedant activity and limited BXD ingestion by aphids reared on the high-BXD cultivar. Such upregulation of detoxification enzymes might indicate they are involved in BXD metabolism in this aphid species, but more studies are necessary to confirm this hypothesis. Aphids also have a wide range of enzymes in the saliva (Madhusudhan *et al.*, 1994), which can potentially be secreted during probing and modify BXDs, as has been proposed for phenols (Urbanska *et al.*, 1998).

Tolerance towards BXDs has also been suggested for *D. v. virgifera*. The transcriptional profiles of larvae feeding on a low-BXD maize mutant and on the high-BXD parent line have been compared (Miller and Zhao, 2015). Differentially expressed genes included a cytochrome P450 and a cathepsin protease. This indicates that *D. v. virgifera* might detoxify BXDs using P450s, but this hypothesis remains to be tested.

Besides detoxification, other strategies such as avoidance and sequestration might contribute to the resistance towards BXDs observed in some insect species. For example, leaf snipping

minimizes plant tissue disruption during feeding and, together with an alkaline gut, can inhibit the activation of plant chemical defenses. These mechanisms allow *Zygaena filipendulae* to limit hydrolysis of cyanogenic glucosides in its host plant and to sequester these compounds to be used against predators (Pentzold *et al.*, 2014). On the other hand, gene expression analyses can reveal potential candidates differentially regulated upon BXD treatment that are involved in BXD metabolism. Feeding assays using purified compounds in artificial diets or plants lacking BXDs are also helpful in assessing the metabolism of individual BXDs, facilitating the quantification and structural elucidation of final products.

Metabolism in mammals

In addition to their importance in assessing the safety and therapeutical uses of BXD derivatives, the metabolism of BXDs in mammals may reveal some common strategies that other animals, including insects, employ to metabolize these compounds. Ingestion of rye bread naturally containing a mixture of BXDs revealed some aspects of BXD metabolism in mammals. In rats and pigs, glucoside hydrolysis, hydroxamic acid reduction to lactam, and aglucone conjugation to glucuronic acid were important metabolic reactions (Adhikari *et al.*, 2012a; Adhikari *et al.*, 2012b). In humans, hydroxamic acid reduction and aglucone conjugation with glucuronic acid and sulfate were observed (Adhikari *et al.*, 2013). However, a considerable part of ingested BXDs was not recovered in the urine and feces in the three studies, indicating that absorption or transformation to unknown metabolites also took place.

Conclusions

Benzoxazinoids constitute a class of plant chemical defenses that display biological activities against a wide range of insect herbivores and other target organisms. The specialized distribution of BXDs among and within plant tissues seem to be crucial for their efficacy against insect herbivores from distinct ecological guilds. A wide range of modes of action derive from the unique structural features and reactivities of BXDs, making them exceptionally interesting from the points of view of evolution and ecology. The studies covered in this review expand our understanding on how BXDs exert toxicity in insect herbivores and influence their physiology, and on the differential susceptibility of different insect species, in addition to give a few first examples of how they have adapted behaviorally and biochemically to cope with these plant defenses. Additionally, the data discussed raise important points concerning experimental aspects of BXD research and expose many other questions that remain to be clarified.

Feeding assays are important tools to assess BXD effects in a dose-dependent way. However, when investigating toxic and antifeedant effects of BXDs, their stability under the bioassay conditions must be considered. Given their reactivity, unstable BXDs can react with other diet components (Argandoña *et al.*, 1982) or degrade to other products such as benzoxazolinones, which might modify the final results. For example, most HDMBOA in an artificial diet degraded within 30 min after HDMBOA-Glc hydrolysis (Glauser *et al.*, 2011) and a similar, albeit slower, decomposition is observed for DIMBOA (Campos *et al.*, 1989). As the degradation of BXD hydroxamic acids and *N*-*O*-methyl derivatives to benzoxazolinones is faster at high pH values (Maresh *et al.*, 2006; Niemeyer *et al.*, 1982), the acidification of diets constitutes an alternative to improve BXD stability during feeding bioassays (Argandoña *et al.*, 1982). Similarly, BXDs applied on plant leaves can also degrade or be metabolized quickly, and their persistence should be assessed when designing experiments. Additionally, the BXD defense system in plants is compartmentalized and depends on temporally and spatially resolved activation by hydrolysis. It is challenge to set up a bioassay that reflects the tissue-specific distribution of BXDs as present in a plant leaf or the gradients of multiple BXDs diffusing through soil. As discussed in the present paper, such aspects might influence insect responses and should be considered when analyzing data from artificial experimental setups.

Studies on the influence of BXDs on food consumption and utilization by insect herbivores are useful to discriminate toxic, digestibility-reducing, and antifeedant factors. In this context, bioassays should take into account the dynamics of the plant-insect interaction, especially when investigating the ecology of such interactions. Induction of BXDs in the plant and upregulation of detoxification genes in the insect both play a role in the natural context and should be accounted for. Artificial diets provide a more controlled way to assess the effects of single compounds on insect physiology but have limitations in representing the plant, since dynamic responses are absent and their nutritional composition is different, which are factors known to modulate toxin activities (Duffey and Stout, 1996). Even in bioassays using plants, the nutritional values and levels of other secondary metabolites in different mutant lines and plant tissues should be considered, in addition to BXD concentrations. These precautions are not meant to discourage studies on how BXDs or other plant defensive chemicals affect consumption and utilization of foods, but are rather factors that should be kept in mind when designing experiments and drawing conclusions from such data.

Future studies on BXD metabolism by insects and other target organisms would benefit from the structural elucidation and quantification of BXD metabolites by modern analytical methods. The development of mutant plant lines with low levels of BXDs facilitates the screening of a wide range of metabolites, especially when allied to metabolomic approaches (Glauser *et al.*, 2011; Maag *et al.*, 2015). In contrast, feeding single compounds via artificial diets facilitates the identification of individual metabolic pathways and their quantification. This is especially important considering the interlinked metabolism of BXDs, with conversion of hydroxamic acids to lactams, and of multiple benzoxazinones to a single benzoxazolinone. The structural elucidation of metabolites gives rise to hypotheses about the enzymatic pathways involved, which can be confirmed *in vitro* and characterized on the gene

level. Finally, knowledge about the genes and enzymes recruited in BXD metabolism enables further studies on their regulation, specificity, and evolution.

The toxicokinetics of BXDs are another important aspect in the description of their biological activities in insects. The absorption, distribution, and excretion of BXDs can be followed with high sensitivity by using radioactive isotopically labelled compounds (Campos *et al.*, 1988, 1989), but these are not readily available. However, careful studies with unlabeled compounds can also provide important insights on those processes and suggest the involvement and location of transporters and detoxification enzymes, and other strategies such as sequestration.

Further understanding of the biological activities and metabolism of BXDs by target organisms require the convergence of many fields such as ecology, evolutionary biology, biochemistry, analytical chemistry, and organic synthesis. The resulting knowledge will provide insight on the ecology of the interactions between BXD-containing plants and other organisms, their coevolution, and can contribute to the development of technological applications such as plant breeding strategies and novel targets for pest control.

Material and methods:

Insects: Larvae of *S. frugiperda* (maize strain) were a generous gift from the Department of Entomology of the Max Planck Institute for Chemical Ecology, and were reared on an artificial diet based on white beans (Bergomaz and Boppré, 1986), under controlled light and temperature conditions (12:12 h light/dark, 20 °C). Eggs from univoltine and bivoltine strains of *O. nubilalis* were obtained from the Agroscope Changins (Switzerland) and were reared under the same conditions described above. The *O. nubilalis* diet was adapted from the literature (Maag *et al.*, 2014), using barley flour instead of wheat germ.

Nutritional indices: Third instar larvae were individually kept on plastic cups and under the rearing conditions described above for 12 days. MBOA was added to the diets during the cooling step of the preparation process as a solution in ethanol (5 mL for each 100 g diet) or pure ethanol for the control treatment. The diets were uniformly poured into Petri dishes, and diet plugs were cut with a cork borer, left for 15 min for ethanol evaporation, and replaced daily. Frass and remaining diet in cups were collected daily, freeze-dried overnight, and weighed in order to quantify ingested food and feces. Larvae were weighed every second day to assess growth. At the end of the experiment and at the end of the life stages studied, larvae were freeze dried and shown to have consistent water content (85%). All larval weights were calculated as dry mass using water content of each individual by the end of the experiment. Overall weight gain, mean weight, ingested food, and feces were calculated in terms of dry mass over the 12 days of the experiment, and nutritional indices were calculated according to the following equations (Waldbauer, 1982):

$$\mathrm{RGR} = \frac{\text{weight gained}}{\text{time} \times \text{mean weight}} \quad \mathrm{CI} = \frac{\text{ingested food}}{\text{time} \times \text{mean weight}} \quad \mathrm{AD} = \frac{\text{ingested food} - \text{feces}}{\text{ingested food}} \times 100$$

$$\mathrm{ECI} = \frac{\text{weight gained}}{\text{ingested food}} \times 100 \quad \mathrm{ECD} = \frac{\text{weight gained}}{\text{ingested food} - \text{feces}} \times 100$$

Enzymatic assays: Gut homogenates for *in vitro* assays were dissected from third to fourth instar larvae of *S. frugiperda* and *O. nubilalis*. The caterpillars were dissected in cold 10 mM phosphate buffer (pH 7.0) and the gut tissue was isolated and its contents removed. The rinsed gut tissues were then transferred to a fresh tube and homogenized with 100 µL of 10 mM phosphate buffer (pH 7.0) per gut. Protein concentrations were determined using the method of Bradford (1976). For the *in vitro* assays 10 µL of gut suspension were incubated with 75 nmol of MBOA and 150 nmol of UDP-glucose in 100 mM phosphate buffer at pH 7.0 (final assay volume: 50 µL). After an incubation period of 60 min at 30 °C the reaction was stopped by adding 50 µL of MeOH/formic acid (50:50, v/v). The samples were centrifuged at 5000 g for 5 min prior to analysis by HPLC-MS/MS. Aliquots of the gut homogenates were heated at 100 °C for 15 min and used for boiled controls.

Chromatographic methods: For analytical chromatography procedures, formic acid (0.05%) in water and acetonitrile were used as mobile phases A and B, respectively, and the column temperature was maintained at 25 °C. The quantitative analysis of MBOA-Glc produced in *in vitro* assays used an XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent Technologies, Boeblingen, Germany) with a flow rate of 1.1 mL min^{-1} and with the following elution profile: 0-0.5 min, 95% A; 0.5-6 min, 95-67.5% A; 6.02-7 min, 100% B; 7.1-9.5 min, 95% A. HPLC-MS/MS analyses were performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany) coupled to an API 3200 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a turbospray ion source operating in negative ionization mode. The ion spray voltage was maintained at −4500 V. The turbo gas temperature was 500 °C, nebulizing gas 60 psi, curtain gas 25 psi, heating gas 60 psi and collision gas 5 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion to product ion conversion with MRM parameters for MBOA-Glc optimized from infusion experiments with a standard (Q1 *m/z*: 372, Q3 *m/z*: 164, DP −15 V, EP −4.5 V, CEP −18 V, CE −20 V, CXP −4 V). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing.

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Manuscript III

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Chemical Ecology

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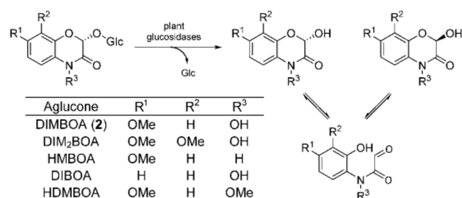
Reglucosylation of the Benzoxazinoid DIMBOA with Inversion of Stereochemical Configuration is a Detoxification Strategy in Lepidopteran Herbivores**

*Felipe C. Wouters, Michael Reichelt, Gaétan Glauser, Eugen Bauer, Matthias Erb, Jonathan Gershenzon, and Daniel G. Vassão**

Abstract: *Benzoxazinoids are chemical defenses against herbivores and are produced by many members of the grass family. These compounds are stored as stable glucosides in plant cells and require the activity of glucosidases to release the corresponding toxic aglucones. In maize leaves, the most abundant benzoxazinoid is (2R)-DIMBOA-Glc, which is converted into the toxic DIMBOA upon herbivory. The ways in which three Spodoptera species metabolize this toxin were investigated. (2S)-DIMBOA-Glc, an epimer of the initial plant compound, was observed in the insect frass, and the associated glucosyltransferase activity was detected in the insect gut tissue. The epimeric glucoside produced by the insect was found to be no longer reactive towards plant glucosidases and thus cannot be converted into a toxin. Stereoselective reglucosylation thus represents a detoxification strategy in Spodoptera species that might help to explain their success as agricultural pests on benzoxazinoid-containing crops.*

To avoid damage by insect herbivores, plants have evolved diverse defense mechanisms, including the production of toxic or deterrent metabolites. In response, many insect species have developed adaptations that enable them to feed on chemically-defended plants without apparent negative effects.[1] These adaptations include the rapid excretion of defense compounds,[2] sequestration,[3] and detoxification.[4] Collectively, these adaptations contribute to the unparalleled ecological success of insect herbivores in terrestrial ecosystems. Adaptations to plant defense traits have also favored the emergence of some species as agricultural pests, as is the case with some lepidopteran caterpillars.[5]

Benzoxazinoids (BXDs) are indole-derived plant defense compounds that are widespread in grasses (Poaceae), including crops like wheat, rye, and maize.[6] They are stored as stable glucosides in plant cells and are hydrolyzed to toxic aglucones upon damage, when they come into contact with specific β-glucosidases (Scheme 1).[7] The aglucones formed



Aglucone	R^1	R^2	R^3
DIMBOA (**2**)	OMe	H	OH
DIM_2BOA	OMe	OMe	OH
HMBOA	OMe	H	H
DIBOA	H	H	OH
HDMBOA	OMe	H	OMe

Scheme 1. Benzoxazinoids are plant defense compounds that are activated by plant glucosidases to release toxic aglucones. Different substitution patterns give rise to the many representatives of this class.

are cyclic hemiacetals that form α-oxo aldehydes through ring opening and thereby become reactive towards a wide range of biological nucleophiles.[8] The quantities and proportions of BXDs in grasses vary between different plant species,[6a] organs, and developmental stages,[9] and after induction by herbivores.[10] The most abundant BXD in young maize (*Zea mays*) leaves is (2*R*)-2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA-Glc, **1**). The corresponding aglucone (DIMBOA, **2**) delays growth and decreases survival in several Lepidopteran species, including *Sesamia nonagrioides*,[11] *Ostrinia nubilalis*,[12] *Ostrinia furnacalis*,[13] *Spodoptera littoralis*,[10] and *Spodoptera exigua*,[14] while *Spodoptera frugiperda* seems to be less affected.[10,14] Moreover, **2** inhibits peptidases in *S. nonagrioides*[11] and detoxification enzymes such as glutathione *S*-transferases and esterases in the aphid *Rhopalosiphum padi*.[15] The ability of herbivores to metabolize **2** could thus facilitate their capacity to feed on maize. Although BXD biosynthesis in plants is well-studied,[7] knowledge of their metabolic fate in herbivores is limited and would provide

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information about detoxification mechanisms and their role in host selection.

To compare BXD metabolism among various Lepidoptera, we analyzed frass (caterpillar feces) samples from *S. frugiperda*, *S. littoralis*, *S. exigua*, *Mamestra brassicae*, and *Helicoverpa armigera*, all of which had previously fed on maize leaves. LC–MS/MS with multiple reaction monitoring (MRM) was used to separate and detect BXD glucosides and aglucones (Figure 1). We observed a peak corresponding to glucoside **1** in samples from *S. frugiperda*, *S. littoralis*, and *S. exigua*, but not from *M. brassicae* and *H. armigera*. This result suggests that the former group of species either excretes intact **1** by inhibiting its hydrolysis or reglycosylates **2** during digestion, as previously hypothesized.[10]

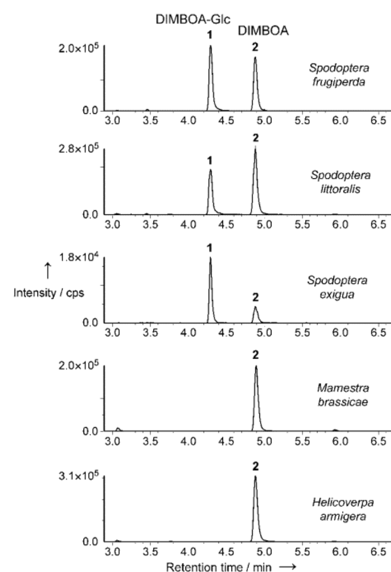

Figure 1. Benzoxazinoid profiles of frass collected from different insect species after they had fed on maize containing DIMBOA-Glc (**1**) demonstrated that this compound is present in *S. frugiperda*, *S. littoralis*, and *S. exigua* after digestion, but not in *M. brassicae* and *H. armigera*. Fourth instar larvae were fed on maize leaves for 48 h. Frass samples were collected, extracted with acidified water/methanol (50:50 v/v, 0.5% formic acid), and analyzed by LC–MS/MS(MRM). Only chromatographic traces for DIMBOA-Glc (**1**) and DIMBOA (**2**) are depicted. The intensities in counts per second (cps) do not reflect quantitative ratios.

To test this putative reglycosylation, we incubated cell-free suspensions from *S. frugiperda* gut tissues with **2** and uridine diphosphate glucose (UDP-glucose). A peak corresponding to **1** confirmed DIMBOA-UDP-glucosyltransferase (UGT) activity (Figure 2A). This activity was highest at pH values around 7.0 (data not shown), thus suggesting the action of an intracellular enzyme, which is in agreement with the membrane association of most insect UGTs.[16]

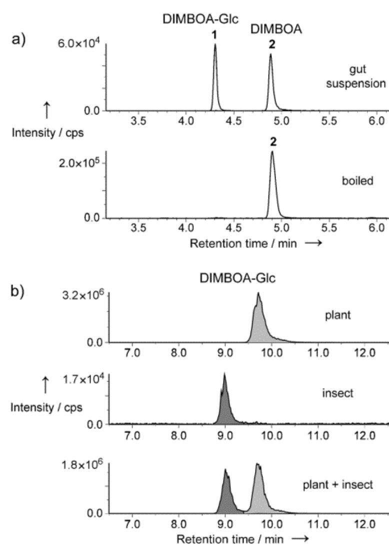

Figure 2. a) Incubation of DIMBOA (**2**) and UDP-glucose with *S. frugiperda* cell-free gut suspensions led to the production of DIMBOA-Glc (**1**; top chromatogram), thus indicating UDP-glucosyltransferase activity that is not observed when using boiled gut preparations (bottom chromatogram). Fourth instar *S. frugiperda* and *S. littoralis* larvae fed either on artificial diet or maize leaves for 48 h were dissected and homogenized, and cell-free gut suspensions were incubated with 3 mM DIMBOA and 6 mM UDP-glucose at pH 4.0, 5.4, 7.0, 8.4, and 10.0 at 30 °C for 30 min, followed by the addition of methanol/formic acid (1:1, v/v), centrifugation, and analysis by LC–MS/MS(MRM). Only assays at pH 7.0 with gut extracts from plant-fed *S. frugiperda* are presented. b) Chromatography under optimized conditions revealed that the DIMBOA-Glc observed in insect frass samples (dark gray) is different from the plant DIMBOA-Glc (light gray). Plant and *S. frugiperda* frass samples were extracted with acidified water/methanol (50:50 v/v, 0.5% formic acid) and analyzed by LC–MS/MS(MRM). The intensities in counts per second (cps) do not reflect quantitative ratios.

A close comparison between chromatograms revealed a small difference in retention time between the peaks corresponding to **1** in samples from plant and insect (both frass and in vitro assays). After optimizing HPLC separation, we confirmed that plant- and insect- derived **1** are indeed different compounds (Figure 2B). Since both compounds present similar fragmentation patterns and MS^2 spectra (Figure S1 in the Supporting Information), we hypothesized that they might differ in terms of stereochemical configuration.

In order to elucidate the structure of insect-derived **1**, we purified it from *S. frugiperda* frass extracts and compared its ^{1}H NMR spectrum (Figure S2) to that of the plant-derived compound (Figure S3). Axial–axial coupling constants of

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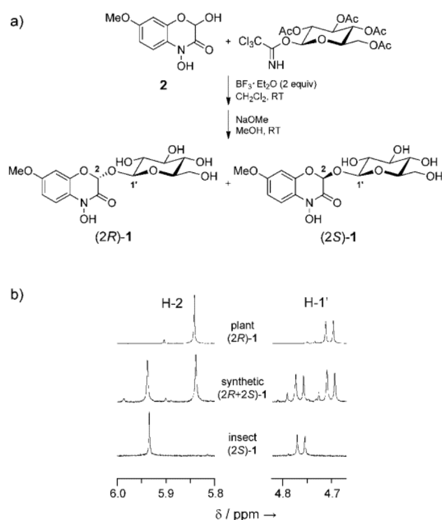
around 8–9 Hz for the glycoside moiety of the insect compound confirmed that it is also a β-glucoside. However, large differences in the chemical shifts for H-2 and H-1′ (Table S1 in the Supporting Information) suggested that plant- and insect-derived **1** may differ in their configuration at C-2. Grasses exclusively accumulate (2*R*)-DIMBOA-Glc [(2*R*)-**1**],[17] which can be synthesized in a diastereoselective fashion.[18] By modifying this published synthetic method, we chemically glucosylated DIMBOA to yield both (2*R*)- and (2*S*)-**1** epimers in a 1:1 ratio (Scheme 2 A). After comparing the NMR spectra from plant-derived, insect-derived, and synthetic samples of **1** (Scheme 2 B), we were able to determine that the insect-derived compound is in fact (2*S*)-DIMBOA-Glc [(2*S*)-**1**].



Scheme 2. a) Chemical glucosylation of DIMBOA (**2**) to yield (2*R*)- and (2*S*)-DIMBOA-Glc (**1**) in a 1:1 ratio by using a method modified from Kluge and Sicker;[18] b) A comparison of partial ^{1}H NMR (500.13 MHz, [D$_6$]acetone) spectra from **1** obtained from the plant, insect frass, and chemical synthesis demonstrates that the insect-derived compound corresponds to (2*S*)-**1**, an epimer of the plant compound.

Since we exclusively observed the (2*S*)-**1** epimer in frass samples, we assessed its abundance relative to (2*R*)-**1** during the course of digestion in the insect gut. We dissected maize-fed insects and analyzed the contents of different regions of the gut by LC–MS/MS. There was a gradual increase in the ratio of (2*S*)-**1** to (2*R*)-**1** upon progression through the foregut and midgut (Figure 3). This ratio markedly increased in hindgut/rectum, and (2*S*)-**1** was the exclusive form excreted in the frass. Furthermore, we observed (2*S*)-**1**, but not (2*R*)-**1** or **2**, in hemolymph from insects feeding on maize leaves (data not shown).

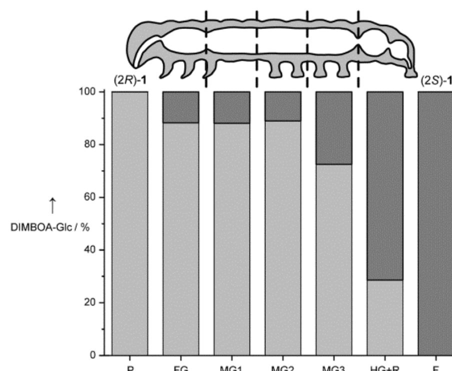


Figure 3. LC–MS analyses of the contents of different gut regions evidence a gradual conversion of (2*R*)-DIMBOA-Glc (**1**; light gray) into (2*S*)-**1** (dark gray) along the course of the insect gut (P plant, FG foregut, MG midgut regions, HG + R hindgut/rectum, F frass). Guts of fourth instar *S. frugiperda* fed on maize leaves for 4 days were dissected and separated into five regions, the contents of which were individually collected, extracted with acidified water/methanol (50:50 v/v, 0.5% formic acid), and analyzed by LC–MS/MS(MRM). Bars represent peak areas for (2*R*)-**1** and (2*S*)-**1** normalized to their sum. The results from one experiment are shown but three replicates showed the same general profile.

Simple reglucosylation to afford a BXD glucoside could be reversed by plant glucosidases, which are likely active along the entire course of the insect gut.[10] This would lead to energy expenditure by the insect instead of effective detoxification. We examined whether the change from (2*R*)-**1** to (2*S*)-**1** affects its suitability as a glucosidase substrate. Insects fed on maize leaves were dissected and the gut contents (containing both epimers of **1** and glucosidases) were incubated in vitro at different pH values. Plant glucosidases were active even after going through the digestion process and displayed striking specificity towards the plant-produced (2*R*)-**1**, whereas the insect-derived (2*S*)-**1** remained unhydrolyzed (Figure 4). Although this glucosidase activity was higher at pH 7.0, it was easily detected after 30 min at pH 10.0. These incubation conditions are realistic considering that lepidopteran guts are highly alkaline[19] and the digestion of plant material averages several hours.[20] In *S. frugiperda*, we measured pH values ranging from 8.5 to 9.2 in all regions of the gut, except in the rectum, where near-neutrality was observed (Figure S4). The glucosidase activity observed at these pH values fits with the corresponding ratios of (2*S*)-**1** to (2*R*)-**1** observed through the gut, and further corroborates previous observations that BXD aglucones are constantly released along the course of the insect gut during digestion.[10] Therefore, the insect UGT not only stabilizes the reactive **2** by glucosylation, but its stereoselectivity additionally renders the new glucoside (2*S*)-**1** inert towards the still-active plant glucosidases, thereby preventing its further hydrolysis to restore **2**.

Since insect UGTs are intracellular and typically membrane-associated, we hypothesize that the reglucosylation of **2**

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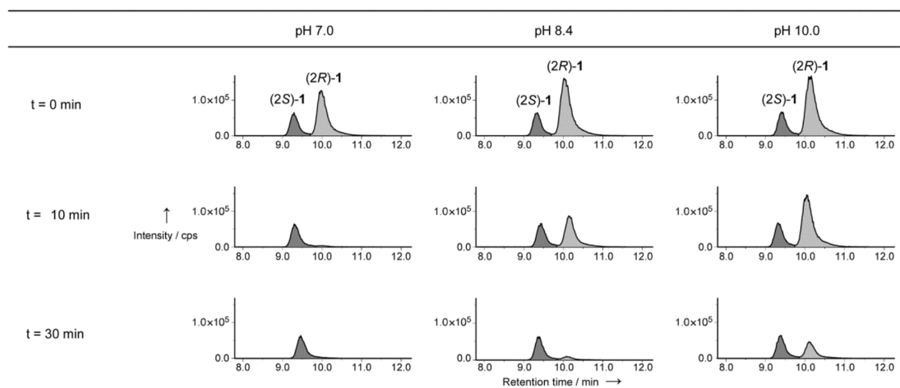

Figure 4. Incubation of the gut contents of maize-fed *S. littoralis*, which contain the plant DIMBOA-Glc (2*R*)-**1** and the insect metabolite (2*S*)-**1**, at different pH values indicates that plant glucosidases are still active under all conditions tested and are specific towards (2*R*)-**1** (light gray). The insect-derived (2*S*)-**1** (dark gray) was not hydrolyzed. Fourth instar *S. littoralis* larvae fed on maize leaves for 8 h were dissected and the total gut contents, which naturally contain both (2*S*)- and (2*R*)-**1**, were incubated at pH 7.0, 8.4, and 10.0 at 30 °C for 10 or 30 min, followed by the addition of methanol/formic acid (1:1, v/v), centrifugation, and analysis by LC–MS/MS(MRM).

takes place inside the gut cells after absorption. As an aglucone, **2** is quickly converted into a racemic mixture by oxo-cyclo-tautomerism[21] and is taken up by the insect UDP-glucosyltransferase, which displays a higher affinity for the aglucone substrate that leads to formation of the 2*S* product. A dynamic kinetic resolution of **2** is thus established through its stereoselective conversion to (2*S*)-**1**. The resulting biologically inert (2*S*)-**1** may be actively transported 1) back to the gut contents and/or 2) to the hemolymph, from which it can be delivered into the hindgut contents later. Subsequently, (2*S*)-**1** is excreted and represents the only epimer of DIMBOA-Glc in the frass.

Our results indicate that the stereoselective reglucosylation of DIMBOA to (2*S*)-DIMBOA-Glc is a detoxification mechanism in the *Spodoptera* species we studied. DIMBOA-UGT activity in *Spodoptera* and other Lepidoptera has already been demonstrated or suggested.[10,22] Our work provides information about the stereoselectivity of this transformation. This strategy may be widespread in the genus *Spodoptera*, which would be consistent with the elevated degree of tolerance towards maize and BXDs in this genus.[10] Glucosylation is an important detoxification pathway that stabilizes toxins and favors excretion.[23,24] To our knowledge, the epimerization of plant defensive compounds by insects has only been described so far for butterflies that use plant pyrrolizidine alkaloids as precursors for pheromone biosynthesis.[25] In this context, our work describes a novel insect herbivore detoxification strategy that is based on the stereochemical inversion of one chiral center to stabilize and deactivate a plant defensive compound. Future characterization of the insect UGT enzyme(s) involved in DIMBOA detoxification should shed light on its efficiency, specificity, evolutionary origin, and value to herbivores feeding on maize. This information and further progress in clarifying how lepidopteran herbivores metabolize BXDs should also increase our understanding of their coevolution with grasses.

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Supporting Information

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Reglucosylation of the Benzoxazinoid DIMBOA with Inversion of Stereochemical Configuration is a Detoxification Strategy in Lepidopteran Herbivores**

*Felipe C. Wouters, Michael Reichelt, Gaétan Glauser, Eugen Bauer, Matthias Erb, Jonathan Gershenzon, and Daniel G. Vassão**

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S2

1. Supporting Methods

1.1. Insects and plants: Larvae of *Spodoptera frugiperda* and *Helicoverpa armigera* were obtained from colonies at the Max Planck Institute for Chemical Ecology, while *Spodoptera exigua* were provided by Andermatt Biocontrol AG (Grossdietwil, Switzerland). Eggs of *Spodoptera littoralis* and *Mamestra brassicae* were provided by Syngenta (Maintal, Germany) and J. Harvey (NIOO Wageningen, The Netherlands) respectively. All insect species were reared on an artificial diet based on white beans,[1] under controlled light and temperature conditions (12:12 h light/dark, 20 °C). Seeds of *Zea mays* were obtained commercially (Kiepenkerl, Germany) and grown under controlled light and temperature conditions (16:8 h light/dark, day-time temperature 22 °C, night-time 20 °C).

1.2. Sample extraction: Plant samples were collected from leaves of 2 week-old maize plants, flash-frozen and stored at −80 °C when necessary. Samples were quickly ground under liquid nitrogen, weighed (around 100 mg) and extracted with a 0.5% formic acid in water/methanol 1:1 (v:v) solution at 10 μL per mg sample. Frass and gut lumen samples were collected after allowing third to fourth instar *S. frugiperda*, *S. littoralis*, *S. exigua*, *M. brassicae* and *H. armigera* individuals to feed on cut maize leaves from 2 week-old plants for 2 days. Frass samples were collected directly and weighed, while gut lumen samples were obtained after dissecting *S. frugiperda* individuals on ice, removing the gut, and separating it into different regions. The contents of the various gut regions were collected separately, weighed and kept on ice. Both frass and gut lumen samples were extracted with 10 μL per mg sample of the same solution used for plant samples. Extracts were vortexed for 2 min, agitated in a paint shaker for 2 min (with 3 mm steel beads), centrifuged at 16,000 g for 5 minutes, and the obtained supernatants were collected and analyzed by LC-MS/MS.

1.3. Chromatographic methods: For all analytical chromatography procedures, formic acid (0.05%) in water and acetonitrile were used as mobile phases A and B, respectively, and the column temperature was maintained at 25 °C. The initial BXD profiling in frass samples used an XDB-C18 column (50 x 4.6 mm, 1.8 μm, Agilent Technologies, Boeblingen, Germany) with a flow rate of 1.1 mL min^{-1} and with the following elution profile: 0-0.5 min, 95% A; 0.5-6 min, 95-67.5% A; 6.02-7 min, 100% B; 7.1-9.5 min, 95% A. In order to achieve better resolution between the two DIMBOA-Glc epimers, a Nucleodur Sphinx RP column (250 x 4.6 mm, 5 μm, Macherey-Nagel, Düren, Germany) was employed at a flow rate of 1.0 mL min^{-1} in isocratic mode: 0-10 min, 85% A; 10.1-12 min, 100 % B; 12.1-15 min, 85% A.

LC-MS/MS analyses were performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany) coupled to an API 3200 tandem spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source operating in negative ionization mode. The ion spray voltage was maintained at −4500 V. The turbo gas temperature was 500 °C, nebulizing gas 60 psi, curtain gas 25 psi, heating gas 60 psi and collision gas 5 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion to product ion conversion with MRM parameters for DIMBOA-

Glc from the literature,[2] except for the Q1 and Q3 *m/z* values of 418 and 372; and with MRM parameters for DIMBOA optimized from infusion experiments with a standard (Q1 *m/z*: 210, Q3 *m/z*: 149, DP −15 V, EP −8 V, CEP −12 V, CE −16 V, CXP −4 V). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing.

HPLC-MS experiments to assess MS^2 spectra of DIMBOA-Glc epimers were performed on Agilent series 1100 equipment coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in negative mode over the range *m/z* 60-1000 with skimmer voltage 40 V, capillary exit voltage 123.4 V, capillary voltage 4000 V, nebulizer pressure 35 psi, drying gas 11 L min^{-1}, and gas temperature 330 °C. The eluate was split off in a 4:1 ratio before reaching the mass spectrometer. Chromatograms were analyzed with the DataAnalysis software from Bruker Daltonics.

1.4. NMR analyses: ^1H NMR (500.13 MHz) spectra were recorded in a Bruker AVANCE-500 NMR spectrometer. Acetone-d_6 was used as solvent and the residual solvent signal (quintet at 2.05 ppm) was used as internal reference standards. NMR spectra were obtained with experiments from the standard Bruker program package.

1.5. HR-ESI-MS analyses: MS analyses of (2*R*)-DIMBOA-Glc and (2*S*)-DIMBOA-Glc samples were performed using the direct infusion method on a Q Exactive Plus-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) in negative ion mode. Capillary temperature and capillary voltage were set to 275 °C and 35 V respectively. Full-scan mass spectra were acquired in a mass range of *m/z* 250–450 at a mass resolution of m/Δm 70000. Data interpretation was accomplished using XCALIBUR (Thermo Fisher Scientific, Waltham, MA, USA).

1.6. Enzymatic assays: Glucosyltransferase activity assays were performed with *S. frugiperda* and *S. littoralis* gut tissues from fourth instar individuals feeding on maize leaves for 4 days. Caterpillars were dissected in cold Tris HCl buffer (pH 8.4, 10 mM). Their guts were collected, emptied and cleaned, and the resultant tissue homogenized in 50 µL of the same buffer per gut. Aliquots of these gut suspensions were boiled for 20 min. Enzymatic assays included 10 µL gut suspension, 2 µL of 75 mM DIMBOA in DMSO (31.7 µg, 0.15 µmol), 4 µL of 75 mM UDP-Glucose in water (183.1 µg, 0.3 µmol) and enough 100 mM buffer solution to give an assay volume of 50 µL. Controls containing boiled gut suspension, and only the gut suspension and buffer were included. The following buffers were also used: citric acid/sodium citrate, pH 4.0 and pH 5.4, 100 mM; phosphate, pH 7.0, 100 mM; and carbonate/bicarbonate, pH 10.0, 100 mM. After incubation at 30 °C for 30 min, the enzymatic reactions were interrupted by adding 50 µL of a methanol/formic acid 1:1 (v:v) solution. Assay tubes were centrifuged at 5,000 g for 5 minutes and the obtained supernatant was collected and analyzed by LC-MS/MS.

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Plant glucosidase activity assays were performed with gut lumen samples from individuals of *S. littoralis* feeding on maize leaves for 8 h. After dissection on ice, guts were separated and the gut lumen collected, weighed and kept on ice. To these gut lumen samples, 10 μL of buffer per mg sample were added and the contents homogenized. The following buffers were used: MOPS, pH 7.0, 10 mM; TAPS, pH 8.4, 10 mM; and CAPS, pH 10.0, 10 mM. Assay tubes were incubated at 30 °C and 50 μL aliquots were taken at time points 0, 10 and 30 min, added to 50 μL of a methanol/formic acid 1:1 (v:v) solution, centrifuged at 5,000 g for 5 min and the resulting supernatants were collected and analyzed by LC-MS/MS.

1.7. Reagents and solvents: DIMBOA for synthesis was kindly provided by Prof. Dieter Sicker (University of Leipzig, Germany). UDP-glucose (Santa Cruz Biotechnology), *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)trichloroacetimidate (Merck), acetone-d_6 (VWR), and UDP-galactose, boron trifluoride etherate, sodium methoxide, dichloromethane and methanol (Sigma-Aldrich) were commercially obtained.

1.8. (2*R*)-DIMBOA-Glc purification: (2*R*)-DIMBOA-Glc was obtained by purification of maize leaf extracts.[3] ^{1}H NMR (500 MHz, acetone-d_6): δ (ppm) 7.27 (d, *J* = 9 Hz, 1H, H-5), 6.70 (dd, J_1 = 2.5 Hz, J_2 = 8.5 Hz, 1H, H-6), 6.76 (d, *J* = 2.5 Hz, 1H, H-8), 5.84 (s, 1H, H-2), 4.70 (d, *J* = 8 Hz, 1H, H-1'), 3.89 (dd, J_1 = 2 Hz, J_2 = 11.5 Hz, 1H, H-6a'), 3.79 (s, 3H, OCH_3), 3.65 (dd, J_1 = 5.5 Hz, J_2 = 11.5 Hz, 1H, H-6b'), 3.36-3.40 (m, 1H, H-5'), 3.39 (dd, J_1 = J_2 = 9 Hz, 1H, H-3'), 3.29 (dd, J_1 = J_2 = 9 Hz, 1H, H-4'), 3.17 (dd, J_1 = J_2 = 8.5 Hz, 1H, H-2'). HR-ESI-MS: *m/z* 372.0939 $[M-H]^-$ (calcd for $C_{15}H_{18}O_{10}N$: 372.0936).

1.9. Synthesis of 2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyloxy)-4-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one:[4] To a suspension of 10.5 mg (0.05 mmol) DIMBOA and 49.3 mg (0.01 mmol) *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)trichloroacetimidate in 3 mL absolute CH_2Cl_2, 13 μL (0.1 mmol) $BF_3{\cdot}OEt_2$ were added with a syringe at RT. The mixture was stirred at RT for 20h under N_2 atmosphere. The reaction was interrupted by adding 5 mL water and the organic phase was separated, dried and evaporated. The resulting oil was directly used for the synthesis of **1** without further purification.

1.10. Synthesis of DIMBOA-Glc (1):[4] To a solution of 0.05 mmol of the tetraacetate derivative (produced above in **1.9.**) in 3 mL anhydrous MeOH, 24.3 mg (0.45 mmol) sodium methoxide were added. The solution was stirred for 30 min at RT and neutralized by the addition of Amberlite IR 120 (H^+), which was removed by filtration. The solvent was evaporated and the resultant residue was directly used for NMR measurements and LC-MS/MS analyses. Small amounts of (2*R*)- and (2*S*)-2-β-D-glucopyranosyloxy-7-methoxy-*2H*-1,4-benzoxazin-3(*4H*)-one (HMBOA-Glc) were observed as side products in the final residue.

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1.11. (2*S*)-DIMBOA-Glc purification: *S. frugiperda* individuals were left to feed on 2 week-old maize leaves for several days and the resulting frass was collected and freeze dried. A total of 5.2 g of dried frass was extracted with 50 mL methanol, agitated in a paint shaker (with 3 mm steel beads) for 10 min, centrifuged at 4,200 g for 10 min and the resulting supernatant was collected. The remaining material was extracted again with additional 50 mL methanol. The combined supernatants were evaporated in vacuo, resuspended in 10 mL water/methanol 1:1 (v:v) and centrifuged at 16000 g for 5 min. After concentration under N_2 flow and methanol addition, 3 mL of a water/methanol 1:1 extract was purified using a Agilent 1100 series equipment coupled to a diode array (DA) detector set on 254 nm wavelength and a SF-2120 fraction collector (Advantec, Dublin, USA). Separation was performed using formic acid (0.005%) in water and acetonitrile as mobile phases A and B, respectively, a Nucleodur Sphinx RP column (250 x 4.6 mm, 5 μm, Macherey-Nagel, Düren, Germany), a flow rate of 1.0 mL min^{-1} and an elution profile as follows: 0-12 min, 90% A; 12.1-15 min, 100% B; 15.1-18 min, 90% A. The fraction corresponding to (2*S*)-DIMBOA-Glc was collected, partially evaporated in vacuo and freeze dried. Chemical shifts for (2*S*)-DIMBOA-Glc were assigned based on (2*R*)-DIMBOA-Glc spectroscopic data. ^{1}H NMR (500 MHz, acetone-d_6): δ (ppm) 7.27 (d, J = 9 Hz, 1H, H-5), 6.71 (dd, J_1 = 2.5 Hz, J_2 = 8.5 Hz, 1H, H-6), 6.69 (d, J = 2.5 Hz, 1H, H-8), 5.93 (s, 1H, H-2), 4.76 (d, J = 8 Hz, 1H, H-1'), 3.89 (dd, J_1 = 2 Hz, J_2 = 11.5 Hz, 1H, H-6a'), 3.78 (s, 3H, OCH_3), 3.67 (dd, J_1 = 6 Hz, J_2 = 11.5 Hz, 1H, H-6b'), 3.40-3.43 (m, 1H, H-5'), 3.39 (dd, J_1 = J_2 = 8.5 Hz, 1H, H-3'), 3.32 (dd, J_1 = J_2 = 9 Hz, 1H, H-4'), 3.13 (dd, J_1 = J_2 = 8 Hz, 1H, H-2'). HR-ESI-MS: *m/z* 372.0938 $[M-H]^-$ (calcd for $C_{15}H_{18}O_{10}N$: 372.0936).

1.12. Gut pH microelectrode measurements: *S. frugiperda* fourth instar larvae were fed on 2 week-old maize leaf slices for 24h and then quickly dissected on ice, without buffer, exposing the gut by carefully removing the cuticle while keeping the head capsule and the cuticle region around the anus. The freshly extracted guts were placed in a micro-chamber on top of a 2-mm layer of 1.5% agarose and covered with 2 mm of 0.5% agarose in Ringer's solution.[5] The pH microelectrode with a tip diameter of 20-30 μm was positioned using a manual micromanipulator (Unisense, Denmark) and the measurements were recorded with a microsensor multimeter (Unisense, Denmark). The reference electrode (Unisense, Denmark) for the pH assessment was placed within the top layer of 0.5% agarose. Calibrations were carried out with standard buffer solutions at pH 4.0, 7.0, and 10.0 before and after each experiment. The pH was recorded in three replicates at 9 equidistant points throughout different gut regions (foregut, midgut, hindgut and rectum). All measurements were performed at ambient temperature (22 ± 1 °C).

S6

2. Supporting Figures

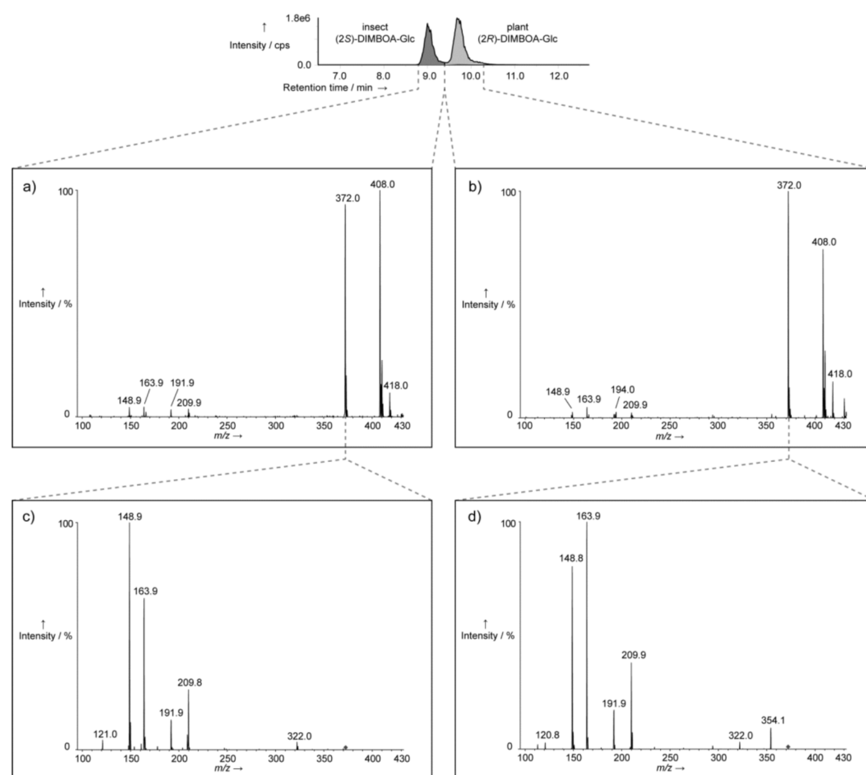


Figure S1. MS spectra of a) insect-derived (2*S*)-DIMBOA-Glc and b) plant-derived (2*R*)-DIMBOA-Glc are strikingly similar, and it is possible to observe the peaks for chloride and formate adducts at *m/z* 408 and 418, respectively. Further fragmentation of the $[M-H]^-$ ion with *m/z* 372 for both c) insect-derived (2*S*)-DIMBOA-Glc and d) plant derived (2*R*)-DIMBOA-Glc gives rise to similar MS^2 patterns that include the common MS fragmentations for the aglucone DIMBOA.[6]

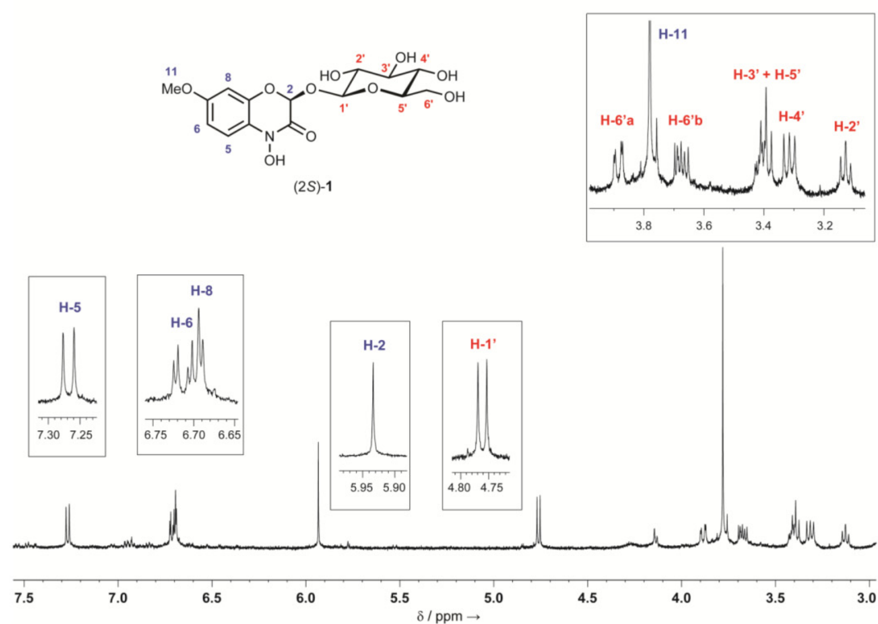


Figure S2. ^{1}H NMR spectrum (500.13 MHz, acetone-d$_6$) of (2*S*)-DIMBOA-Glc, (2*S*)-**1**, isolated from *S. frugiperda* frass, after feeding on maize leaves. Characteristic axial-axial coupling constants for H-1' and H-4' confirm that the compound possesses a β-glucose moiety. In accordance with previous discussions in the literature,[4] the signal for H-2 is shifted around 0.1 ppm downfield in the (2*S*)-diastereomer of DIMBOA-Glc, compared to the plant-derived (2*R*)-diastereomer.

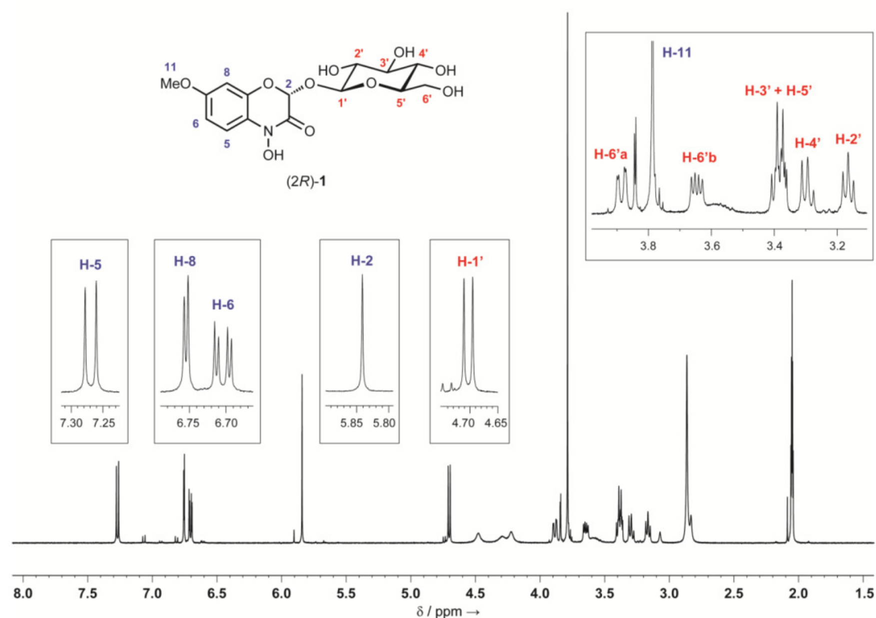
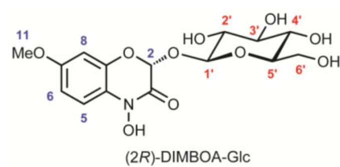


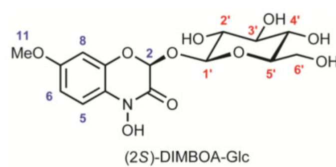
Figure S3. ^{1}H NMR spectrum (500.13 MHz, acetone-d$_6$) of (2*R*)-DIMBOA-Glc, (2*R*)-**1**, isolated from maize leaves. Spectroscopic data are in agreement with those described in the literature for the plant-derived compound.[3,7] Trace amounts of 2-β-D-glucopyranosyloxy-4-hydroxy-7,8-dimethoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIM$_2$BOA-Glc) are present as a contaminant from the purification process.

Table S1: ^{1}H NMR data of (2*R*)-**1** and (2*S*)-**1** (in acetone-d_6, δ values in ppm, *J* values in Hz)

Position	δ_H (multiplicity, *J*)	
	(2*R*)-DIMBOA-Glc	(2*S*)-DIMBOA-Glc
2	**5.84 (s)**	**5.93 (s)**
5	7.27 (d, 9.0)	7.27 (d, 9.0)
6	6.70 (dd, 8.5, 2.5)	6.71 (dd, 8.5, 2.5)
8	6.76 (d, 2.5)	6.69 (d, 2.5)
11	3.79 (s)	3.78 (s)
1’	**4.70 (d, 8.0)**	**4.76 (d, 8.0)**
2’	3.17 (d, 8.5, 8.5)	3.13 (dd, 8.0, 8.0)
3’	3.39 (dd, 9.0, 9.0)	3.39 (dd, 8.5, 8.5)
4’	3.29 (dd, 9.0, 9.0)	3.32 (dd, 9.0, 9.0)
5’	3.36-3.40 (m)	3.40-3.43 (m)
6’a	3.89 (dd, 11.5, 2.0)	3.89 (dd, 11.5, 2.0)
6’b	3.65 (dd, 11.5, 5.5)	3.67 (dd, 11.5, 6.0)



(2*R*)-DIMBOA-Glc



(2*S*)-DIMBOA-Glc

S10

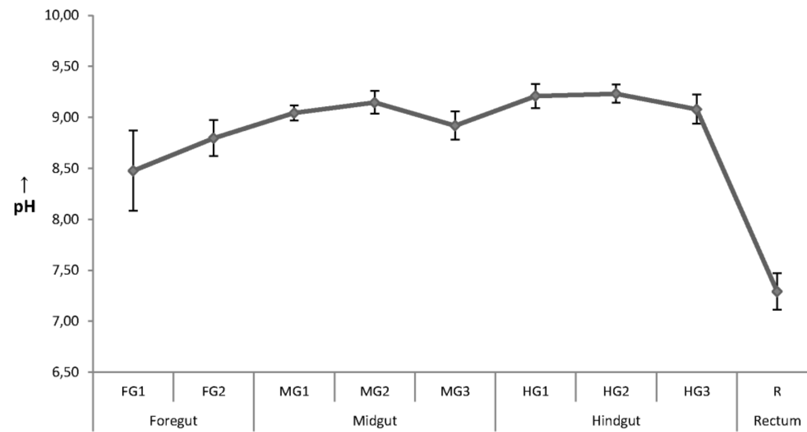


Figure S4. pH measurements along the *S. frugiperda* gut. Individuals feeding on maize leaves maintain near-constant alkaline pH values along most of the gut lumen, with a rapid shift to near-neutral conditions towards the rectum (FG, foregut regions; MG, midgut regions; HG, hindgut regions; R, rectum). Each point represents the mean pH value ±SE on each gut region for three replicate guts.

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Manuscript IV

Screening of UDP-glucosyltransferases responsible for benzoxazinoid detoxification in *Spodoptera frugiperda*

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***Abstract**:*

Insect herbivores are often faced with toxic plant chemical defenses. In order to detoxify these compounds, they have evolved diverse biochemical pathways, including conjugation with glucose, catalyzed by UDP-glucosyltransferases (UGT). This reaction increases the polarity of xenobiotics, diminishes their toxicity, and facilitates their excretion, playing an important role in insect resistance towards plant allelochemicals. Benzoxazinoids are chemical defenses against herbivores used by grasses such as maize, wheat and rye. Despite their high toxicity to a broad range of insects, some species like the fall armyworm (*Spodoptera frugiperda*) display considerable resistance to benzoxazinoids. Although glucosylation reactions have been implicated in benzoxazinoid metabolism by *S. frugiperda*, no UGT candidates have been associated with such activities. In this work we identified and functionally screened *S. frugiperda* UGTs in order to determine activities towards benzoxazinoids. We took advantage of RNA sequencing technology to explore the transcriptome of *S. frugiperda* and identify putative UGTs. We identified and expressed 25 UGT candidates in *Trichoplusia ni* cell cultures and screened for activity towards DIMBOA and MBOA. We found a total of 5 candidates with activities towards BXDs: one towards DIMBOA producing (2*S*)-DIMBOA-Glc, three towards MBOA producing MBOA-Glc, and one towards both DIMBOA and MBOA, producing

(2*R*)-DIMBOA-Glc and MBOA-Glc, respectively. Moreover, we investigated the induction of UGT activities towards DIMBOA and MBOA upon ingestion of benzoxazinoids and compared these activities among gut, Malpighian tubules, fat bodies, testes, and cuticle of *S. frugiperda* larvae. With these data, we provide a better understanding of the regulation and compartmentalization of *S. frugiperda* DIMBOA and MBOA glucosylation, and report candidate genes encoding enzymes potentially acting on benzoxazinoid detoxification that can be further explored on the molecular level.

Keywords: chemical ecology, plant defenses, detoxification, *Spodoptera frugiperda*, UDP-glycosyltransferase, transcriptomics

Abbreviations: BXDs (benzoxazinoids), UGT (UDP-glycosyltransferases)

1. Introduction:

Insect herbivores must deal with a range of plant chemical defenses in order to use them as a food source. To do so, they have developed numerous strategies such as avoidance behavior, rapid excretion, sequestration, and detoxification of xenobiotics (Després *et al.*, 2007). The metabolic pathways used to detoxify xenobiotics include functional group activation by cytochrome P450 monooxygenases and esterases (Phase I metabolism), and conjugation with glutathione and sugars (Phase II metabolism) (Kennedy and Tierney, 2013). Within the latter strategy, the glycosylation of xenobiotics in insects is assisted by UDP-glycosyltransferase (UGT) enzymes that use uridine diphosphate glucose (UDP-glucose) as a sugar donor (Ahn *et al.*, 2012). Glycosylation increases the polarity and hydrophilicity of metabolites, restricting their diffusion across membranes and facilitating their recognition and excretion by transport systems (Phase III metabolism). Therefore, the glycosylation of plant secondary metabolites by insects represents an effective way to control their stability, subcellular localization, bioavailability, and activity (Meech *et al.*, 2012), and constitutes an important route for their detoxification.

In insects, UGTs are involved in cuticle formation (Hopkins and Kramer, 1992; Kramer and Hopkins, 1987), pigmentation (Daimon *et al.*, 2010), and olfaction (Wang *et al.*, 1999). Insect viruses possess genes encoding ecdysteroid glucosyltransferases that are assumed to be derived from insect UGT genes, and are involved with larval molting delay during infection (O'Reilly and Miller, 1990), and behavior modulation, as observed in *Lymantria dispar* tree top disease (Hoover *et al.*, 2011). In addition, UGTs are expected to play important roles in insect metabolism of xenobiotics, including plant defenses (Heidel-Fischer and Vogel, 2015) and insecticides (Bull and Whitten, 1972). In the silkworm *Bombyx mori*, BmUGT1 (most similar to genome sequence UGT40A1) has been characterized and shown to be active towards a wide range of substrates, including flavonoids and phenols (Luque *et al.*, 2002). Capsaicin glucosylation activity was detected in various tissues of *Helicoverpa armigera*, *H. zea*, and *H. assulta*, representing a probable detoxification mechanism (Ahn *et al.*, 2011). In *Spodoptera littoralis*, UGT40R3 and UGT46A6, which are expressed mostly in

antennae, showed potential function in odorant and insecticide clearance (Bozzolan *et al.*, 2014). The UGT33A1 from the Burnet moth *Zygaena filipendulae* is responsible for *de novo* synthesis of cyanogenic glycosides used as chemical defenses by the larvae (Jensen *et al.*, 2011). Recently, UGT41B3 and UGT40D1 have been suggested to detoxify gossypol in *H. armigera* and *Heliothis virescens* (Krempl *et al.*, 2016). With the increasing availability of published full genomes and access to transcriptomic studies, there has been more interest in investigating insect UGTs on a molecular level. The recent identification of all putative UGTs from *B. mori* and *H. armigera*, together with a comparative analysis of 310 UGTs from genome databases, provided unprecedented insights on the phylogeny of insect UGTs (Ahn *et al.*, 2012; Huang *et al.*, 2008). Similarly to human UGTs, insect UGTs are membrane-bound and present a relatively conserved *C*-terminal domain responsible for binding to the sugar donor, and a more variable *N*-terminal domain, which contains the two catalytic residues and is responsible for substrate specificity (Ahn *et al.*, 2012; Krempl *et al.*, 2016). Nevertheless, characterization of insect UGTs at both the molecular and functional level is still scarce in the literature, and this superfamily of detoxifying enzymes is not as well studied as others such as cytochrome P450 monooxygenases, glutathione-*S*-transferases (GSTs), and carboxylesterases (Després *et al.*, 2007).

Many grasses (Poaceae), including crops such as wheat, rye, and maize, produce benzoxazinoids (BXDs) in order to deter herbivores (Niemeyer, 2009; Sicker and Schulz, 2002). These indole-derived compounds are stored as inert glucosides and, upon tissue damage for example by a chewing herbivore, are hydrolyzed by specific plant β-glucosidases, releasing toxic aglucones (Frey *et al.*, 2009). We have recently described that *Spodoptera* species reglucosylate the aglucone DIMBOA derived from the most abundant BXD in maize leaves, (2*R*)-DIMBOA-Glc, as a detoxification strategy (Wouters *et al.*, 2014). The stereoselectivity of this conjugation renders the new glucoside, (2*S*)-DIMBOA-Glc, inert towards plant glucosidases, which can only hydrolyze the plant-derived (2*R*)-DIMBOA-Glc. That is, both insect- and plant-derived UGTs use BXDs and UDP-Glc as substrates, but perform their glucosylation reactions to produce a different final stereochemistry. Glucosylation of BXDs had been previously proposed for several Lepidopteran species such as *S. frugiperda*, *S. littoralis*, *Mythimna separata* and *Ostrinia furnacalis* (Glauser *et al.*, 2011; Kojima *et al.*, 2010; Phuong *et al.*, 2015; Sasai *et al.*, 2009). Furthermore, *N*-glucosylation of MBOA, a toxic spontaneous degradation product of other BXDs, has been described as a detoxification mechanism in *S. frugiperda* and *S. littoralis* (Maag *et al.*, 2014). However, the insect genes and enzymes associated with these BXD metabolism pathways (Fig. 1) have not been investigated so far. In the present work, we generated and analyzed a transcriptome of *S. frugiperda* combined with other public databases for this species in order to identify putative UGT genes, and used heterologous expression in insect cells to screen for glucosylation activity towards DIMBOA and MBOA. We identified 39 putative UGTs from *S. frugiperda*, successfully expressed 25 of these in *Trichoplusia ni* cells, and screened them for BXD-UGT activity. We detected activity towards BXDs in 5 expressed enzymes, from which SfUGT6 is likely responsible for the DIMBOA glucosylation reaction observed *in vivo*, whereas SfUGT22, SfUGT32 and SfUGT35 are promising candidates for MBOA *N*-glucosylation. We also explored the UGT activity towards DIMBOA and MBOA in different *S. frugiperda* tissues and

their induction by BXD treatment. Structural and functional aspects of *S. frugiperda* UGTs in comparison to other known UGTs are also discussed.

2. Material and Methods:

2.1. Insects and plants:

Eggs and larvae of *Spodoptera frugiperda* (maize strain) were obtained from colonies at the Max Planck Institute for Chemical Ecology, and eggs of *Spodoptera littoralis* were a generous gift from Syngenta Crop Protection (Stein, Switzerland). All insects were reared on an artificial diet based on white beans (Bergomaz and Boppré, 1986), under controlled light and temperature conditions (12:12 h light/dark, 20 °C). Seeds of *Zea mays* were obtained commercially (Badischer Gelber variety, Kiepenkerl, Germany), *bx1* mutant seeds were obtained from the Maize Genetics Cooperation Stock Center of the USDA/ARS at the University of Illinois, Urbana/Champaign (http://maizecoop.cropsci.uiuc.edu, stock 428G), and grown under controlled light and temperature conditions (16:8 h light/dark, day-time temperature 22 °C, night-time 20 °C).

2.2. Insect cell cultures:

Spodoptera frugiperda Sf9 cells and *Trichoplusia ni* High Five® cells were cultured in Sf-900 II serum-free medium (Gibco) and ExpressFive serum-free medium (Gibco), respectively. Adherent cultures were maintained at 27 °C, and sub-cultured every 3-4 days.

2.3. RNA extraction and reverse transcription:

For transcriptome sequencing experiments, third to fourth instar larvae of *S. frugiperda* were fed maize leaf slices (2 weeks old plants, L4 stage) for 48h, then quickly dissected on cold phosphate buffer (pH 7.0, 10mM), having their guts and integument collected separately, briefly washed cleaned from gut lumen, and stored in RNAlater (Sigma) overnight at 4°C before RNA extraction. Tissue samples from two individuals were pooled together for RNA extraction. Samples of Sf9 cell cultures (obtained during sub-culturing at full confluency) were centrifuged at 500 g for 5 min, the culture medium was discarded, and the fresh pellets were directly used for RNA extraction.

Total RNA was extracted from gut, skin, and Sf9 cell samples using the innuPREP RNA Mini Kit (Analytik Jena) including a DNase treatment to eliminate genomic DNA from the samples following the manufacturer's protocol. RNA concentrations were measured with the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). RNA quality was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies). First Strand cDNA was synthesized from 1 µg total RNA using SuperScript® III Reverse Transcriptase and OligodT primers from Invitrogen.

2.4. *cDNA library construction and Illumina sequencing:*

cDNA was prepared from total RNA using NEBNext Ultra™ Directional RNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer's instructions. Library construction and sequencing were performed by the Max Planck Genome Center Cologne, Germany (http://mpgc.mpipz.mpg.de/) using the manufacturers' protocols. TruSeq libraries were generated from poly-A enriched mRNA. The library was sequenced with an Illumina HiSeq2500 sequencer, and after demultiplexing 8-11 million of 100 bp paired-end reads per sample were obtained.

2.5. *RNA-Seq data analysis:*

Short 100 bp paired-end Illumina reads were quality-trimmed and filtered for duplicates using default parameters and then assembled de novo using the CLC Genomics Workbench software package (CLC bio Qiagen), using bubble size = 65. Consensus sequences were extracted to give 49,836 contigs.

2.6. *Bioinformatics analysis:*

Contigs obtained from RNA-Seq analyses were used as a database for tblastx queries from *Helicoverpa armigera* UDP-glucosyltransferase sequences (Ahn *et al.*, 2012). The resultant 63 contigs were considered putative *S. frugiperda* UGTs, whose sequences were further assembled using the short Illumina reads until they reached the expected size for an insect UGT (1500-1600 bp) and resemblance to described *H. armigera* UGT domains. After discarding redundant contigs and sequences that did not show the typical UGT structure, the resulting 36 full sequences were confirmed by comparison with the published *S. frugiperda* draft genome (Kakumani *et al.*, 2014) (NCBI:JQCY00000000) and transcriptome databases (Kakumani *et al.*, 2015; Legeai *et al.*, 2014; Nègre *et al.*, 2006) (NCBI:GCTM00000000, Lepidodb:TR2012b [downloadable at http://www6.inra.fr/lepidodb/Downloads/TR2012b], and Spodobase [http://bioweb.ensam.inra.fr/spodobase/]), and were used to design full sequence primers used for heterologous expression. Another 3 sequences could not be completed by these means. Geneious 9.1.3 software was used for sequence data treatment.

2.7. *Cloning and heterologous expression:*

Full sequence primers were designed based on the UGT full sequences retrieved from RNA-Seq analysis, covering 3-15 bases before the start codon and lacking the stop codon (Supplementary

Table 1). From the 36 UGT candidates with full sequences retrieved, 25 candidates were successfully amplified from *S. frugiperda* gut cDNA samples using Phusion® High Fidelity DNA Polymerase (New England Biolabs) (PCR protocol: 30 s at 98 °C; 35 cycles of 10 s at 98 °C, 20 s at 60 °C, 45 s at 72 °C; and 5 min at 72 °C). The resulting amplified products were purified with a PCR cleanup kit (Qiagen), and incubated with GoTaq® DNA polymerase (Promega) for 15 minutes at 72 °C in order to add A overhangs. The products were directly cloned into the pIB/V5-His-TOPO® (Life Technologies) vector and transformed into TOP10® (Life Technologies) cells, which were plated on selective LB agar medium containing 100 µg/mL ampicillin and incubated overnight at 37 °C. Positive colonies were identified by PCR using vector-specific primers OpIE2, subcultured overnight at 37 °C in liquid LB medium containing 100 µg/mL ampicillin, and used for plasmid DNA purification with the NucleoSpin® Plasmid kit (Macherey-Nagel). Concentration and purity of the obtained constructs were assessed by a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) and the correct insertion of the PCR products was confirmed by DNA sequencing.

For transfection, *T. ni* cells (HighFive®, Life Technologies) were sub-cultured at full confluency to a 6-well plate in a 1:3 dilution, and left overnight to adhere to the flask surface. The medium was replaced and transfections were carried out using FuGENE® HD Transfection reagent (Promega) on a 1:3 plasmid/lipid ratio (1.7 µg plasmid and 5.0 µL lipid for 3 mL total medium). Cells were incubated for 48 h at 27 °C, resuspended in fresh medium, and divided in three aliquots, which were used for reseeding, a UGT enzymatic assay, and Western blot analysis. Reseeded cells were selected by growing in culture medium containing 50 µg/mL blasticidin for 2 weeks and tested for UGT activity again. Transfected cells that showed activity towards DIMBOA and/or MBOA were maintained at 10 µg/mL blasticidin as stable cell cultures.

2.8. Enzymatic assays:

For comparative UGT assays with gut and integument, *S. frugiperda* fourth instar individuals feeding on artificial diet were used. Caterpillars were dissected in cold phosphate buffer (pH 7.0, 10 mM), and their gut tissues and integument were collected separately, and cleaned from gut lumen. Tissue samples were homogenized in 50 µL of the same buffer per gut and 100 µL per integument. Samples from Sf9 cell cultures were collected, centrifuged at 500 g for 5 min, and pellets were resuspended and homogenized in phosphate buffer (pH 7.0, 100 mM). Aliquots of these enzyme preparations were boiled for 20 min. Enzymatic assays for gut and integument tissues included 10 µL tissue homogenate, 2 µL of 12.5 mM DIMBOA or MBOA in DMSO (25 nmol), 4 µL of 12.5 mM UDP-glucose in water (50 nmol), and enough phosphate buffer (pH 7.0, 100 mM) solution to give an assay volume of 50 µL. Enzymatic assays for Sf9 cells included 44 µL cell homogenate, 2 µL of 12.5 mM DIMBOA or MBOA in DMSO, and 4 µL of 12.5 mM UDP-glucose in water. Controls containing either boiled enzymatic preparation, or only the protein suspension and buffer were included. After incubation at 30 °C for 60 min, the enzymatic reactions were interrupted by adding 50 µL of a

methanol/formic acid 1:1 (v:v) solution. Assay tubes were centrifuged at 5,000 g for 5 minutes and the obtained supernatant was collected and analyzed by LC-MS/MS.

For testing the induction of UGT activity, *S. frugiperda* and *S. littoralis* neonates were used. Eggs were kept in plastic containers without any food and, upon hatching, larvae were transferred to cups with maize leaf slices (2 week-old plants, fourth leaf forming) from WT or *bx1* mutant plants. Larvae were kept for 2 days on the lab bench (23°C, no humidity control), and 10 individuals were pooled together for each of 5 replicates. Pooled individuals were weighed, and whole bodies were homogenized in 100 µL cold phosphate buffer (pH 7.0, 100 mM). Enzymatic assays were performed as described above for larval tissues. For enzymatic assays with *S. frugiperda* tissues, third to fourth instar individuals were used. In order to assess induction by BXDs, leaf slices from maize plants (2 weeks old, L4 stage) were used as treatments. After feeding on WT maize leaves (BXD+), mutant *bx1* maize leaves (BXD−) or artificial bean diet for 24h, individuals were dissected on cold phosphate buffer (pH 7.0, 10 mM). Guts, Malphigian tubules, fat bodies, testes, and cuticle were collected separately to produce 3 samples of each tissue, pooled from 3 individuals each. Tissue samples were homogenized in phosphate buffer (pH 7.0, 100 mM; 100 µL for Malpighian tubules and testes, 300 µL for guts and fat bodies, and 500 µL for cuticles) and enzymatic assays were performed as described above for larval tissues. For measuring specific DIMBOA and MBOA-UGT activities of neonates and larval tissues, samples were quantified for DIMBOA-Glc and MBOA-Glc by LC-MS/MS, and each homogenate had protein levels estimated by Bradford method (Bradford, 1976) and activity levels normalized accordingly.

For testing activity of heterologously expressed UGTs, samples of transiently or stably expressed cultures were centrifuged at 500 g for 5 min, and resuspended in 100 µL phosphate buffer (pH 7.0, 100 mM). Enzymatic assays were carried as described above for larval tissues.

2.9. Western blot:

Samples from transient cultures of transfected cells, together with non-transfected controls, were centrifuged at 500 g for 5 min. After the medium was removed, pellets were resuspended in 100 µL hypotonic buffer (20 mM Tris, 5 mM EDTA, 1 mM DTT, pH 7.5). After 10 min incubation at room temperature, samples were centrifuged at 1,200 g for 5 min, the supernatant was discarded, and the pellets were resuspended in 50 µL hypotonic buffer. These suspensions were analyzed by SDS-PAGE, and proteins were transferred electrophoretically to nitrocellulose membranes, which were incubated with Anti-V5-HRP antibody (Life Technologies) overnight and developed colorimetrically using diazoaminobenzidine and hydrogen peroxide.

2.10. Sample extraction:

Leaf slice samples used for neonate caterpillar feeding were collected from leaves of 2 week-old maize plants, flash-frozen and stored at −80 °C until extraction. Samples were quickly ground

under liquid nitrogen, quickly transferred to tubes, freeze dried, weighed (around 8 mg) and extracted with 0.5% formic acid in water/methanol (1:1, v:v) at 50 µL per mg sample. Extracts were vortexed for 2 min, agitated in a paint shaker for 2 min (with 3 mm steel beads), centrifuged at 16,000 g for 5 minutes, and the obtained supernatants were collected and analyzed by LC-MS/MS.

2.11. Chromatographic methods:

For all analytical chromatography procedures, formic acid (0.05%) in water and acetonitrile were used as mobile phases A and B, respectively, and the column temperature was maintained at 25 °C. Analyses of enzymatic assays and plant samples used an XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent Technologies, Boeblingen, Germany) with a flow rate of 1.1 mL min^{-1} and with the following elution profile: 0-0.5 min, 95% A; 0.5-6 min, 95-67.5% A; 6.02-7 min, 100% B; 7.1-9.5 min, 95% A. For enzymatic assays that formed DIMBOA-Glc, resolution between the two epimers was achieved using a Nucleodur Sphinx RP column (250 x 4.6 mm, 5 µm, Macherey-Nagel, Düren, Germany) at a flow rate of 1.0 mL min^{-1} with the following gradient: 0-10 min, 85% A; 10.1-12 min, 100 % B; 12.1-15 min, 85% A.

LC-MS/MS analyses were performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany) coupled to an API 3200 tandem spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a turbospray ion source operating in negative ionization mode. The ion spray voltage was maintained at −4500 V. The turbo gas temperature was 500 °C, nebulizing gas 60 psi, curtain gas 25 psi, heating gas 60 psi and collision gas 5 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion to product ion conversion with parameters from the literature for DIMBOA (Pedersen *et al.*, 2011) and DIMBOA-Glc (Wouters *et al.*, 2014). MRM parameters were optimized from infusion experiments with standard samples for MBOA-Glc (Q1 *m/z*: 372, Q3 *m/z*: 164, DP −15 V, EP −4.5 V, CEP −18 V, CE −20 V, CXP −4 V). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Absolute quantities of the measured benzoxazinoids were determined using standard curves obtained from purified samples of DIMBOA (1-100 µM), (2*R*)-DIMBOA-Glc (1-10 µM), and MBOA-Glc (0.1-5 µM). A (2*R*)-DIMBOA-Glc standard curve was used for quantification of both (*2R*) and (2*S*) epimers. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing.

2.12. Reagents and solvents:

DIMBOA for UGT assays, and BXD standards ((2*R*)-DIMBOA-Glc and MBOA-Glc) were kindly provided by Prof. Dieter Sicker (University of Leipzig, Germany), and Dr. Gaétan Glauser (University of Neuchâtel, Switzerland) respectively. MBOA (Sigma Aldrich), UDP-glucose (Santa Cruz Biotechnology) and blasticidin and other cell culture media and reagents (Gibco) were commercially obtained.

3. **Results**:

3.1. UGT activity induction in neonate larvae and comparison among tissues

As a first trial to identify differential UGT activity in *S. frugiperda* and *S. littoralis*, neonate larvae were fed on WT maize leaves containing regular amounts of BXDs (BXD+), and *bx1* mutants, which lack the first biosynthetic enzyme in the BXD pathway and therefore show low levels of these compounds (BXD−) (Frey, 1997). Specific UGT activity of whole body extracts towards DIMBOA and MBOA are summarized in Fig. 2a. Upon BXD feeding, induction of DIMBOA-UGT activity in *S. frugiperda* (1.3 fold) and in *S. littoralis* (1.8 fold), and MBOA-UGT activity in *S. littoralis* (1.8 fold) was observed, but not of MBOA-UGT activity in *S. frugiperda* larvae. The average larval weights in all groups were the same by the end of the feeding (data not shown), which suggests that the small observed differential activities are indeed due to enzyme induction rather than differences in development between treatment groups. Plant material used in this experiment was analyzed for DIMBOA and DIMBOA-Glc content (Supplementary Table 2). Mutant *bx1* maize mutant contained around 100-fold less DIMBOA and DIMBOA-Glc in leaf slices than WT at the beginning of the feeding. After 2 days of feeding, considerable amounts of BXDs were still present in leaf slices (>80% DIMBOA and DIMBOA-Glc), which assures that larvae ingested BXDs throughout all the experiment.

Specific DIMBOA- and MBOA-UGT activities from *S. frugiperda* gut and integument homogenates, as well as Sf9 cells, are shown in Fig. 2b. The specific activities towards both substrates follow opposite trends when all three enzyme preparations are compared, with gut tissues giving intermediate values. For DIMBOA, UGT activity is higher in Sf9 cells (2.1 fold from gut) and lower in skin tissue (6.6 fold from gut), whereas for MBOA, it is higher in skin tissue (4.0 fold from gut) and lower in Sf9 cells (2.0 fold from gut). Compared to neonate BXD treatment, these tissues possessed higher differences in levels of *in vitro* BXD-UGT activities between each other and were therefore used for RNAseq.

3.2. Identification of UGT candidates

UGT candidates from *S. frugiperda* were retrieved from a transcriptome analysis of larvae gut and skin tissue, and Sf9 cells. From all the contigs generated after assembly, 63 partial sequences were selected as putative UGTs based on similarity with UGTs identified in *H. armigera* (Ahn *et al.*, 2012). Further assembly of these sequences aided by comparison to publicly available *S. frugiperda* sequences generated 36 full and 3 incomplete sequences for putative UGTs. All obtained full UGT sequences show the size and structure expected for insect UGTs (Fig. 3). These sequences probably represent only a part of all the UGTs present in *S. frugiperda*, as some other members of this superfamily might have been missed due to low expression, or specificity to certain tissues or

developmental stages. As a comparison, the lepidopteran species *H. armigera* and *B. mori* encode 42 and 45 UGTs, respectively (Ahn *et al.*, 2012). *S. frugiperda* candidates were assigned to their UGT families by similarity between their amplified sequences and UGTs described for *H. armigera* and *B. mori* (Fig. 4).

3.3. Heterologous expression of UGTs and screening towards BXDs

From the 36 full UGT sequences retrieved, 25 UGT candidate genes were successfully amplified from cDNA from *S. frugiperda* gut tissue and cloned into a pIB/V5-His-TOPO expression vector. Constructs were transfected into *Trichoplusia ni* cell cultures and screened for UGT activity towards DIMBOA and MBOA. Results are summarized in Table 1. Three candidates (SfUGT22, SfUGT32, and SfUGT35) showed activity towards MBOA. One candidate (SfUGT3) showed activity towards both DIMBOA and MBOA, but with the former substrate it produces the (*2R*) epimer of DIMBOA-Glc. One candidate (SfUGT6) showed activity towards DIMBOA, yielding (2*S*)-DIMBOA-Glc, the detoxification metabolite detected in S*. frugiperda* frass and *in vitro* assays with gut suspensions. SfUGT6 was also able to glucosylate HMBOA (data not shown). All transiently expressed cell cultures were analyzed by SDS-PAGE and Western blot, which confirmed the presence of the V5 epitope on protein bands with the expected size for the UGT candidates (60-80 kDa).

3.4. BXD-UGT activity comparison among larval tissues

Specific activities for DIMBOA and MBOA glucosylation were assessed in several tissues of third to fourth instar *S. frugiperda* larvae: gut, Malpighian tubules, fat bodies, testes, and cuticle. The measurements were performed on larvae feeding on WT maize leaves (BXD+), mutant *bx1* maize leaves (BXD−), and artificial diet. The results are displayed in Fig. 5. The activities among tissues varied consistently between treatments, with gut tissue presenting the highest DIMBOA-UGT activity (16 fold higher than cuticle in BXD+ treatment), and fat bodies presenting the highest MBOA-UGT activity (10 fold higher than cuticle in BXD+ treatment). An apparent 2.5 fold induction in DIMBOA-UGT activity was observed upon BXD+ treatment, compared to BXD−, but an intermediate activity was given by treatment with artificial diet, which does not contain BXDs. No significant induction was observed for MBOA-UGT activity.

4. **Discussion**:

4.1. BXD-UGT activity is not strongly induced upon BXD exposure

DIMBOA- and MBOA-UGT activities were weakly induced in *S. frugiperda* and *S. littoralis* neonate larvae feeding on BXDs (Fig. 2a). On the other hand, a comparison between tissues in *S. frugiperda* showed that DIMBOA-UGT specific activity is remarkably higher in gut tissue than in the integument, which would be expected for detoxification enzymes, whereas MBOA-UGT specific activity is higher in the integument than it is in gut tissue (Fig. 2b). The latter observation suggests that this activity might be associated with other constitutive functions in the insect, such as cuticle formation or pigmentation. Surprisingly, Sf9 cells, which are derived from *S. frugiperda* ovarian tissue, are able to perform DIMBOA glucosylation *in vitro* more efficiently than gut tissue, but have lower MBOA-UGT activities than gut and integument tissues. It is not known how Sf9 cells have adapted to culture conditions, but this might have involved differential UGT gene regulation that resulted in the observed activities. On the other hand, we can not rule out the possibility that different genes and enzymes are responsible for the same activity in different tissues. The distinct profiles observed for DIMBOA- and MBOA-UGT activities among tissues indicate that these reactions involve at least two different enzymes. Attempts to use RNAseq expression data to filter candidates according to their differential expression between tissues, predicting their specificities towards DIMBOA or MBOA, were not successful, likely due to the lack of sample replication during short-read and transcriptome generation.

4.2. Structure of UGT candidates

The alignment of the 25 expressed UGTs described in this work (representative sequences displayed in Fig. 3) reveals the same conserved features as other insect and mammal UGTs. The C-terminal domain presents the UGT signature motif [FVA]-[LIVMF]-[TS]-[HQ]-[SGAC]-G-x(2)-[STG]-x(2)-[DE]-x(6)-P-[LIVMFA]-[LIVMFA]-x(2)-P-[LMVFIQ]-x(2)-[DE]-Q (amino acids that can occur at a given position are listed inside brackets; x indicates any amino acid) (Mackenzie *et al.*, 1997) and other residues responsible for sugar donor binding, while the *N*-terminal domain contains the two conserved catalytic residues (H44 and D165 in the alignment in Fig. 3).

In accordance with *H. armigera* and *B. mori* (Ahn et al., 2012), UGT33 and UGT40 are the largest UGT families identified in *S. frugiperda* in this work, with 9 and 8 members among the expressed candidates, respectively. The phylogenetic tree in Fig. 4 displays the relationships between the 25 expressed SfUGTs and their families.

4.3. Activity of expressed UGTs

Five of the 25 expressed UGTs were able to glucosylate DIMBOA and/or MBOA. Unfortunately, there is not enough data in the literature in order to make general predictions about which UGT families are preferably involved in xenobiotic detoxification, if any. The five candidates that showed activity towards BXDs in this work belong to four different families: UGT33 (SfUGT3 and SfUGT6), UGT40 (SfUGT22), UGT42 (SfUGT32), and UGT46 (SfUGT35) (Fig. 4).

The candidate SfUGT6 showed specific activity towards DIMBOA, forming (2*S*)-DIMBOA-Glc, in agreement with the detoxification reaction observed *in vivo*. Among the expressed candidates, this is the most likely to be responsible for the reported DIMBOA reglucosylation in *S. frugiperda* (Wouters *et al.*, 2014). Interestingly, SfUGT6 also catalyzes the glucosylation of HMBOA yielding HMBOA-Glc, which is another important product of DIMBOA metabolism by *S. frugiperda* (Glauser *et al.*, 2011 and Wouters *et al.*, in preparation). This suggests that insect UGTs might be active towards many BXD aglucones with similar structures. In contrast, SfUGT3 showed activity towards both DIMBOA and MBOA, which was not expected considering that *in vitro* data suggested the involvement of different enzymes for these two activities. Moreover, SfUGT3 yields (2*R*)-DIMBOA-Glc, which is not the epimer observed *in vivo*. It is important to note that *S. frugiperda* gut, integument, and Sf9 cells formed exclusively (2*S*)-DIMBOA-Glc in the *in vitro* UGT assays showed in Fig. 2b. Therefore, SfUGT3 seems to have inappropriately low substrate specificity, and its expression levels estimated preliminarily by RNAseq are too low for it to be relevant in BXD metabolism *in vivo*. However, it would be interesting to investigate which protein structural features dictate the stereochemistry observed in the DIMBOA-Glc products formed by SfUGT6 and SfUGT3.

Candidates SfUGT22, SfUGT32, and SfUGT35 are able to metabolize MBOA, but their relevance in the overall *in vivo* MBOA glucosylation needs further assessment. The MBOA glucosylation reaction is particularly interesting, as it produces an *N*-glycoside. In humans, individual UGTs have clear preferences towards either *O*- or *N*-glucuronidation. Human enzymes such as UGT1A1, UGT1A3, UGT1A4, UGT1A9, UGT2B4, UGT2B7, and UGT2B10 are considered to play an important role in *N*-glucuronidation of xenobiotics (Kaivosaari *et al.*, 2011). Most importantly, UGT2B10 seems to be “specialized” in *N*-glucuronidation, with no *O*-glucuronidation reactions reported. Based on studies with human UGTs, the residues from the UGT catalytic dyad (H44 and D165 in the alignment in Fig. 3) are considered to have opposite effects on *O*- and *N*-glycosylation transition states. While the histidine residue (assisted by the aspartate residue) is required to deprotonate *O*-nucleophiles in order to increase their nucleophilicity, *N*-nucleophiles do not need deprotonation in order to attack the glycosyl donor, but rather require a negatively-charged aspartate residue to stabilize the resultant positively-charged transition state (Patana *et al.*, 2008). Accordingly, this highly conserved histidine residue (H44 in alignment) in human UGTs is substituted by proline and leucine in UGT1A4 and UGT2B10, respectively, both of which possess high specificities towards *N*-glucuronidation. Moreover, site directed mutagenesis studies succeeded in switching between the *O*- and *N*-glucuronidation specificity of human UGTs based on the presence or absence of this

histidine residue, respectively (Kerdpin *et al.*, 2009). However, the presence of this catalytically important histidine residue might still contribute to *N*-glycosylation by directing and orienting the nucleophilic attack, not necessarily involving substrate deprotonation (Brazier-Hicks *et al.*, 2007), and the differences between *O*- and *N*-nucleophiles are based on their general structural features. The four *S. frugiperda* UGT candidates that showed activity towards MBOA (SfUGT3, SfUGT22, SfUGT32 and SfUGT35) all possess a conserved histidine residue at the catalytic site. One possible explanation is that the relatively high acidity of benzoxazolinones (pK_a = 12.1 for BOA in DMSO, Bravo and Weiss-Lopez, 1999) might favor a glycosylation mechanism involving deprotonation by histidine prior to nucleophilic attack to the glycoside donor. On the other hand, the two candidates that do not possess this conserved histidine (SfUGT34 with asparagine, and SfUGT27 with leucine) did not display UGT activity towards MBOA.

It is important to mention that all candidates were amplified without their original stop codon. Therefore, the translated proteins possess a His-tag and a V5 epitope on the *N*-terminal domain, derived from the vector used for their expression. The presence of such tags could influence the selectivity and activity of the expressed UGTs.

Future experiments will provide information on the substrate specificity of the five active enzymes described in this article. By assessing the UGT activity of these expressed enzymes towards a range of biologically relevant aglucones, we intend to better understand their function and possible involvement in other pathways, which is especially relevant for the observed MBOA-UGT activity. Further studies are necessary to assess the importance of such UGT activities on the overall detoxification capacity of the insect and their impact on insect fitness, for example via *in vivo* silencing of individual UGT genes.

4.4. BXD-UGT profile differs among S. frugiperda tissues

In third to fourth instar *S. frugiperda* larvae, feeding on high BXD containing maize did not strongly induce BXD-UGT activity compared to feeding on low-BXD mutant maize and artificial diet. The small apparent increase of DIMBOA-UGT activity in BXD+ treatment compared to BXD− treatment suggests weak induction upon feeding on BXDs. However, an intermediate DIMBOA-UGT activity in artificial diet treatment, with no BXDs, indicates that this activity is mostly constitutive and possibly regulated by compounds other than BXDs. In all three treatments, both DIMBOA and MBOA-UGT activities follow consistent profiles among tissues. DIMBOA-UGT activity is highest in gut tissue, while MBOA-UGT activity is highest in fat bodies. This last observation suggest that the high MBOA-UGT activity observed in the integument in the previous experiments was due to fat bodies that were still attached and were not thoroughly cleaned from the tissue sample. The localization of DIMBOA-UGT activity mostly in gut tissue agrees with its important role in xenobiotic detoxification. However, the higher levels of MBOA-UGT activity in fat bodies compared to gut tissue are not completely understood. Two hypotheses can be built from this scenario: (i) since MBOA is much more lipophilic

than DIMBOA, its permanence in gut cells is lower and it diffuses more easily into the fat bodies, where it is detoxified; or (ii) the enzymes responsible for MBOA glucosylation do not perform this reaction as its primary function, but rather carry out other physiological functions in the fat bodies, yet still possess a broad substrate specificity that allows them to metabolize MBOA.

Evaluation of the expression of the genes encoding the five UGTs active towards BXDs described here by qPCR is underway. Their expression profiles among tissues, combined with the *in vitro* activities displayed in Fig. 5 will provide information on the relevance of each candidate to the observed *in vivo* reactions of DIMBOA and MBOA glucosylation.

In the present work we explored for the first time both molecular and functional aspects of *S. frugiperda* UGTs. We successfully expressed 25 *S. frugiperda* UGT candidates and found 5 of them to have activity towards DIMBOA and/or MBOA. By investigating the tissue distribution of these activities and their induction upon BXD feeding, we furthered our understanding of the mechanisms that cause BXD resistance in *S. frugiperda*. The molecular data presented here can be used for future studies to investigate the functional roles of *S. frugiperda* UGTs not only in BXD metabolism, but also in detoxification of other plant defenses and xenobiotics, as well as on physiological processes in this species. This comprehensive exploration of *S. frugiperda* UGTs also expands our knowledge on this enzyme superfamily in insects, and provides information for future comparative studies with other lepidopteran species and insect pests in general.

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Figures:

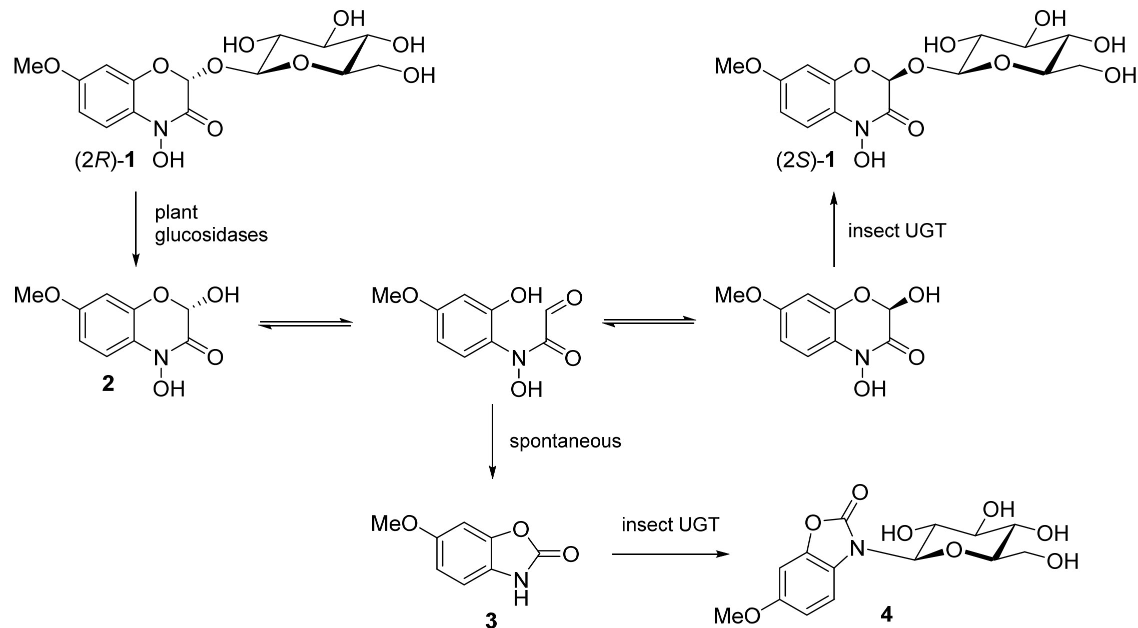

Fig. 1. Glucosylation reactions used by *Spodoptera* species in order to detoxify the benzoxazinoids DIMBOA (**2**) and MBOA (**3**)

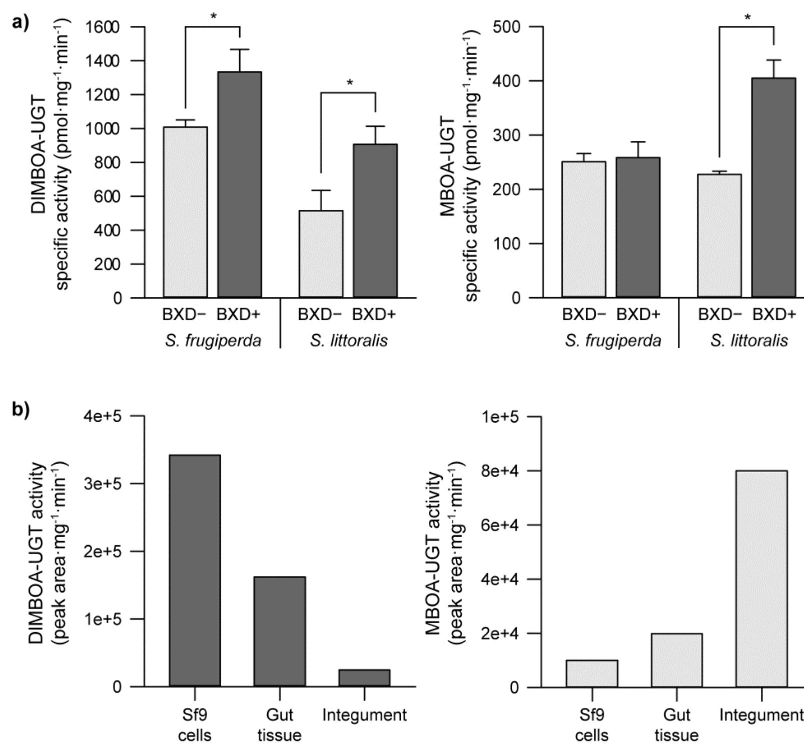

Fig. 2. *in vitro* UGT activities towards DIMBOA and MBOA in (a) body homogenates of *S. frugiperda* and *S. littoralis* neonates feeding on BXDs (N=5, t-test, *P<0.05), and (b) homogenates of Sf9 cells and *S. frugiperda* gut and integument tissue

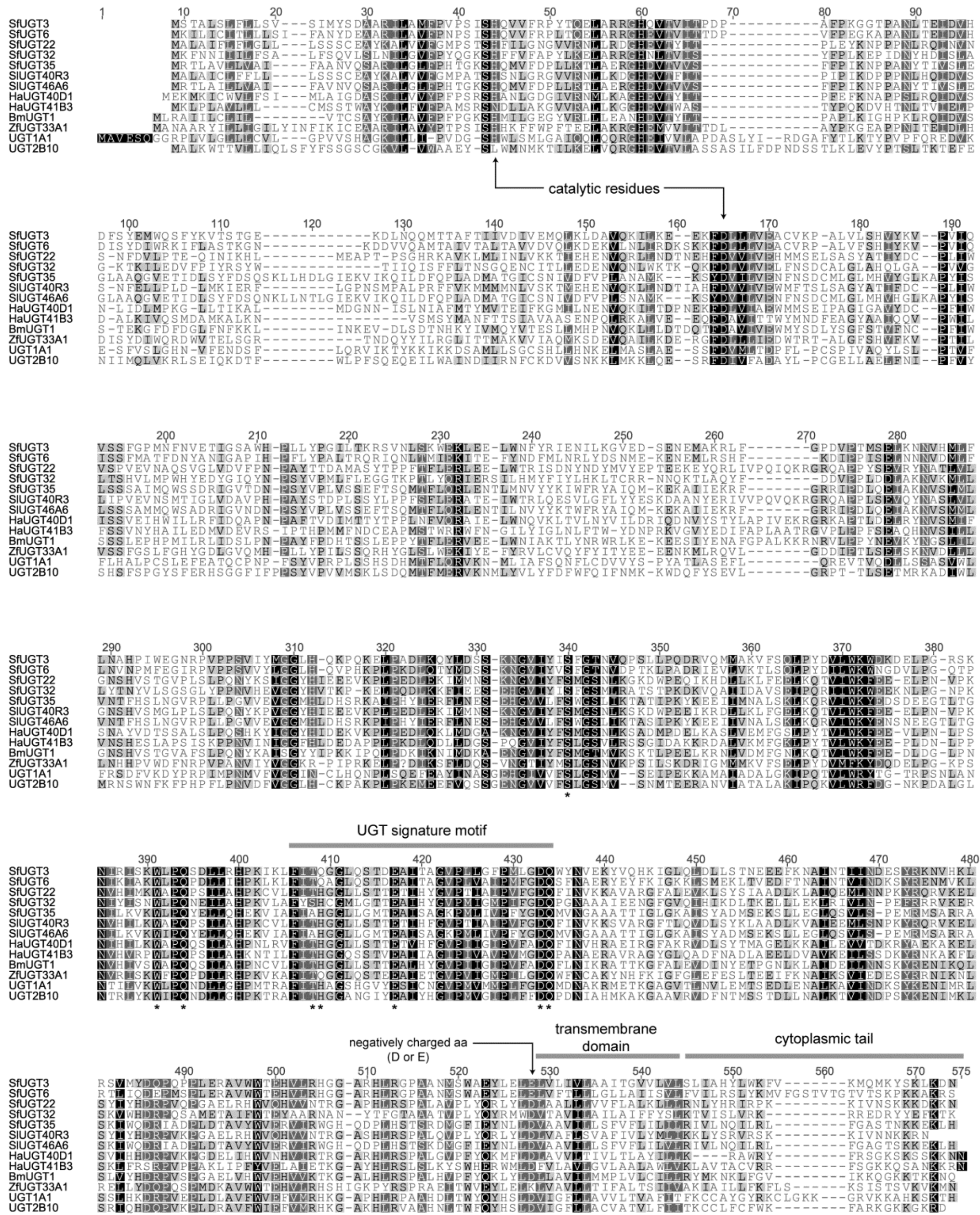

Fig. 3. Alignment of *S. frugiperda* UGT sequences described in this work (5 candidates with activity towards BXDs) with representative UGTs from *S. littoralis*, *H. armigera*, *B. mori*, *Z. filipendulae*, and *Homo sapiens*. Residues responsible for binding with the sugar donor are marked with an asterisk (*) (Ahn *et al.*, 2012).

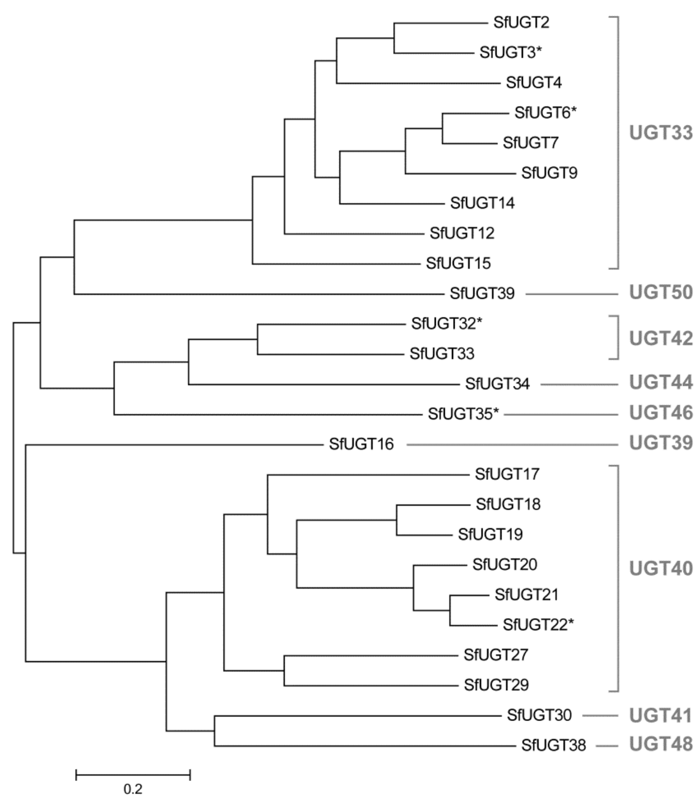

Fig. 4. Phylogenetic tree of *S. frugiperda* UGTs and their families; only the 25 heterologously expressed UGTs are represented. *enzymes with activity towards BXDs.

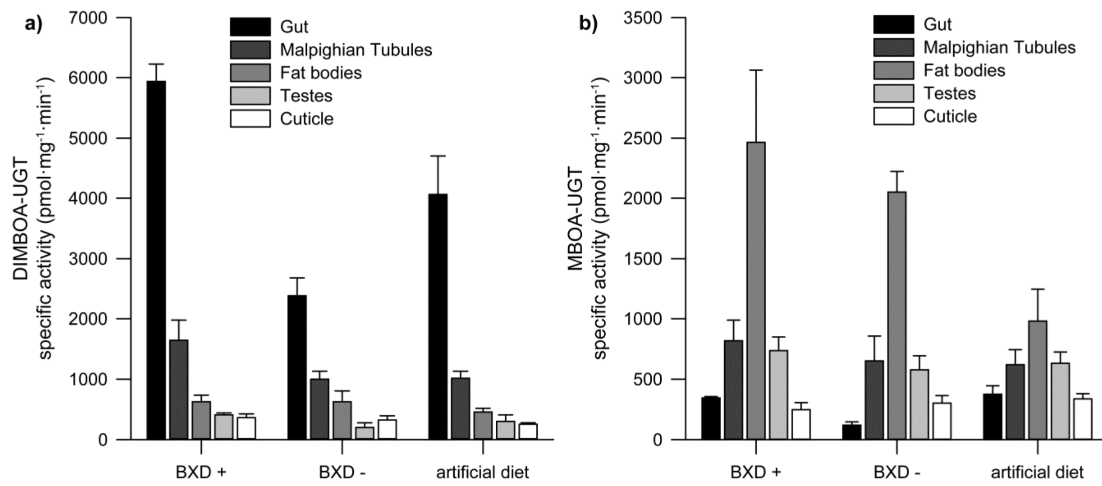

Fig. 5. Comparison of *in vitro* UGT activities towards (a) DIMBOA and (B) MBOA among tissues from S. frugiperda larvae feeding on WT maize leaves (BXD+), mutant bx1 maize leaves (BXD-), and artificial diet. (N=3 pooled tissue samples from 3 individuals each)

Tables:

Table 1: UDP-glucosyltransferases (UGTs) identified in *Spodoptera frugiperda* transcriptome, and activity towards the benzoxazinoids DIMBOA and MBOA

Name	Family	UGT size (amino acids)	DIMBOA activity*	MBOA activity*
SfUGT2	UGT33	516	nd	nd
SfUGT3	UGT33	515	✓ (2R)	✓
SfUGT4	UGT33	518	nd	nd
SfUGT6	UGT33	525	✓ (2S)	nd
SfUGT7	UGT33	521	nd	nd
SfUGT9	UGT33	519	nd	nd
SfUGT12	UGT33	520	nd	nd
SfUGT14	UGT33	521	nd	nd
SfUGT15	UGT33	520	nd	nd
SfUGT16	UGT39	521	nd	nd
SfUGT17	UGT40	524	nd	nd
SfUGT18	UGT40	522	nd	nd
SfUGT19	UGT40	524	nd	nd
SfUGT20	UGT40	520	nd	nd
SfUGT21	UGT40	520	nd	nd
SfUGT22	UGT40	523	nd	✓
SfUGT27	UGT40	522	nd	nd
SfUGT29	UGT40	523	nd	nd
SfUGT30	UGT41	520	nd	nd
SfUGT32	UGT42	508	nd	✓
SfUGT33	UGT42	516	nd	nd
SfUGT34	UGT44	526	nd	nd
SfUGT35	UGT46	526	nd	✓
SfUGT38	UGT48	530	nd	nd
SfUGT39	UGT50	544	nd	nd

*nd: not detected;

Supplementary tables:

Supplementary Table 1: Primers used for full sequence amplification

Gene	Forward primer	Reverse primer
SfUGT2	5'-CGTTCGATAATGTTCTTCCC	5'-ATTCTTCTTAACTTTCTTCTGCTTTG
SfUGT3	5'-TTGCGTGTAATCATGTCTACCG	5'- ATTGTCTTTTAACTTACTATATTTCATTTG
SfUGT4	5'-CTCAAGATGGTTGGGTTT	5'-ACTACTCTTCACTTTAGTCTTAATTACATAGTT
SfUGT6	5'-TAGAAAATGAAGATATTAATTTGTATAACTCTG	5'-ACTACGTTTGGCTTTTTTAGGTT
SfUGT7	5'-AGCCAGACAATGAGGTATTTTAT	5'-ACTACGTTTTGATTTTGATTTTTTAA
SfUGT9	5'-ATAAAAATGTTATCTTTTGTGTACATAATA	5'-ATTATTTTTAACTTTAGGAGTAGAAACAATACT
SfUGT12	5'-AGGACACGTGCAATGTCGTTC	5'-ACTCCTTTTAACCTTACCAGCG
SfUGT14	5'-CGCGTTACAATGTCGGTATTACTC	5'-ATTCCGTTTCTTCTTCACACTAGTTTT
SfUGT15	5'-TCGATCACAATGTCTGTGC	5'-ACTCCGTTTAATCTTAACATCACTAA
SfUGT16	5'-AAAATGGACCCCCTAAAAATAAT	5'-ATGTTTCTTCTGTTTTTTCGTTG
SfUGT17	5'-CTGTCAAAAATGGAGATAATTAAATAC	5'-ATTCTTCTTAAGTTTTGGGCTCT
SfUGT18	5'-ATTGAGAATATGGAAAGAATGAAG	5'-GTTGTTCTTCTTTTGGCTTCC
SfUGT19	5'-TCAGCCATCATGCAACG	5'-ATTCTTCTTCTTTTTTCCTCCTGTAT
SfUGT20	5'-ATTTGTAAAATGGCATTAGCAATAT	5'-ATTCCTCTTCTTACTGTCTACAATTTT
SfUGT21	5'-GTGCCGATAATGGCCTTAGTATTA	5'-ATTCCTTTTTTTACTATTTACAATTTTCTT
SfUGT22	5'-TTGTGCGTGCCAATAATGGCG	5'-ATTCTTCTTATCCTTTTTCTTACTATTTAC
SfUGT27	5'-TGTGTCACAATGAGGTTGCCA	5'-ATTAGTTTTCTTCTGATCATATTTTGTTGC
SfUGT29	5'-AGCAATAAAATGAACAAATGGATT	5'-ACTCCTTTTCTTCTTTTTATCTTCAC
SfUGT30	5'-ACAATGAAGCTAAGTATCCTGTTACTC	5'-ATTCCTTTTCTTTTTACTAGATTTCTCTTT
SfUGT32	5'-AATAAAATGAAGTTTAATAATATAATAATCTTG	5'-CTTAGTCTTGAACTCATAATAACGATCT
SfUGT33	5'-TCCATAATGAAGCCCGGC	5'-CGTTCGCTTCGCCTTTTT
SfUGT34	5'-GCATTCAAAATGACAAAACAGACA	5'-TTCGAACTTCACTCTTTTTGAGGA
SfUGT35	5'-TTGCCCACGATGCG	5'-ATGCAACTTCTCCTTCTTATTCG
SfUGT38	5'-GTAGCTGACATGAGGCGG	5'-TTCGTTTTTCTTGTCTTCAAAATA
SfUGT39	5'-GCAAAAATGCATAGGTGGA	5'-CAATTTCTTCTTTGTTGCCG

Supplementary Table 2: DIMBOA and DIMBOA-Glc levels on maize plants used for *S. frugiperda* and *S. littoralis* feeding (in µg/mg dry weight (±SEM), N= 6)

		DIMBOA-Glc	**DIMBOA**
bx1	day 1	161 (±63)	46 (±18)
	day 3	49 (±16)	16 (±6)
WT	day 1	16455 (±905)	5111 (±486)
	day 3	15018 (±2193)	2750 (±622)

Manuscript V

Metabolic fate of benzoxazinoids in *Spodoptera frugiperda*

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Abstract – Benzoxazinoids are defensive metabolites in crops such as maize, wheat, and rye. (2*R*)-DIMBOA-Glc, the major benzoxazinoid in maize aerial parts, is hydrolyzed to the toxic aglucone DIMBOA upon herbivory. The mixture deployed by the plant also includes other benzoxazinoids such as HMBOA and MBOA, which collectively exert toxicity towards many insect herbivores. As a response, some insect species have developed mechanisms to avoid benzoxazinoid toxicity, such as the agricultural pest *Spodoptera frugiperda*. However, the metabolic basis of its resistance to benzoxazinoids remains relatively unknown. In the present work we describe the glucosylation of HMBOA to (2*S*)-HMBOA-Glc, a new detoxification reaction by *S. frugiperda*, confirming the identity of the product by synthesis and NMR analyses. In order to quantitatively investigate the metabolism of DIMBOA, MBOA, (2*R*)-DIMBOA-Glc, and (2*R*)-HMBOA-Glc by *S. frugiperda*, we took advantage of feeding assays with purified compounds followed by metabolic screening in frass and hemolymph. We found that *S. frugiperda* metabolizes DIMBOA by partially reducing it to the lactam HMBOA and degrading it to MBOA, followed by glucosylation reactions yielding (2*S*)-DIMBOA-Glc, (2*S*)-HMBOA-Glc, and MBOA-Glc. We also detected these glucosides in hemolymph up to 6 hours after DIMBOA ingestion, which suggests a path of transport for their excretion. Additionally, we observed that *S. frugiperda* partially hydrolyzes the plant glucosides (2*R*)-DIMBOA-Glc and (2*R*)-HMBOA-Glc. Our findings represent the first quantitative evaluation of the contribution of several metabolic pathways employed by *S. frugiperda* to cope with individual benzoxazinoids.

Key words–Benzoxazinoids, Plant defenses, Detoxification, *Spodoptera frugiperda*, Excretion, Glucosylation

1. Introduction:

Plants rely on a diverse arsenal of defensive secondary metabolites in order to protect themselves from herbivory. Some insect herbivores, on the other hand, have evolved countermeasures to cope with such toxic compounds, being able to use chemically well-defended plants as a food source without strong ill-effects. The range of adaptations that insects developed to deal with plant defenses includes their rapid excretion, sequestration, and detoxification (Després *et al.*, 2007; Heckel, 2014; Heidel-Fischer and Vogel, 2015). One example of such compounds, benzoxazinoids (BXDs), constitute an important plant defense family widespread in grasses (Poaceae), including important crops such as maize, wheat and rye (Niemeyer, 2009). BXDs are stored in the plant as stable glucosides (Frey *et al.*, 2009) that upon cell damage (e.g. by chewing herbivores) are activated by β-glucosidases yielding toxic aglucones (Scheme 1). The electrophilicity of BXD aglucones is considered to play a role in their toxicity, promoting reactions with nucleophilic thiol and amine groups from amino acid residues in proteins (Cuevas *et al.*, 1990; Niemeyer *et al.*, 1982a; Pérez and Niemeyer, 1989a, b). Furthermore, hydroxamic acid BXD aglucones such as DIMBOA further degrade spontaneously to benzoxazolinones such as MBOA, which are also toxic towards insect herbivores (Houseman et al., 1992). The most abundant BXD in maize aerial parts, (2*R*)-DIMBOA-Glc ((2*R*)-2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one), has been implicated in resistance towards many insect herbivores, such as the European corn borer, *Ostrinia nubilalis* (Robinson *et al.*, 1978), and the aphids *Metopolophium dirhodium* (Argandoña *et al.*, 1980) and *Schizaphis graminum* (Corcuera *et al.*, 1982). On the other hand, some species such as the the fall armyworm *Spodoptera frugiperda* seem to be more tolerant towards BXD toxicity (Glauser *et al.*, 2011; Kohler *et al.*, 2015; Rostás, 2006).

Controlled feeding assays with plants and purified compounds have clarified some important aspects of the metabolism, detoxification, transport, and sequestration of plant defenses such as glucosinolates (Schramm *et al.*, 2012; Stauber *et al.*, 2012), cyanogenic glycosides (Zagrobelny and Møller, 2011), phenol and terpenoid glycosides (Discher *et al.*, 2009), and pyrrolizidine alkaloids (Lindigkeit *et al.*, 1997) by insects, although more research is still necessary. Similarly, the metabolism of benzoxazinoids by lepidopteran herbivores is currently not fully understood and quantified. Experiments using radioactive ^{3}H-labeled DIMBOA and MBOA showed that *O. nubilalis* rapidly excretes the label in the frass and pupal case, but the identity of the metabolites was not investigated (Campos *et al.*, 1988, 1989). Humans, pigs, and rats metabolized BXDs mainly through glycoside hydrolysis, reduction of hydroxamic acid to lactams, and conjugation to glucuronic acid (Adhikari *et al.*, 2012a; Adhikari *et al.*, 2013; Adhikari *et al.*, 2012b). However, although previous research has started to shed light onto the metabolic fates of BXDs in *S. frugiperda* , the pathways involved have not yet been fully characterized. The mechanisms employed by this species to avoid BXD toxicity likely contribute to its status as an agricultural pest, and knowledge about its BXD metabolism can provide new approaches to pest control. Recently, we and others have reported that *S. frugiperda* efficiently detoxifies BXDs (Glauser *et al.*, 2011), including the metabolism of MBOA by

N-glucosylation to give MBOA-Glc (Maag *et al.*, 2014) and of DIMBOA to give (2*S*)-DIMBOA-Glc, an epimer of the original plant compound that is not prone to hydrolysis by plant β-glucosidases (Wouters *et al.*, 2014). However, the relative importance of these pathways to the overall BXD metabolism was not assessed in a quantitative fashion.

In the present work, we investigated the metabolism of BXDs in *S. frugiperda* in a quantitative fashion for the first time. In this study, we have confirmed that *S. frugiperda* glucosylates HMBOA forming (2*S*)-HMBOA-Glc, analogously to the characterized detoxification of DIMBOA (Wouters *et al.*, 2014). Furthermore, by feeding individual purified BXDs to *S. frugiperda* larvae, we were able to evaluate the metabolic fate of DIMBOA, MBOA, (2*R*)-DIMBOA-Glc, and (2*R*)-HMBOA-Glc. Surprisingly, the glucosides (2*R*)-DIMBOA-Glc and (2*R*)-HMBOA-Glc were observed to be partially hydrolyzed by the insect. After DIMBOA feeding, most of the BXDs recovered in frass were represented by (2*S*)-DIMBOA-Glc, (2*S*)-HMBOA-Glc, and MBOA-Glc, result of partial DIMBOA reduction to HMBOA and degradation to MBOA, followed by glucosylation reactions. Additionally, these glucosides were detected in the hemolymph after administration, suggesting a route of transport for their excretion.

2. Material and Methods:

2.1. Insect rearing

Larvae of *Spodoptera frugiperda* (maize strain) were obtained from colonies at the Max Planck Institute for Chemical Ecology, and were reared individually in plastic cups, on an artificial diet based on white beans (Bergomaz and Boppré, 1986), under controlled light and temperature conditions (12:12 h light/dark, 20 °C).

2.2. Enzymatic assays

Fourth to fifth instar *S. frugiperda* larvae were dissected in cold phosphate buffer (pH 7.0, 10 mM), and their guts were collected and cleaned from gut lumen. Gut tissue samples were homogenized in 50 µL of the same buffer per gut. Aliquots of these enzyme preparations were boiled for 20 min. Enzymatic assays included 10 µL tissue homogenate, 2 µL of 75 mM HMBOA in DMSO (0.15 µmol), 4 µL of 75 mM UDP-glucose in water (183.1 µg, 0.3 µmol), and enough phosphate buffer (pH 7.0, 100 mM) solution to give an assay volume of 50 µL. Controls containing boiled enzymatic preparation, and only the gut suspension and buffer were included. After incubation at 30 °C for 30 min, the enzymatic reactions were interrupted by adding 50 µL of a methanol/formic acid 1:1 (v:v) solution. Assay tubes were centrifuged at 5,000 g for 5 minutes and the supernatant was collected and analyzed by LC-MS/MS. A larger scale reaction was performed using 3 mg HMBOA (15 µmol in 20 µL DMSO), 21.9 mg UDP-glucose (30 µmol in 80 µL water) and 300 µL gut suspension, completed

with phosphate buffer (pH 7.0, 100 mM) solution to a final volume of 400 µL. After overnight incubation at 30 °C, the reaction was interrupted by adding 400 µL of a methanol/formic acid 1:1 (v:v) solution, centrifuged at 5,000 g for 5 minutes and the obtained supernatant was collected and fractionated by semipreparative HPLC.

2.3. Droplet feeding assays

Individual solutions containing approximately 10 mM of DIMBOA, 5 mM of MBOA, 20 mM of (2*R*)-DIMBOA-Glc, and 20mM (2*R*)-HMBOA-Glc were prepared by dissolving the BXDs in DMSO (5% of the final volume) and diluting them in 10% sucrose solution in water. Fourth to fifth instar *S. frugiperda* larvae were starved for 24h, stimulated with forceps immediately before the feeding in order to regurgitate, and dried with paper towel. With the assistance of forceps and a microscope, BXD solutions (2 µL) were administered directly to larval mouthparts using a micropipette. Approximately half of the individuals used were receptive to droplet feeding, by actively and completely sucking the BXD solution from the pipette tip. Individuals that regurgitated or moved causing losses of the solution during the feeding were discarded. Individuals for frass collection were then left to feed on artificial diets and kept under the regular rearing conditions for 24 h. After this time, the frass samples in the plastic cups were collected thoroughly, and extracted with 1 mL of a water/methanol 1:1 (v:v) solution. After adding 3 mm steel beads, extracts were vortexed for 2 min, agitated in a paint shaker for 2 min, and centrifuged at 16,000 g for 5 minutes. This extraction was repeated two more times and the combined supernatants were dried in a centrifugal vacuum concentrator (Eppendorf Concentrator 5301, Eppendorf, Hamburg, Germany), resuspended in 200 µL of 0.5% formic acid in water/methanol 1:1 (v:v) solution, and analyzed by LC-MS/MS. Droplet samples (2 µL) from the administered solutions were diluted and quantified by LC-MS/MS in order to normalize the ingested amount of each BXD.

Individuals for hemolymph collection (N=3) were kept feeding on artificial diet on the bench after droplet feeding. After 1, 2, 3, 6, and 12 h, individuals were anesthetized by -20 °C treatment for 5 min, and had one proleg severed with dissecting scissors without damaging the gut tissue. With a micropipette, 10 µL of the hemolymph droplet were collected and immediately transferred to a tube containing 40 µL of 0.5% formic acid in water/methanol 1:1 (v:v) solution on ice. The resulting samples were centrifuged at 5,000 g for 5 minutes and the supernatant was collected and analyzed by LC-MS/MS.

2.4. Chromatographic methods:

For all analytical chromatography procedures, formic acid (0.05%) in water and acetonitrile were used as mobile phases A and B, respectively, and the column temperature was maintained at 25 °C. Analyses of enzymatic assays and insect samples used an XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent Technologies, Boeblingen, Germany) with a flow rate of 1.1 $mL \cdot min^{-1}$ and with the

following elution profile: 0-0.5 min, 95% A; 0.5-6 min, 95-67.5% A; 6.02-7 min, 100% B; 7.1-9.5 min, 95% A. For discrimination of DIMBOA-Glc and HMBOA-Glc epimers, resolution was achieved using a Nucleodur Sphinx RP column (250 x 4.6 mm, 5 µm, Macherey-Nagel, Düren, Germany) at a flow rate of 1.0 mL min^{-1} in isocratic mode: 0-10 min, 15% B; 10.1-12 min, 100 % B; 12.1-15 min, 15% B.

Semipreparative HPLC procedures used formic acid (0.005%) in water and acetonitrile as mobile phases A and B, respectively, an XTerra MS C18 column (150 x 10 mm, 5 µm, Waters Corporation, Milford, MA, USA) with a flow rate of 4 mL·min^{-1}, and the column temperature was maintained at 25 °C. The fractionation of DIMBOA and HMBOA-Glc epimers employed the following gradient: 0-14 min, 12.5% B; 14-17 min, 100% B; 17-20 min, 12.5% B. For DIMBOA-Glc epimers, the following gradient was employed: 0-10 min, 15% B; 10-13 min, 100% B; 13-16 min, 15% B. For HMBOA, the following gradient was employed: 0-10 min, 20-35% B; 10-13 min, 100% B; 13-16 min, 20% B. The semipreparative fractionation was performed on an Agilent 1100 HPLC system (Agilent Technologies, Boeblingen, Germany) coupled to an Advantec SF-2120 fraction collector (Advantec MFS Inc., Dublin, CA, USA). The crude products from synthesis were dissolved in MeOH/H_2O 1:1 and successive 100 µL injections were fractionated according to the UV chromatogram peaks observed at 254 nm.

LC-MS/MS analyses were performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany) coupled to an API 3200 tandem spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a turbospray ion source operating in negative ionization mode. The ion spray voltage was maintained at −4500 V. The turbo gas temperature was 500 °C, nebulizing gas 60 psi, curtain gas 25 psi, heating gas 60 psi and collision gas 5 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion to product ion conversion with parameters from the literature for DIMBOA, HMBOA (Pedersen *et al.*, 2011), and DIMBOA-Glc (Wouters *et al.*, 2014). MRM parameters for HMBOA-Glc were the same as for DIMBOA-Glc, except for the Q1 and Q3 *m/z* values of 402 and 356; and were optimized from infusion experiments with standard samples for MBOA (Q1 *m/z*: 164, Q3 *m/z*: 149, DP −20 V, EP −7 V, CEP −12 V, CE −20 V, CXP −4 V) and MBOA-Glc (Q1 *m/z*: 372, Q3 *m/z*: 164, DP −15 V, EP −4.5 V, CEP −18 V, CE −20 V, CXP −4 V). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Absolute quantities of the measured benzoxazinoids were determined using standard curves obtained from purified samples of DIMBOA, HMBOA (1-100 µM), MBOA (1-50 µM), (2*R*)-DIMBOA-Glc, (2*R*)-HMBOA-Glc, (2*S*)-HMBOA-Glc (1-10 µM), and MBOA-Glc (0.1-5 µM). Both (2*R*) and (2*S*) epimers of HMBOA-Glc gave similar MS responses. (2*S*)-DIMBOA-Glc could not be obtained in adequate quantity and purity for preparation of standard samples, therefore the (2*R*)-DIMBOA-Glc standard curve was used for quantification of both epimers. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing.

3. **Results**:

3.1. Synthesis of benzoxazinoids

We obtained the (2*S*) and (2*R*) epimers of DIMBOA-Glc and HMBOA-Glc as described in Scheme 2, using methodologies adapted from the literature (Atkinson *et al.*, 1991; Sicker and Schulz, 2002). First, methyl methoxyacetate (**6**) was brominated with NBS and coupled to the potassium phenoxide **8**, yielding the nitro compound **9.** The conditions for the following reductive cyclization step are critical for driving the reaction to the formation of the hydroxamic acid **10** or the lactam **11**. Hydrogenation over sulfided platinum on activated carbon in acetic acid promoted complete reduction of the nitro group, followed by cyclization, forming the lactam **11**, without the rearrangement observed in the reaction of the dioxo compound originally described (Hartenstein and Sicker, 1993). Alternatively, reduction with palladium on activated carbon and sodium borohydride in the presence of water (Atkinson *et al.*, 1991) drove the cyclization of the partially reduced intermediate, yielding the hydroxamic acid **10** as the major product, with some of the lactam **11** as a side product. Both methyl ethers **10** and **11** were demethylated by boron tribromide, yielding DIMBOA (**3**) and HMBOA (**4**), respectively. Due to the strong reactivity of boron tribromide, demethylation of the aromatic methoxy group also took place, especially for the methyl ether **11**, but was slower than the formation of the hemiacetal. Subsequently, DIMBOA (**3**) and HMBOA (**4**) were purified by semipreparative HPLC. The aglucones DIMBOA (**3**) and HMBOA (**4**) were further glycosylated using a modified thrichloroacetimidate method (Kluge and Sicker, 1996; Wouters *et al.*, 2014), yielding both (2*R*) and (2*S*) epimers. The individual epimers were purified by semipreparative HPLC, and (2*R*)- and (2*S*)-HMBOA-Glc were characterized by ^{1}H, ^{13}C, HSQC, HMBC, and NOESY NMR. Detailed synthetic procedures and NMR spectral data can be found in the Supplementary material.

*3.2. Insects produce the epimer (2*S*)-HMBOA-Glc*

The incubation of *S. frugiperda* gut tissue homogenate with HMBOA and UDP-glucose resulted in the formation of HMBOA-Glc, analogously to the glucosylation of DIMBOA promoted by this species (Wouters *et al.*, 2014). To elucidate the stereochemistry of the HMBOA-Glc formed by the insect, we performed a larger scale reaction and purified the product by semipreparative HPLC. The NMR spectral data for this product matched those obtained for the synthetic (2*S*)-HMBOA-Glc. Therefore, the insect gut tissue is able to enzymatically glucosylate the aglucone HMBOA yielding (2*S*)-HMBOA-Glc, an epimer of the glucoside produced by plants (Nagao *et al.*, 1985).

3.3. Metabolic profiling of frass after BXD feeding

Initial attempts to produce radioactive ^{14}C labeled BXDs by feeding maize plants with labeled precursors resulted in low yields and accumulation of side products. Likewise, application of BXD glucosides onto the surface of mutant maize leaves was unsuccessful. We therefore evaluated the metabolism of several BXDs in *S. frugiperda* by feeding droplets of their solutions in 10% sucrose in water (5% DMSO) to fourth to fifth instar larvae, and analyzing their frass after additional feeding on artificial diet without BXDs for 24 h.

The profiles of BXD metabolites in frass after DIMBOA, MBOA, (2*R*)-DIMBOA-Glc, and (2*R*)-HMBOA-Glc feeding are summarized in Fig. 1. Samples from the administered BXD solutions were analyzed and used for normalization of the ingested amounts. Frass extracts were quantified by LC-MS/MS, using multiple reaction monitoring (MRM) parameters that cover the most important natural BXD aglucones and their glucosides. The molar amount of recovered BXDs in frass was calculated, together with the corresponding fractions of individual BXD metabolites. The recovery of BXD derivatives observed in the frass ranged from 30% to 60%. In frass collected between 24 and 48 h after administration, no BXD derivatives were detected (data not shown).

Upon DIMBOA feeding, (2*S*)-DIMBOA-Glc (44%), (2*S*)-HMBOA-Glc (28%), MBOA-Glc (13%), and DIMBOA (13%) were observed in the frass, together with trace amounts of HMBOA and MBOA (1% each). Feeding of MBOA resulted almost exclusively in MBOA-Glc (99%). Feeding of (2*R*)-DIMBOA-Glc resulted mostly in unchanged (2*R*)-DIMBOA-Glc (94%), in addition to DIMBOA and (2*S*)-HMBOA-Glc (3% each). Feeding of (2*R*)-HMBOA-Glc resulted in excretion of the unchanged compound (63%) accompanied by (2*S*)-HMBOA-Glc (29%), HMBOA (6%), and (2*R*)-DIMBOA-Glc (2%). The latter compound is likely a result from impurities in the solution used for feeding (96% (2*R*)-HMBOA-Glc and 2% (2*R*)-DIMBOA-Glc), rather than a metabolism product. Taken together, these data indicate that DIMBOA is reduced to HMBOA and degrades to MBOA, with all three compounds being mostly glucosylated by *S. frugiperda*. Additionally, *S. frugiperda* seems to possess endogenous glucosidase activity that can accept BXDs as substrates and is responsible for the small amounts of observed hydrolysis products from (2*R*)-DIMBOA-Glc) and (2*R*)-HMBOA-Glc.

3.4. Benzoxazinoid glucosides are transported through the hemolymph

To obtain information about the distribution and excretion dynamics of BXD metabolites, we analyzed the hemolymph of *S. frugiperda* larvae at various time points after oral administration of DIMBOA. The variation of the levels of (2*S*)-DIMBOA-Glc, (2*S*)-HMBOA-Glc, and MBOA-Glc over time are displayed in Fig. 2. The highest concentration of these glucosides was observed at the earliest time point, 1 h after DIMBOA ingestion. After 6 h, these compounds were almost completely absent in hemolymph samples. Only the insect-derived (2*S*) epimers of DIMBOA-Glc and HMBOA-Glc were detected in the hemolymph, which is in agreement with the data obtained for DIMBOA

metabolites in frass, and no aglucones were detected. These data suggest that BXD aglucones are glucosylated by *S. frugiperda* gut tissues and are transported through the hemolymph before being excreted.

4. Discussion:

The present work provides insight into the metabolism of BXDs by larvae of the relatively BXD-resistant species *S. frugiperda*. We have shown that this species is able to detoxify HMBOA by glucosylating it stereoselectively to (2*S*)-HMBOA-Glc. With feeding assays, we were able to quantify the relative contribution of multiple pathways involved in the metabolism of individual BXDs by *S. frugiperda*. Additionally, we present evidence that the excretion of glucosylated BXD detoxification products by *S. frugiperda* involves their transport through the hemolymph.

We demonstrated that *S. frugiperda* gut tissues are able to catalyze the glucosylation of HMBOA to (2*S*)-HMBOA-Glc, which has been characterized for the first time in the present work. Although the glucosylation of HMBOA has already been observed in the literature (Glauser et al., 2011), the stereochemistry of the product had not yet been assigned. A similar glucosylation of DIMBOA to (2*S*)-DIMBOA-Glc has been previously reported by our group (Wouters *et al.*, 2014). The formation of (2*S*)-DIMBOA-Glc represents a strategy to detoxify the aglucone DIMBOA and to prevent its reactivation by hydrolysis, since plant glucosidases are not able to hydrolyze this epimer. The same scenario can be suggested for the HMBOA stereospecific glucosylation described here. Due to the similarity between structures, such glucosylation reactions can also take place with other BXD hydroxamic acids and lactams. Indeed, current work indicates that a UDP-glucosyltransferase from *S. frugiperda* is able to glucosylate both DIMBOA and HMBOA to their (2*S*) glucosides *in vitro* (Wouters *et al.*, in preparation).

The administration of individual BXDs to *S. frugiperda* larvae followed by metabolite screening in the frass elucidated important quantitative aspects of their metabolism. The simplest scenario was provided by the feeding of MBOA, which was shown to be excreted almost exclusively as its glucoside MBOA-Glc. This metabolite has been described as a detoxification product of *S. frugiperda* and *S. littoralis*, and high MBOA-Glc/MBOA ratios were also observed in frass (Maag *et al.*, 2014). Feeding of DIMBOA reveals multiple metabolic fates for this compound, as summarized in Scheme 3. The most abundant compound (2*S*)-DIMBOA-Glc originated from glucosylation of DIMBOA by *S. frugiperda*, while some DIMBOA was excreted unchanged. The second most abundant compound, (2*S*)-HMBOA-Glc, is likely derived from glucosylation of HMBOA formed by reduction of DIMBOA. The reduction of the hydroxamic acid DIMBOA yielding the lactam HMBOA can be promoted by thiols such as cysteine and mercaptoethanol (Atkinson *et al.*, 1991; Niemeyer *et al.*, 1982b), and might represent an additional strategy to diminish DIMBOA toxicity, since HMBOA is considered less toxic to insects (Escobar *et al.*, 1999). On the other hand, reactions between DIMBOA and glutathione, an abundant free thiol in biological systems, produced a range of spiroadducts that were not considered

in the present study (Dixon *et al.*, 2012). Further experiments are necessary to establish whether the formation of HMBOA from DIMBOA involves reduction by thiols or other compounds, and if this reaction is catalyzed by enzymes. Another major compound, MBOA-Glc, results from *N*-glucosylation of MBOA, a degradation product of DIMBOA. BXD hydroxamic acids such as DIMBOA have been shown to spontaneously degrade to benzoxazolinones, and the rate of this reaction substantially increases at high pH values (Niemeyer *et al.*, 1982). The alkaline gut contents *S. frugiperda*, with pH around 9 in the foregut and midgut (Wouters *et al.*, 2014), likely accelerates the degradation of DIMBOA to MBOA, but the excretion of unmodified DIMBOA shows that this reaction is not quantitative in the time scale of digestion in these larvae. Very low amounts of the intermediates HMBOA and MBOA were detected, suggesting that the degradation and reduction reactions of DIMBOA forming these compounds are slower than the corresponding glucosylation reactions.

Feeding of (2*R*)-DIMBOA-Glc indicated that this compound was excreted mostly unchanged. A small fraction of formed DIMBOA (3%) and (2*S*)-HMBOA-Glc (3%) implies a weak glucosidase activity responsible for (2*R*)-DIMBOA-Glc hydrolysis to DIMBOA, partially followed by its reduction to HMBOA and glucosylation to (2*S*)-HMBOA-Glc. However, no (2*S*)-DIMBOA-Glc was observed, suggesting that DIMBOA reglucosylation did not play an important role in this context. The (2*R*)-DIMBOA-Glc used for such feeding assays was of high purity (>99%), excluding the possibility of pre-existing contamination with HMBOA-Glc. Finally, feeding of (2*R*)-HMBOA-Glc showed that this compound was excreted mostly unchanged, but the presence of its epimer (2*S*)-HMBOA-Glc suggests that the initial compound is either epimerized by the insect, or hydrolyzed to HMBOA with following reglucosylation to the (2*S*) epimer. The presence of a small amount of HMBOA in the frass supports the latter hypothesis. In addition to the major β-glucoside, the (2*R*)-HMBOA-Glc sample used for this feeding assay contained the α-glucoside as a side product from synthesis (14%), which might show a different stability towards insect glucosidases and could have contributed to the observed hydrolysis. However, the absolute amounts of HMBOA and (2*S*)-HMBOA-Glc observed in the frass exceeded the amount of administered (2*R*)-HMBOA-α-glucoside, meaning that hydrolysis of the plant compound (2*R*)-HMBOA-β-glucoside indeed took place.

The molar amounts of BXDs recovered in the frass varied between 30% and 60%. Although care was taken in order to guarantee complete droplet ingestion by the larvae and extensive extraction from frass, the losses associated with the employed methodology are unknown. Furthermore, the targeted analysis of BXD metabolites in frass extracts excluded metabolites other than natural BXD aglucones and their monoglucosides. Nevertheless, the observed hydrolysis of glucosides to reactive aglucones suggests that BXD excretion in the frass is not quantitative for any of the treatments. Higher recovery rates in frass were associated with the more stable glucosides (2*R*)-DIMBOA-Glc and (2*R*)-HMBOA-Glc, while lower recovery was observed for the more reactive and lipophilic aglucones DIMBOA and MBOA. Part of the losses observed for DIMBOA might be associated to its irreversible reaction with nucleophilic residues of proteins, as has been suggested to be its mode of action (Niemeyer, 2009). Despite the fact that DIMBOA and its toxic metabolites HMBOA and MBOA are only partially detoxified by glucosylation, this strategy seems to contribute to the BXD resistance observed in *S. frugiperda*. Another interesting hypothesis is that *S. frugiperda*

larvae absorb part of the ingested BXDs and use them as nutrients. In agreement with this, *S. frugiperda* has been suggested to use BXDs as foraging cues (Kohler e*t al.*, 2015), and showed improved growth (Glauser *et al.*, 2011) and feeding stimulation associated to DIMBOA (Rostás, 2006). Further studies using isotopically-labeled BXDs or focusing on how BXDs affect food consumption and utilization would be useful to test the potential nutritional values of these plant defenses to insects.

Finally, the transport of BXD glucosides via the hemolymph gives us hints about the toxicokinetics of DIMBOA. The UGT activities of *S. frugiperda* towards DIMBOA and HMBOA are higher in gut tissues (Wouters *et al.*, in preparation), and present an optimal pH around 7 (Wouters *et al.*, 2014). Therefore, we hypothesize that the glucosylation of DIMBOA and HMBOA occurs inside the gut cells, once these aglucones diffuse through the epithelial membranes. The presence of the (2*S*)-DIMBOA-Glc and (2*S*)-HMBOA-Glc (and not the aglucones and (2*R*) epimers) in the hemolymph suggests that the glucosides produced by the insect are actively transported out of the gut cells to the hemolymph. Since no MBOA was found in the hemolymph, a similar scenario can be proposed for this aglucone, despite its high lipophilicity that potentially facilitates its diffusion across membranes. Subsequently, the glucosides present in the hemolymph are then excreted into the hindgut lumen and defecated. Accordingly, the proportions of (2*S*)-DIMBOA-Glc, (2*S*)-HMBOA-Glc, and MBOA-Glc observed in the hemolymph match the ones observed in frass samples. The time needed for BXD disappearance in the hemolymph is similar to the observed for *O. nubilalis* after topical application of ^{3}H labeled DIMBOA (Campos *et al.*, 1989).

Notably, the transport of (2*S*) BXD glucosides via hemolymph in *S. frugiperda* seems to contradict the stereospecificity of these detoxification reactions. We have previously suggested that *S. frugiperda* produces (2*S*) epimers of BXD glucosides in order to avoid reactivation of these compounds in the gut lumen, since they are inert towards plant β-glucosidases that are still present in the food bolus (Wouters *et al.*, 2014). On the other hand, the allocation of such metabolites to the hemolymph already protects them from the plant glucosidase activity located in the gut lumen. One hypothesis accounting for the development of both (2*S*) glucosylation and hemolymph transport strategies is that the final transfer of insect-derived BXD glucosides from the hemolymph into the hindgut lumen for excretion could lead to their reactivation, since this region has a near neutral pH and plant glucosidases are still active in the larval digestive tract Moreover, the specificity of the transport from gut cells to hemolymph and of the insect endogenous glucosidase activity towards both epimers of BXD glucosides are not known. Such factors could direct the evolution of the stereospecificity of BXD glucosylation reactions. It would be interesting to search for transporters involved with BXD distribution and excretion in *S. frugiperda*, and compare their evolutionary origin with those of UDP-glucosyltransferases responsible for BXD glucosylation. Feeding experiments with other BXDs and analyses of BXD concentrations in specific tissues over time should provide more detail on how these compounds are transported and excreted.

In summary, the data presented enable a better understanding of the mechanism of BXD resistance observed in *S. frugiperda*. The quantification of the different metabolic pathways in which

individual BXDs are transformed in the insect helps to clarify the processes taking place in the natural context. To date, the metabolism of BXDs in insects is not well understood, and the results observed for *S. frugiperda* represent an important advance in our knowledge of detoxification and especially excretion of plant secondary metabolites. This plays an important role in understanding the ecology of plant-herbivore interactions and the coevolution of plant defenses and insect detoxification strategies. Likewise, the mechanisms that confer resistance towards BXDs in *S. frugiperda* might be present in other species, especially agricultural pests that feed on BXD-containing plants, and their further exploration could aid in the development of novel and sustainable pest control approaches.

5. Acknowledgements:

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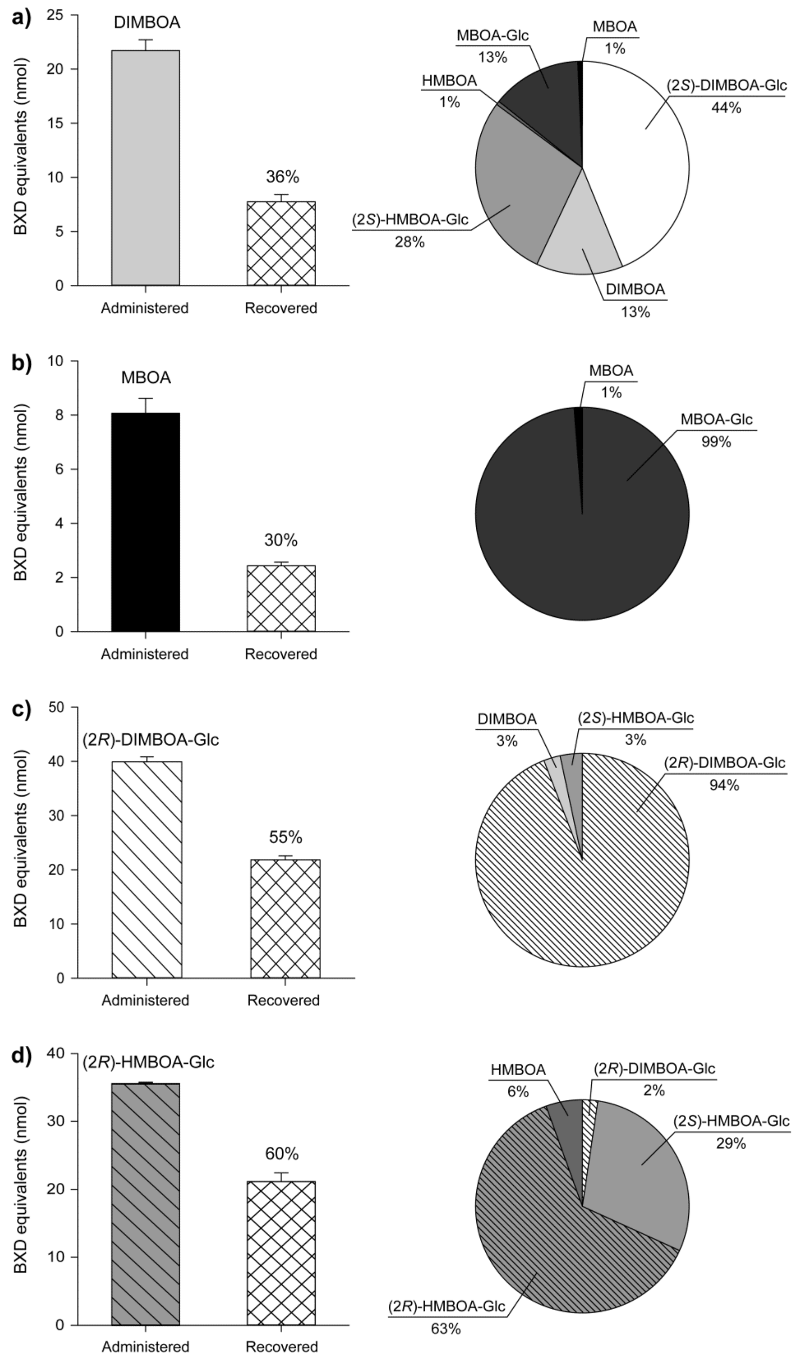


Fig. 1 Recovery rates and quantification of BXD metabolites recovered in *S. frugiperda* frass after oral administration of (a) DIMBOA, (b) MBOA, (c) (2*R*)-DIMBOA-Glc, and (d) (2*R*)-HMBOA-Glc (N=5-6, ±SEM)

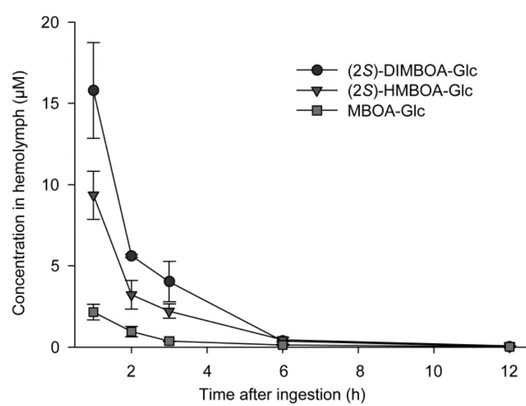
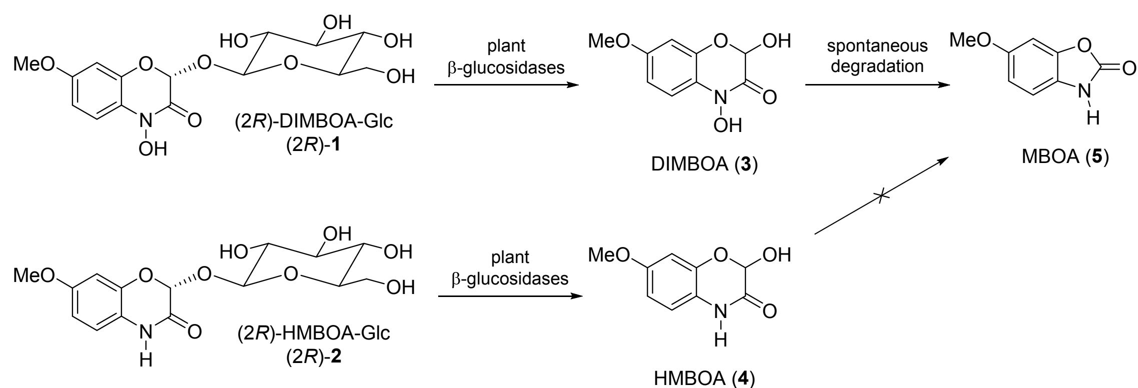
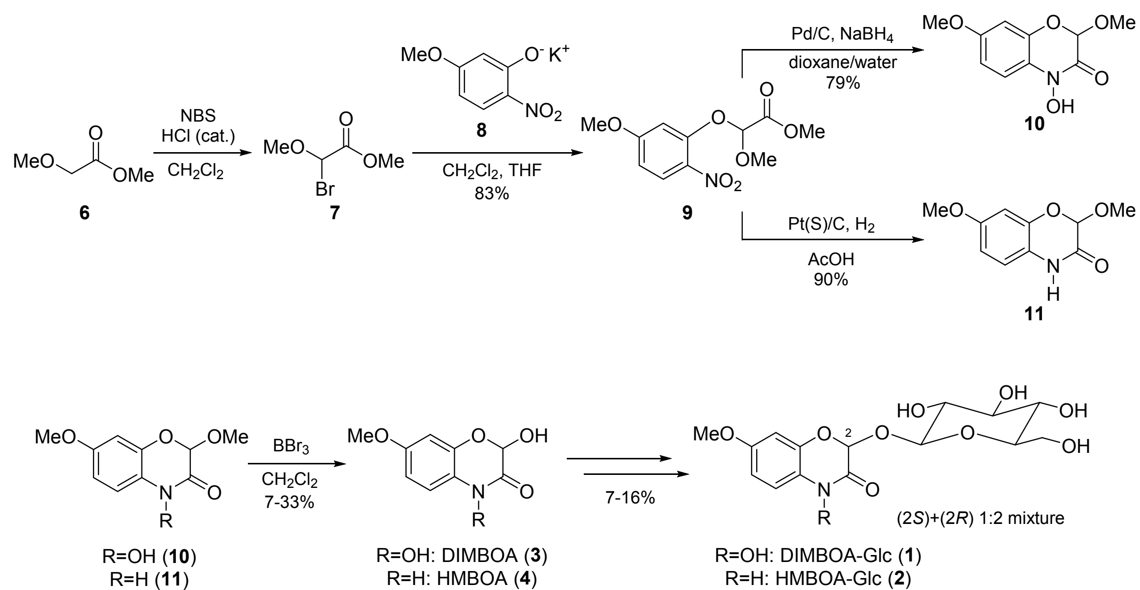


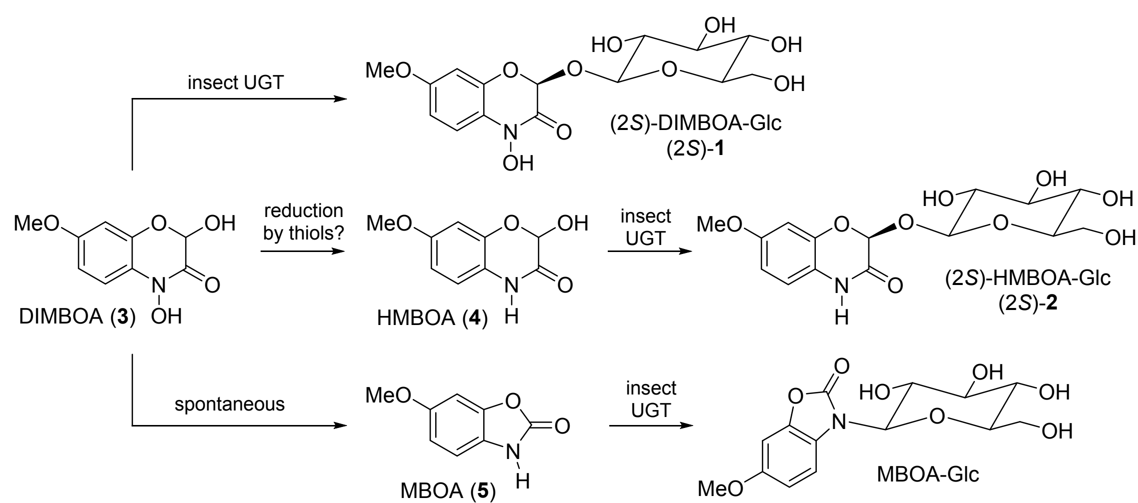
Fig. 2 Quantification of BXD glucosides in *S. frugiperda* hemolymph after oral administration of DIMBOA (N=3, ±SEM)



Scheme 1. Activation of benzoxazinoid glucosides by hydrolysis, representative for hydroxamic acids (**1**) and lactam (**2**) derivatives.



Scheme 2. Synthetic route to the benzoxazinoids DIMBOA (**3**), HMBOA (**4**), and their epimeric glucosides (**1**, **2**)



Scheme 3. Summary of metabolic fate of DIMBOA and derivatives in *S. frugiperda*.

Supplementary materials and methods

Reagents and solvents

All reagents and chemicals were purchased from Sigma Aldrich, except where indicated. Anhydrous solvents were commercially obtained and directly used. MBOA (Sigma Aldrich), methyl methoxyacetate (Alfa Aesar), 2-nitro-5-methoxyphenol (Acros Organics), UDP-glucose (Santa Cruz Biotechnology), *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)trichloroacetimidate (Merck), and acetone-d_6 (VWR) were commercially obtained. MBOA-Glc standard for quantification was kindly provided by Dr. Gaétan Glauser (University of Neuchâtel, Switzerland).

NMR analyses

NMR spectra were recorded on an Avance III HD 700 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a resonance frequency of 700.13 MHz for ^{1}H and 175.75 MHz for ^{13}C NMR measurements. The spectrometer was equipped with a 1.7 mm Bruker TCI microcryoprobe. Standard Bruker pulse sequences as implemented in TopSpin ver.3.2 were used to record NMR spectra in MeOH-d_4 at 298 K. Spectra were referenced to the residual solvent signals at δ 3.31 for ^{1}H NMR data and δ 49.15 for ^{13}C NMR data, respectively.

Synthetic procedures

Methyl-2-bromo-2-methoxyacetate (**7**): In a 100 mL round-bottom flask connected to a condenser, 495 µL (520 mg, 5 mmol) methyl methoxyacetate, 979 mg (5.5 mmol) *N*-bromosuccinimide were suspended in 15 mL dichloromethane. A droplet of concentrated hydrochloric acid was added and the system was heated to reflux by an oil bath. After 5 h of stir and reflux, the initially bright orange solution became almost colorless. At this point, the solution was cooled down and submitted to vigorous stirring and nitrogen flux in order to remove residual HBr. As the solution became cold, succinimide precipitation was observed. The solution was quickly filtered directly to the flask containing the potassium phenoxide.

Potassium 5-methoxy-2-nitrophenoxide (**8**): In a 100 mL round-bottom flask, 862 mg (5.1 mmol) 5-methoxy-2-nitrophenol were suspended in 5 mL absolute ethanol. To this suspension, 10 mL of a 0.5 M potassium hydroxide solution was added. The resulting suspension was evaporated to dryness in a rotary evaporator and directly used in the following reaction.

Methyl 2-methoxy-2-(5-methoxy-2-nitrophenoxy)acetate (**9**): To the round-bottom flask containing the potassium phenoxide, 10 mL THF were added. The dichloromethane solution from the methyl methoxyacetate bromination was filtered onto the potassium phenoxide suspension, and the succinimide precipitate was washed with cold dichloromethane. The mixture was stirred at room temperature for 18 h. The orange slurry was poured into a separatory funnel with 20 mL dichloromethane and washed three times with 10% solution of Na_2CO_3, once with water, once with brine, and dried over Na_2SO_4 and evaporated, yielding 1.127 g (83% yield) of a pale yellow oil that slowly crystallized on standing.

*4-hydroxy-2,7-dimethoxy-2*H*-1,4-benzoxazin-3(4*H*)-one* (DIMBOA methyl ether, **10**): To a 50 mL round-bottom flask, 152 mg (4.0 mmol) sodium borohydride and 15 mg palladium (5%) on activated carbon were added. Under ice bath, a few droplets of dioxane were added to the system, followed by 6mL dioxane/water 1:1 (v/v). The ice bath was exchanged for a water bath and 271 mg (1.0 mmol) of the nitro compound **9** dissolved in 2 mL dioxane were added in small portions to the $NaBH_4$/Pd-C suspension. Upon addition of the nitro compound, bubble evolution could be observed and a yellow coloration, which faded after a few seconds. The water bath was removed and the mixture was stirred at room temperature for 2h. Activated carbon was removed by filtering the mixture through filter paper and the resulting solution was acidified with HCl 1 M until foaming stopped and the pH was approximately 4, as measured by pH paper. The solution was extracted three times with ethyl acetate, and the organic phase was washed with brine, dried with Na_2SO_4, filtered, and evaporated, yielding 178 mg (79% yield of impure product) of a red oil, which was used on the following step without purification

*2,7-dimethoxy-2*H*-1,4-benzoxazin-3(4*H*)-one* (HMBOA methyl ether, **11**): In a 50 mL round-bottom flask, 135 mg (0.5 mmol) of the nitro compound **9** and 10 mg sulfide platinum (5%) on activated carbon were suspended in 3 mL acetic acid. The system was purged with nitrogen, followed by a purge with hydrogen gas. A balloon filled with hydrogen was attached to the flask and the mixture was stirred at room temperature for 48 h, with occasional purge with H_2. The activated carbon was removed by filtering the mixture through filter paper and most of the acetic acid was removed by evaporation at reduced pressure. The resulting oil was dissolved in ethyl acetate and washed twice with saturated solution of $NaHCO_3$ and once with water. The organic phase was dried with Na_2SO_4, filtered, and evaporated, yielding 95 mg (90% yield of impure product) of a brown oil, which was used on the following step without purification.

*2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one* (DIMBOA, **3**): In a 50 mL round-bottom flask, 360 mg (theor. 1.6 mmol) unpurified DIMBOA methyl ether **10** was dissolved in 5 mL dichloromethane. Under a dry ice/acetone bath (-78°C), 3.4 mL (3.4 mmol, 1 M solution in dichloromethane) boron tribromide were added. The mixture was stirred at -78°C for 30 min, and the

dry ice/acetone bath was substituted for an ice bath. The reaction was stirred for 30 min more and quenched by adding 10 mL water. After adding 5 mL ethyl acetate, the organic phase was collected and the aqueous phase was extracted 6 times with ethyl acetate. The combined organic phase was washed with brine, dried with Na_2SO_4, filtered, and evaporated, yielding 284 mg of a purple oil, which was purified by semipreparative HPLC to yield 22 mg DIMBOA in the form of a white solid (7% yield).

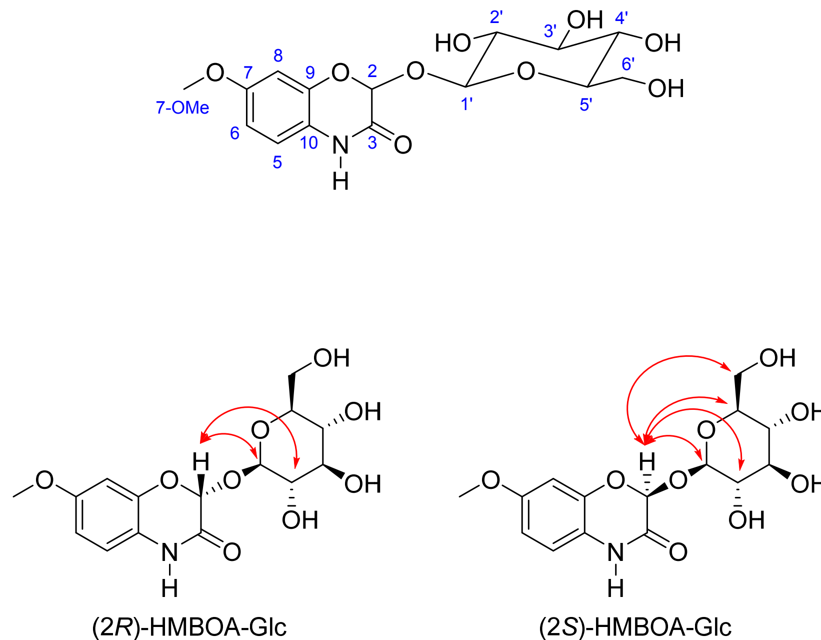
*2-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one* (HMBOA, **4**): A similar procedure as described above for DIMBOA was applied to142 mg (theor. 0.68 mmol) unpurified HMBOA methyl ether **11**, with the difference that the reaction was stirred for 30 min at -78°C and then 1 h more on an ice bath. The crude product (166 mg) was purified by semipreparative HPLC to yield 44 mg HMBOA in the form of a white solid (33% yield).

General procedure for formation of tetraacetyl glucosides: In a 25 mL round-bottom flask, 100 mg molecular sieves 3Å were activated by 5 min microwave at 600W and another 5 min at 800W (stopping every 30 s to stir and avoid overheating). After cooling down under high vacuum, the flask was kept under a nitrogen atmosphere, and 0.1 mmol aglucone (21 mg DIMBOA, **3**, or 19 mg HMBOA, **4**) and 98 mg (0.2 mmol) *O*-(2,3,4,6-tetra-*O*-acetyl-β-D glucopyranosyl)trichloroacetimidate were added and dissolved into 3 mL anhydrous dichloromethane. At room temperature, 25 µL (0.2 mmol) boron trifluoride etherate were added. The reaction was stirred under nitrogen and room temperature and the progress was followed by TLC (ethyl acetate/hexane 2:1 v:v) and HPLC-MS analyses. Another 25 µL $BF_3 \cdot Et_2O$ were added at 24 h, and other 50 mg trichloroacetimidate were added at 24 h and 48 h. The reaction was finished at 72 h by adding 3 mL water. The organic phase was collected, dried with Na_2SO_4, filtered, and evaporated, yielding the crude tetraacetyl glucoside, which was used in the following step without purification.

General procedure for deacetylation of tetraacetyl glucosides (DIMBOA-Glc, **1**, and HMBOA-Glc, **2**): The unpurified tetraacetyl glucoside was dissolved in 3 mL methanol, and 10 mg (0.2 mmol) sodium methoxide were added. The reaction was stirred at room temperature for 2 h, and neutralized by adding Amberlite IR 120 (H^+). The resin was removed by filtration through a cotton plug in a pipette, and the filtrate was evaporated to dryness to yield a mixture of (2*R*) and (2*S*) epimers of the glucosides **1** or **2**. The crude products were fractionated by HPLC and yielded 2.7 mg total DIMBOA-Glc (**1**, 7% yield) and 5.8 mg HMBOA-Glc (**2**, 16% yield).

Supplemental Table 1. ^{1}H and ^{13}C NMR chemical shifts of (2*R*)-HMBOA-Glc and (2*S*)-HMBOA-Glc in MeOD (700 MHz).

Position	(2*R*)-HMBOA-Glc ((2*R*)-**X**)		(2*S*)-HMBOA-Glc ((2*S*)-**X**)	
	δ ^{1}H (*J* in Hz)	**δ ^{13}C**	**δ ^{1}H (*J* in Hz)**	**δ ^{13}C**
2	**5.72 (s)**	96.3	**5.83 (s)**	93.0
3	-	162.2	-	162.6
5	6.84 (d, 8.6)	117.0	6.85 (d, 8.7)	117.2
6	6.60 (dd, 8.6/2.7)	109.7	6.61 (dd, 8.7/2.7)	109.7
7	-	157.9	-	157.9
8	6.72 (d, 2.7)	104.6	6.70 (d, 2.7)	104.9
9	-	142.5	-	142.4
10	-	120.0	-	120.5
7-OMe	3.72 (s)	56.1	3.76 (s)	55.9
1‘	**4.68 (d, 7.9)**	103.8	**4.74 (d, 7.9)**	99.8
2‘	3.20 (dd, 9.2/7.9)	74.6	3.12 (dd, 9.2/7.9)	74.3
3‘	3.37 (dd, 9.2/8.5)	77.7	3.36 (dd, 9.2/9.2)	77.9
4‘	3.30 (dd, 8.5/9.5)	70.8	3.28 (dd, 9.5/9.2)	71.3
5‘	3.34 (ddd, 9.5/5.2/2.2)	78.2	3.37 (ddd, 9.5/6.1/2.4)	78.1
6‘a	3.87 (dd, 11.9/2.2)	62.2	3.68 (dd, 12.1/2.4)	62.6
6‘b	3.69 (dd, 11.9/5.2)		3.69 (dd, 12.1/6.1)	



Supplemental Scheme 1. NOE correlations observed for (2*R*)-HMBOA-Glc and (2*S*)-HMBOA-Glc.

4. Discussion

Insect herbivores have developed diverse strategies in order to avoid the detrimental effects of plant defensive secondary metabolites. In this context, the aim of my dissertation was to explore how lepidopteran herbivores metabolize and detoxify BXDs present in maize. To do so, I focused on the detoxification of DIMBOA, the most abundant BXD in maize aerial parts, by larvae of *S. frugiperda*, which is an economically important maize pest with remarkable resistance to BXDs. In **manuscript III**, I described how *S. frugiperda*, *S. littoralis*, and *S. exigua* are able to stabilize and deactivate the toxic compound DIMBOA through reglucosylation, forming (2*S*)-DIMBOA-Glc. In **manuscript IV**, I searched for the genes encoding enzymes responsible for BXD glucosylation in *S. frugiperda*. In **manuscript V**, I performed quantitative analyses of the overall metabolism of several BXDs, assessing the biological relevance of individual detoxification pathways. In order to contribute to the theoretical framework of BXD research, I summarized the knowledge about the chemistry of BXDs and how they relate to their modes of action in **manuscript I**. And to facilitate a comparative assessment of insect resistance towards BXDs, I reviewed the literature on the biological effects of BXDs on insect herbivores from different guilds, as well as the current knowledge about the metabolism of BXDs in insects in **manuscript II**.

4.1. BXD glucosylation across lepidopteran species: evolution and biological relevance

The detoxification of DIMBOA by (re)glucosylation was described in *S. frugiperda*, *S. littoralis* and *S. exigua*, but it does not seem to occur in *Mamestra brassicae* and *Helicoverpa armigera* (**Manuscript III**), being a pathway restricted to the genus *Spodoptera*. Thus, the glucosylation of DIMBOA can be considered a specialized strategy since it is not widespread in lepidopterans, and is dedicated to chemical defenses that are relatively restricted to Poaceae and a few dicot plant species. Surprisingly, however, this detoxification reaction is also performed by *S. littoralis* and *S. exigua*, which are highly polyphagous and considered generalist herbivores (Lee *et al.*, 2002; Merkx-Jacques *et al.*, 2008). In contrast, despite its broad host range of more than 80 plant species from different families, *S. frugiperda* displays a high preference for grasses (Hardke *et al.*, 2015). Accordingly, although all three *Spodoptera* species are relatively tolerant to BXDs, *S. frugiperda* seems to be more resistant to their detrimental effects when compared to *S. littoralis* and *S. exigua* (Glauser *et al.*, 2011; Rostás, 2006). This quantitative difference also agrees with the fact that *S. frugiperda* uses BXDs as foraging cues, stimulating feeding, while for *S. littoralis* and *S. exigua*, these same compounds have antifeedant effects (Kohler *et al.*, 2015; Rostás, 2006). Based on these observations, it can be speculated that the ability to glucosylate DIMBOA (and other benzoxazinones) by *Spodoptera* spp. was not developed as a specific adaptation to feed on BXD-containing plants, but likely contributed to the expansion of the host range towards grasses in *S. littoralis* and *S. exigua*, and even to the preference of *S. frugiperda* for this plant family.

The glucosylation of MBOA, on the other hand, has been described not only in *S. frugiperda* and *S. littoralis*, but also in the BXD-susceptible species *O. nubilalis* (Maag *et al.*, 2014), besides being detected in *S. exigua*, *M. brassicae*, and *H. armigera* (data not shown). As opposed to its DIMBOA equivalent, the glucosylation of MBOA seems to be widespread in lepidopteran species, regardless of their specialist/generalist status and their preference for BXD-containing plants. Therefore, MBOA glucosylation is not likely to have evolved in response to the ingestion of benzoxazolinones by insect herbivores. However, as with DIMBOA glucosylation, quantitative differences of MBOA glucosylation capacity between species are also observed. As discussed in **manuscript II**, the specific MBOA-UGT activity in gut tissue is around 10-fold higher in *S. frugiperda* than in *O. nubilalis* (both univoltine and bivoltine strains). These differences in enzymatic activity were also reflected in the amounts of MBOA-Glc excreted after feeding on a MBOA-containing artificial diet (Maag *et al.*, 2014). This suggests that the glucosylation of MBOA, though not so important as the glucosylation of its DIMBOA equivalent, might also have contributed to the high resistance and even preference of *S. frugiperda* towards BXD-containing plants.

As presented in **manuscript IV**, the profiles of enzymatic activity responsible for the glucosylation of DIMBOA and MBOA in different caterpillar tissues indicate that at least two different enzymes are involved in such metabolic pathways, namely DIMBOA-UGT and MBOA-UGT. Moreover, the DIMBOA-UGT activity is more abundant in gut tissues, while MBOA-UGT activity is highest in fat bodies. The enzymes responsible for the detoxification of ingested plant chemical defenses are expected to be excreted in the gut lumen or highly expressed in the gut tissue, to rapidly deactivate plant toxins before they penetrate further into the organism. This might explain the preferential localization of DIMBOA-UGT activity, but it does not apply to MBOA-UGT activity. It is not clear whether the higher activity of MBOA glucosylation in fat bodies supports the hypothesis that the corresponding enzyme(s) might have other primary functions. Alternatively, the highest MBOA-UGT activity being in fat bodies might be simply related to the high lipophilicity of MBOA when compared to that of DIMBOA (discussed in more detail in the next section). These strategies might represent specific adaptations of *S. frugiperda* in order to deploy MBOA-UGT activity to maximum advantage. It would be interesting, for instance, to investigate whether this differential distribution of MBOA-UGT activity is found in other lepidopteran species as well.

In the corn-strain of *S. frugiperda*, DIMBOA- and MBOA-UGT gene expression and protein activities do not seem to be highly and specifically induced by BXD feeding, as discussed in **manuscript V**. This is consistent with the fact that this strain prefers to feed on BXD-rich maize plants, and therefore constitutively require BXD detoxification enzymes. The detoxifying capabilities of the rice-strain of *S. frugiperda* have not been assessed in detail, but are expected to be similar to the corn-strain, since the less closely related species of *S. littoralis* and *S. exigua* remain capable of such detoxifying reactions. However, because the rice-strain does not encounter BXDs as often as the corn-strain, the rice-strain of *S. frugiperda* might possess a more inducible pattern of BXD-UGT activities when compared to the strong constitutive pattern found in the corn-strain.

The molecular aspects of the detoxification of BXDs in lepidopteran species are essential to understand the evolution of such metabolic pathways. The sequence data from *S. frugiperda* UGTs potentially involved in these reactions, as covered in **manuscript IV**, greatly contribute to further research on their origin and regulation. It would be interesting to search for the presence of homologs of *S. frugiperda* UGTs with activity towards BXDs in other lepidopteran species displaying various degrees of tolerance to BXDs. This would shed light on the evolutionary history of such genes and their encoded enzymes, and whether the genetic variation of BXD-UGTs contributes to the host plant preferences of a larger group of herbivores. Moreover, the expression and functional screening of lepidopteran UGTs would also give hints on their substrate specificity and possible original functions other than detoxification. Taking into account the occurence of DIMBOA- and MBOA-UGTs across different herbivore species, the corresponding sequences seem to be derived from different evolutionary periods. The sequences of DIMBOA-UGTs are more recent, and apparently restricted in occurrence to the radiation of the *Spodoptera* genus (Noctuidae) and possibly close sister groups; with the exception of *H. armigera* and *M. brassicae*, Noctuidae members who do not have DIMBOA-UGTs (Mitchell *et al.*, 2005 and **manuscript III**). In contrast, MBOA-UGTs seem to have a more ancestral origin, and are conserved in species as distant as *O. nubilalis* (Crambidae) – the common ancestor of Crambidae and Noctuidae dates from more than 100 million years ago (Wahlberg *et al.*, 2013). Moreover, as demonstrated in **manuscript IV**, the relevant UGTs identified to have activity towards DIMBOA and MBOA in *S. frugiperda* are grouped in distinct families, further indicating their distant relationship: DIMBOA-UGT sequences are present in family UGT33, and MBOA-UGT sequences, in families UGT40, UGT42, and UGT46. Apart from analyzing BXD-UGT activity in other lepidopteran species according to their host plant preferences, the investigation of the actual enzymatic activities in other *Spodoptera* spp., as well as sister groups, could support the more recent status of DIMBOA glucosylation. Likewise, the presence of MBOA glucosylation activity in more distantly related lepidopterans would validate its more ancestral status suggested by the phylogenetic tree. Naturally, there is the possibility of independent evolution of UGTs in BXD resistance across Lepidoptera since scattered species from other genera, such as *Ostrinia furnacalis* (Crambiade) and *Mythimna separata* (Noctuidae), are also resistant to BXDs (Kojima *et al.*, 2010; Phuong *et al.*, 2015; Sasai *et al.*, 2009). In **manuscript IV**, I present not only the gene and protein sequences, but also the associated BXD glucosylation activity of *S. frugiperda*. Looking for similarities in other lepidopterans, as well as in other families, as mentioned above, would explore how these adaptations have been conserved or modified over evolutionary time.

The glucosylation of HMBOA, the lactam equivalent of DIMBOA, was also described in **manuscript V**. Notably, the most promising UGT candidate responsible for DIMBOA glucosylation in *S. frugiperda* (SfUGT6, **manuscript IV**) also possessed activity towards HMBOA, yielding (2*S*)-HMBOA-Glc, the same epimer observed *in vivo*. Due to their similar chemical structures, DIMBOA and HMBOA are likely glucosylated by the same enzyme, and both reactions are quantitatively relevant in the metabolism of BXDs, as shown in **manuscript V**. Other BXD aglucones structurally similar to DIMBOA and HMBOA might also be glucosylated in a similar fashion, likely by the same or a closely related enzyme. Furthermore, *S. frugiperda*, *S. littoralis* and *S. exigua* are resistant to many

insecticides (Hardke *et al.*, 2015), and are also able to feed on a wide range of host plants, some of which accumulate other classes of defensive compounds. These herbivore traits might be related to the UGT activities found in the larvae and other potential detoxification pathways. Future screening experiments to define the substrate specificity of *S. frugiperda* UGTs including a broad range of plant defenses can help to determine their relevance in the metabolism of other xenobiotics.

4.2. The physiology of BXD metabolism in *S. frugiperda*

As a complementary investigation to the results presented in **manuscript III**, in **manuscript V** I explored the relevance of BXD glucosylation in *S. frugiperda* by studying the *in vivo* metabolism and toxicokinetics of DIMBOA. The glucosides (2S)-DIMBOA.Glc, (2S)-HMBOA-Glc and MBOA-Glc represent most of the BXD derivatives recovered from the frass, indicating that such detoxification pathways are quantitatively significant to the overall metabolism of DIMBOA.

The enzymatic activities associated with the glucosylation of DIMBOA and MBOA in *S. frugiperda* show optimal pH values near neutrality (**manuscript III** and Maag *et al.*, 2014), which suggest that these reactions take place inside the gut cells, rather than in the highly alkaline gut lumen. The highly lipophilic DIMBOA and MBOA aglucones likely diffused through the membranes of the gut cells, where these compounds possibly exert toxicity. The glucosylation activity of DIMBOA (and HMBOA) is expected to be relevant within this context, since the highest DIMBOA-UGT specific activity was detected in the gut tissues (**manuscript V**). On the other hand, the highest MBOA-UGT activity is found in fat bodies of *S. frugiperda*. One possible scenario is that the MBOA-UGT activity in gut tissues, despite not being the highest, is sufficient to convert all the MBOA locally available (both from diffusion into the cell and from the degradation of DIMBOA). In this case, the high MBOA-UGT activity found in fat bodies would likely mediate other physiological functions other than the detoxification of MBOA. In a second scenario, the highly lipophilic MBOA diffuses through gut cell membranes and accumulates in the lipophilic fat bodies faster than DIMBOA, where it is efficiently glucosylated. In this case, the differential tissue-specificity of MBOA-UGTs could be considered an adaptation in order to maximize the detoxification of MBOA. Almost no MBOA aglucone is excreted in the frass probably due to its lipophilic character, which supports its rapid and thorough absorption by *S. frugiperda* tissues. Moreover, after DIMBOA administration, only MBOA-Glc is detected in the hemolymph, and no MBOA aglucone. This means that either (i) MBOA is completely glucosylated inside the gut cells before reaching the hemolymph, or that (ii) MBOA is not available in the hemolymph, probably due to its poor hydrophilicity.

As discussed in **manuscript V**, the transport of insect-derived BXD glucosides to the hemolymph might represent a strategy to keep these metabolites separated from plant BXD β-glucosidases, so as to avoid their reactivation by hydrolysis. The hemolymph fate of BXD glucosides seems to complement the stereochemical control of the glucosylation reactions of DIMBOA and HMBOA. The products formed by the insect-derived BXD glucosides are (2*S*) epimers, which are not recognized and hydrolyzed by plant glucosidases present in the lumen. The stereochemical

configuration of the products determined by the insect's detoxifying glucosidases is likely more important when the (2*S*) glucosides are delivered back into the hindgut lumen in order to be excreted. As demonstrated in **manuscript III**, the hindgut lumen has a pH close to neutrality, and the BXD glucosidases from the digested plant material are still active. However, the (2*S*) configuration prevents a reactivation of those BXD glucosides back to toxic aglucones, since plant glucosidases can only act on (2*R*) glucosides. As another possibility, the transport of insect-derived BXD glucosides out of the gut cells might release them partially back into the gut lumen, in which case it would also be advantageous that (2*S*) glucosides (non-hydrolyzable by plant glucosidases) are formed. The identification of transporters of insect-derived BXD glucosides could tell us about their selectivity and evolutionary history, which could be in turn associated with the insect UGT enzymes responsible for BXD glucosylation.

The reduction of the hydroxamic acid DIMBOA to the lactam HMBOA has also been reported in **manuscript V**. This reaction has been suggested to happen in *S. frugiperda* (Glauser *et al.*, 2011), and has been observed *in vitro* by incubating DIMBOA with thiols (Atkinson *et al.*, 1991; Niemeyer *et al.*, 1982). It is not yet clear whether this reduction is a spontaneous reaction of DIMBOA with biological thiols, or whether it is assisted by *S. frugiperda* enzymes; nor if it takes place in the gut lumen, or inside the gut cells. The reduction of DIMBOA to HMBOA can, nevertheless, represent a detoxification strategy, since BXD lactams are considered less toxic to insects (Escobar *et al.*, 1999). It would be interesting to investigate if other species also possess this metabolic pathway, and whether the lack of DIMBOA to HMBOA conversion imposes fitness costs.

Another important observation from **manuscript V** is the capacity of *S. frugiperda* to hydrolyze (2*R*)-DIMBOA-Glc and (2*R*)-HMBOA-Glc. Since the droplet feeding assays were followed by feeding on artificial diets, and no plant material was ingested, the BXD glucosidase activity observed is solely due to insect enzymes. Counterintuitively, such insect endogenous hydrolytic activities would enhance the toxicity of plant (2*R*) BXD glucosides, accelerating their activation; however more studies are required in order to verify the biological relevance of such traits from the insect's perspective. For instance, it is not known if the endogenous insect BXD glucosidase activity is present in the gut lumen or elsewhere. The administered BXD glucosides are highly hydrophilic and are not likely to diffuse into the gut cells without the involvement of membrane transporters, and insects are expected to possess glucosidases in the gut lumen, where they have digestive functions (Terra and Ferreira, 2012). These observations support the BXD glucoside hydrolysis in the gut lumen, but more evidence is needed in order to confirm the localization of endogenous BXD glucosidase activity in the insect. The screening of *S. frugiperda* digestive β-glucosidases (Marana *et al.*, 2000) for activity towards (2*R*) and (2*S*) epimers of different BXD glucosides would reveal more about the biological relevance of these reactions. In a more realistic scenario, the endogenous hydrolysis of BXD glucosides by the insect is likely outweighed by the hydrolysis via plant glucosidases, thus not representing a relevant detrimental effect to the insect. Alternatively, upon feeding on BXD-containing plants, *S. frugiperda* might down-regulate the expression of digestive β-glucosidases with activity towards BXD glucosides, similarly to what has been observed in other insect herbivores feeding on defenses activated by glucosidases (Pentzold *et al.*, 2014). The data

also suggest that (2*R*)-HMBOA-Glc is more easily hydrolyzed by the insect than (2*R*)-DIMBOA-Glc. This implies that the diversity of BXD structures produced by the plant might originate from their differential suitability as substrates to the insect enzymes, and therefore be evolutionary retained. Even though HMBOA is not as toxic as DIMBOA, the former could be a more effective defense when both are deployed as glucosides in the absence of plant BXD glucosidases. This might not be biologically relevant to *S. frugiperda*, but the use of more labile BXD glucosides can have an effect on other insect herbivores, such as aphids, whose feeding behavior avoids the ingestion of plant BXD β-glucosidases (**manuscript II**). Moreover, the possibility of BXD glucosidase activity associated with BXD sequestration does not seem probable for *S. frugiperda*, since the reglucosylated BXD products are readily excreted.

An interesting point raised in the discussion of **manuscript V** is the possibility of the utilization of BXDs as nutrients by *S. frugiperda* larvae. The partial recovery rates of 30-60% of BXD metabolites in the frass can indicate that *S. frugiperda* absorbs some of the ingested BXDs. The effects of MBOA on the consumption and utilization of food by *S. frugiperda*, presented in **manuscript II**, suggest that this compound enhances the efficiency of conversion of digested food into larval biomass (ECD). This is consistent with a scenario in which MBOA is used as a nutrient, possibly due to its nitrogen content, which is an important nutritional requirement for insects. Accordingly, MBOA-Glc has been shown to improve growth in *O. nubilalis* (Maag *et al.*, 2014), and this could be indirectly linked to the observations that low concentrations of DIMBOA improve growth in *S. frugiperda* (Glauser *et al.*, 2011). This would agree with the feeding stimulant effects that BXDs have on *S. frugiperda*, meaning that this herbivore would benefit from a certain level of BXD in the diet (Kohler *et al.*, 2015; Rostás, 2006). However, further experiments are needed in order to determine if and how BXDs can be used as nutrients by insect herbivores. The low recoveries of BXD metabolites in frass only refer to the known insect-derived glucosides targeted in our analysis, and unknown metabolites might have been disregarded. It is also possible that a fraction of the reactive BXDs administered or formed in the digestive tract reacted with other components of the diet or insect targets, fulfilling their role as digestibility-reducer or toxic compounds. Such other metabolic fates of BXDs could be traced by feeding experiments using radioactive isotopically labeled BXDs followed by scintillation analyses of frass and insect tissues in order to account for the overall distribution of the radioactive tracer.

In summary, the absorption, distribution, metabolism and excretion of BXDs in *S. frugiperda* seem to involve several distinct events. After ingestion, plant (2*R*) BXD glucosides are slowly hydrolyzed along the larval digestive tract by plant β-glucosidases, which are resistant to the digestion process and are still active even in the frass. Free BXD aglucones in the gut lumen potentially undergo non-specific reactions with diet components, decreasing food nutritional value, or reactions with insect digestive exoenzymes, impairing digestibility. At the same time, free BXD aglucones (hydroxamic acids and *N*-*O*-methyl derivatives) in the gut lumen are also prone to degradation into benzoxazolinones, whose rate of formation is greatly enhanced by high pH values. In this context, the alkaline foregut and midgut of *S. frugiperda* (pH around 9.0) are responsible for not only decreasing the activity of plant BXD glucosidases, but also possibly accelerating their degradation to less toxic benzoxazolinones, thus representing a **first line of defense** towards the BXD defense system.

Lipophilic BXD aglucones (both benzoxazinones and benzoxazolinones) in the gut lumen then diffuse through cell membranes into the gut cells, where they can exert their toxicity by reacting with intracellular enzymes and damaging organelles. Either in the gut lumen or inside the gut cells, reactive BXD aglucones can be converted to less toxic equivalents, as is the case for the reduction of the hydroxamic acid DIMBOA to the lactam HMBOA. Such transformations minimize BXD toxicity and represent a **second line of defense** to their detrimental effects. Inside the gut cells, BXD aglucones can be glucosylated to their (2*S*) glucosides by specific *S. frugiperda* UGTs, probably located in the endoplasmic reticulum. After glucosylation, these metabolites are no longer able to exert toxicity and are stable towards hydrolysis, even if they encounter plant BXD glucosidases. Therefore, BXD glucosylation constitutes a **third line of defense** towards BXDs. The resulting (2*S*) BXD glucosides, along with MBOA-Glc, can then be transported out of the gut cells into the hemolymph. From the hemolymph, BXD glucosides and intermediates are transported and delivered back into the hindgut lumen, allowing their excretion in the frass. During the retention of (2*S*) BXD glucosides in the hindgut until their excretion, plant glucosidases still present and active in the digested plant material are not able to hydrolyze and reactivate such detoxification end products. Therefore, the (2*S*) stereochemical control of the detoxification reaction is critical in order to effectively deactivate and stabilize the toxic aglucones. Other mechanisms and pathways likely take part into this scenario, and further studies on BXD toxicokinetics should provide insights into their importance and interrelationships. Since free DIMBOA aglucone is still detected at considerable amounts in the frass, the diffusion into gut cells is not completely efficient. In this context, *S. frugiperda* is anyway protected from DIMBOA toxicity just by having glucosylation activities that match the amount of DIMBOA aglucone that successfully enters the gut cells. Hydroxamic acid and lactam BXDs seems to be successfully detoxified by glucosylation, but *N*-*O*-derivatives, like HDMBOA, may be too unstable to be detoxified by UGTs. In addition, HDMBOA is much less stable than DIMBOA in terms of degradation to MBOA, and the alkaline gut of *S. frugiperda* would play a more central role in accelerating its conversion to a less toxic metabolite. However, this does not seem to be sufficient to reduce toxicity *in vivo*, given that the toxic and antifeedant effects of HDMBOA are effective even against the well-adapted *S. frugiperda* (Glauser *et al.*, 2011).

4.3. Future directions for BXD metabolism research

The retrieval of molecular data retrieved from *S. frugiperda* (**manuscript IV**) is a key step towards a more detailed understanding of the regulation and tissue localization of BXD-UGT activities in larvae. The expression profiles of individual UGT candidates can be explored in different larval stages and treatments by qPCR experiments. Another approach would involve the specific silencing of individual UGT candidates in *S. frugiperda* by RNA interference techniques. Such experiments would provide stronger evidence supporting the involvement of individual UGTs in BXD glucosylation reactions, and ultimately test the impact of such reactions on insect performance when reared on BXD-rich *versus* BXD-poor diet.

The controlled feeding assays described in this thesis (**manuscript V**) represent the first attempt to quantitatively investigate the overall metabolism and toxicokinetics of BXDs by *S. frugiperda*. Based on BXD presence observed in frass and hemolymph, additional experiments can now be performed in order to detect further enzymatic activities associated with BXD detoxification. For instance, the reduction of DIMBOA to HMBOA can be mediated by specific enzymes, or simply by non-specific and spontaneous reactions with biological thiols. Careful dissection and analyses of larval tissues can be used to assess the levels of enzymatic activity and their associated metabolites over time, and under different diets. Injections of BXDs directly into *S. frugiperda* hemolymph or topical applications might provide further insights into metabolite transport and allocation in the insect. Furthermore, feeding assays with radioactive isotopically-labeled BXDs represent a sophisticated tool to better determine the fates, modes of action and overall role of individual BXDs, whether they react non-specifically to proteins or are used by the insect as a nutrient, and would enable us to identify mechanisms not considered in this thesis.

Although many studies have explored potential mechanisms by which BXDs exert their biological effects, their modes of action are not well defined and might change according to the target organism (**manuscript I**). Furthermore, many individual aspects of plant-insect interactions, such as herbivore feeding behavior, seem to influence BXD activities (**manuscript II**). Taking into account the chemistry and differential biological effects of BXDs covered in **manuscript I** and **manuscript II**, robust experiments can be designed in order to further clarify the chemical and physiological modes of actions associated with BXDs in insects. By using diverse synthetic BXD analogs in feeding assays, one can explore structure-activity relationships and evaluate the contribution of individual structural features that modulate BXD toxicity, digestibility-reduction, and antifeedant effects. Associated with such experiments, the investigation of how different BXDs affect consumption and utilization of food would expand our knowledge about the physiological modifications underlying their general observed effects on growth and performance.

From a technological perspective, a better understanding on how insect herbivores absorb, distribute, metabolize, and excrete BXDs provides promising targets for innovative pest control approaches. Insect UGTs are responsible for BXD detoxification, and could be down-regulated by RNA interference technologies or selective inhibitors, which could be delivered either directly or via the plant to insects. Further investigation about transporters involved in BXD allocation and excretion in insects could also reveal targets to reduce insect BXD resistance. Alternatively, plant breeding programs could benefit from knowing the differential biological activities of BXDs and their modes of action depending on the insect target, focusing on BXD traits that are more selective and efficient to the system of interest. A deeper knowledge about the modes of action and metabolism of BXDs also brings the possibility to search for novel rationally designed synthetic BXD structures able to circumvent existing insect detoxification strategies.

4.4. Conclusions

Insects employ diverse strategies to avoid the detrimental effects of plant defenses, including metabolic detoxification pathways. BXDs play an important role in the chemical defense of various grasses, including maize, but some insect species have developed adaptations that allow them to successfully feed on BXD-containing plants. Despite the importance of BXDs in the interaction between economically relevant crop plants and insect pests, the biochemical and molecular bases of herbivore resistance to BXDs has remained largely unexplored. In this work, I used a combination of chemical, genetic and physiological perspectives in order to better understand how *S. frugiperda* copes with the biological effects of maize BXDs. I described the importance of glucosylation reactions for the detoxification of BXDs and their rigorous control over the stereochemistry of the resulting metabolites, and identified potential UGT enzymes associated to these pathways. I also explored the intricate network of biochemical reactions and dynamic allocation of metabolites that *S. frugiperda* uses to metabolize BXDs. These results demonstrate that insect herbivores rely on fine-tuned and sophisticated strategies to avoid BXD toxicity.

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5. Summary

In order to fend off insect herbivore attack, plants have evolved a diverse arsenal of mechanisms, including defensive secondary (or specialized) metabolites. In response, insects must cope with such toxic chemicals if they are to use well-defended plants as a food source. To do this, insects have developed various strategies to counteract the toxic, digestibility-reducing, and antifeedant activities of these plant allelochemicals. In this context, the metabolism and detoxification of plant chemicals represent a major adaptation in insect herbivores, and greatly contributes to their resistance towards specific classes of plant defenses.

Benzoxazinoids (BXDs) represent one such class of plant defensive secondary metabolites, including benzoxazinone compounds containing the 2-hydroxy-*2H*-1,4-benzoxazin-3(*4H*)-one skeleton and derivatives such as benzoxazolinones. These compounds are derived from indole and are widespread in grasses (Poaceae), being produced by important crops such as maize, wheat and rye, as well as by few dicot species. BXDs are two-component defenses (or phytoanticipins) that are stored as stable glucosides in the plant cell, spatially separated from their activating β-glucosidases. Upon cell damage, for example by herbivore attack, these glucosides are hydrolyzed to toxic aglucones, which are highly reactive electrophilic species capable of reacting unspecifically with biological nucleophiles such as thiol, hydroxy, and amino groups in protein amino acid residues. Some insects, however, show remarkable resistance towards the detrimental effects of BXDs and can successfully use BXD-containing plants as food, sometimes becoming economically relevant agricultural pests. One such example is the fall armyworm *Spodoptera frugiperda*, but many other insect herbivores also display different tolerance levels to BXDs. The aim of my dissertation is to investigate the metabolism and detoxification mechanisms employed by lepidopteran herbivores in order to cope with BXDs present in maize.

As a first exploration of the BXD detoxification pathways across several lepidopteran species, I analyzed the BXD profiles of larval frass after feeding on maize leaves. I found that reglucosylation of DIMBOA is a strategy employed by *S. frugiperda*, *S. littoralis* and *S. exigua*, but not *Mamestra brassicae* and *Helicoverpa armigera*. I detected the UDP-glucosyltransferase (UGT) activity responsible for this reaction in gut tissues of *Spodoptera* spp. and characterized the product as (2*S*)-DIMBOA-Glc, which is an epimer of the glucoside produced by the plant, (2*R*)-DIMBOA-Glc. I further demonstrated that the plant β-glucosidases are unable to hydrolyze the insect-derived (2*S*) product due to its differential stereochemistry. Therefore, I described the reglucosylation of DIMBOA with overall inversion of stereochemical configuration as a detoxification reaction that efficiently stabilizes the toxic aglucone DIMBOA and prevents its further activation in the larval digestive tract.

I further analyzed the molecular basis of BXD detoxification reactions by looking for putative UGT-encoding genes in the transcriptome of *S. frugiperda* and functionally screening them for activity towards DIMBOA and MBOA. I successfully identified 25 UGT candidates and heterologously expressed them in *Trichoplusia ni* cells. From these candidates, I identified 5 *S. frugiperda* UGTs that

are able to glucosylate DIMBOA and/or MBOA. Only one candidate is able to glucosylate DIMBOA to (2*S*)-DIMBOA-Glc, and I suggest that this is the enzyme responsible for the detoxification reaction observed *in vivo*, together with other 3 UGTs that are potentially implicated in the formation of MBOA-Glc. I also analyzed the DIMBOA and MBOA-UGT specific activities in *S. frugiperda* neonates and several tissues of mature larvae. These are not strongly induced upon BXD feeding and are the highest in gut tissue and fat bodies, respectively.

Lastly, in order to calculate the relative importance of each detoxification route in the overall metabolism of BXDs and to investigate their toxicodynamics, I performed controlled feeding assays with *S. frugiperda* larvae using individual BXDs and quantified the metabolites recovered in the frass. I assessed the proportions of final metabolites relative to the ingested amounts of DIMBOA, MBOA, (2*R*)-DIMBOA-Glc, and (2*R*)-HMBOA-Glc. I first detected another glucosylation reaction in *S. frugiperda*, the transformation of the BXD lactam HMBOA into (2*S*)-HMBOA-Glc, and characterized the product by chemical synthesis and NMR analyses. I showed that DIMBOA metabolism consists mainly in its partial reduction to the lactam HMBOA and degradation to MBOA, followed by glucosylation reactions yielding (2*S*)-DIMBOA-Glc, (2*S*)-HMBOA-Glc, and MBOA-Glc. Moreover, I found that shortly after oral administration of DIMBOA, these 3 glucosides are present in the hemolymph, suggesting an excretion mechanism that involves this transport strategy. I further demonstrated that MBOA is mainly excreted as MBOA-Glc, and the glucosides (2*R*)-DIMBOA-Glc and (2*R*)-HMBOA-Glc are partially hydrolyzed by *S. frugiperda*. Overall, I obtained recovery rates of BXD metabolites in the frass between 30 and 60%, indicating that a considerable fraction of the administered BXDs reacts with components of the diet, is absorbed by the insect as nutrients, and/or is transformed to yet unknown metabolites.

In summary, through the combined use of analytical, organic synthesis, molecular biology, and biochemical techniques, I explored the metabolism and detoxification of maize BXDs by *S. frugiperda* and other lepidopteran herbivores. I identified glucosylation reactions promoted by UGTs as important detoxification pathways and explored them at the chemical and molecular level. The results contribute to our general understanding of BXD metabolism by insects since the strategies described in this dissertation might also be employed by other species. Furthermore, I provide data that allows for the further exploration of the distribution, evolution, and regulation of enzymes responsible for BXD glucosylation, as well as deeper investigations on the modes of action associated with BXDs. From a more general perspective, the findings presented in this dissertation contribute to our knowledge on how insects cope with plant secondary defensive metabolites and how both partners might have influenced the evolution of each other. The results also contribute to the further exploitation of BXDs for control of agricultural pests by stimulating efforts to minimize detoxification processes.

6. Zusammenfassung

Um Befall durch herbivore Insekten abzuwehren, haben Pflanzen ein diverses Arsenal von Verteidigungsmechanismen entwickelt, zum Beispiel die Synthese von sekundären (oder spezialisierten) Metaboliten. Insekten hingegen müssen solche giftigen Chemikalien bewältigen, wenn sie gut verteidigte Pflanzen als Nahrungsquelle verwenden wollen. Dazu haben Insekten verschiedene Strategien entwickelt, um den toxischen und fressabwehrenden Aktivitäten der pflanzlichen Allelochemikalien entgegenzuwirken. In diesem Zusammenhang stellt der Metabolismus und Entgiftung von Pflanzenchemikalien eine wichtige Anpassung von herbivoren Insekten dar und trägt in hohem Maße zu ihrer Resistenz gegenüber spezifischen Klassen von pflanzlichen Verteidigungsmetaboliten dar.

Benzoxazinoide (BXDs) sind eine solche Klasse von Pflanzensekundärmetaboliten, welche Benoxazionone mit der 2-Hydroxy-*2H*-1,4-benzoxazin-3(*4H*)-on-Grundstruktur und deren Derivate wie Benzoxazolinon beinhaltet. Diese Verbindungen leiten sich von Indol ab und sind weit verbreitet in der Pflanzenfamilie der Süßgräser (Poaceae), zu welcher landschaftlich wichtige Kulturpflanzen wie Mais, Weizen und Roggen, sowie einige zweikeimblättrige Arten gehören. BXDs bilden eine Zwei-Komponenten-Abwehr, die als stabile Glucoside in der Pflanzenzelle räumlich getrennt von ihren aktivierenden β-Glucosidasen gespeichert sind. Bei Beschädigung der Pflanzenzelle, beispielsweise durch Pflanzenfresser, werden die Glucoside zu toxischen Aglyconen hydrolysiert. Aglycone sind eine hoch reaktive elektrophile Spezies und können unspezifisch mit biologischen Nukleophilen wie Thiol-, Hydroxy- und Amino-Gruppen der Aminosäuren in Proteinen reagieren. Einige Insekten zeigen jedoch bemerkenswerte Resistenz gegenüber den schädlichen Wirkungen von BXDs und nutzen BXD-haltige Pflanzen erfolgreich als Lebensmittel, wodurch sie sich zu landwirtschaftlich relevanten Schädlingen entwickeln. Ein solches Beispiel ist der Eulenfalter *Spodoptera frugiperda* (Lepidoptera), aber auch viele andere herbivore Insekten haben eine Resistenz gegen BXDs entwickelt. Das Ziel meiner Arbeit war es, den Stoffwechsel und die Entgiftungsmechanismen BXD-fressenden Lepidoptera-Herbivoren zu untersuchen.

Als Erstes erforschte ich die BXD-Entgiftungswege über mehrere Lepidoptera-Spezies und analysierte die BXD-Profile im Larvenkot, nachdem diese auf Maisblättern fraßen. Ich habe herausgefunden, dass die Glykosylierung des im Mais enthaltenen BXD DIMBOA eine Entgiftungsstrategie ist, die von *S. frugiperda, S. littoralis* und *S. exigua*, aber nicht von *Mamestra brassicae* und *Helicoverpa armigera* angewandt wird. Ich ermittelte die Aktivität von UDP-Glucosyltransferase (UGT), welche verantwortlich für diese Reaktion in Darmgewebe der *Spodoptera* spp. sind, und charakterisierte das Glykosylierungsprodukt als (*2S*)-DIMBOA-Glc, das ein Epimer des Mais-Glucosid (*2R*) -DIMBOA-Glc ist. Weiterhin zeigte ich, dass die pflanzlichen β-Glucosidasen nicht in der Lage sind, das vom Insekt gebildete (*2S*)-Produkt aufgrund seiner differenziellen Konfiguration zu hydrolysieren. Schlussfolgernd ist die Glykosylierung von DIMBOA mit Inversion der

stereochemischen Konfiguration eine Entgiftungsreaktion, die das toxische Aglycon DIMBOA effizient stabilisiert und die weitere Aktivierung im Larvenverdauungstrakt verhindert.

Anschließend habe ich die molekulare Basis der BXD-Entgiftungsreaktionen untersucht. Im Transkriptom von *S. frugiperda* suchte ich mutmaßliche UGT-kodierende Gene und führte ein funktionelles Screening nach Aktivität gegen DIMBOA und MBOA durch. Ich identifizierte erfolgreich 25 UGT-Kandidaten und exprimierte diese heterolog in *Trichoplusia ni*-Zellen. Von diesen Kandidaten waren fünf *S. frugiperda* UGTs in der Lage, DIMBOA und/oder MBOA zu glykosylieren. Ich identifizierte nur einen Kandidaten, der DIMBOA zu (*2S*)-DIMBOA-Glc umwandeln konnte. Ich schlussfolgerte, dass dieses Enzym für die *in vivo* beobachtete Entgiftungsreaktion verantwortlich ist, zusammen mit drei weitere UGTs, die möglicherweise bei der Bildung von MBOA-Glc beteiligt sind. Weiterhin analysierte ich die DIMBOA- und MBOA-UGT spezifischen Aktivitäten in frisch geschlüpften *S. frugiperda* Larven und in verschiedenen Geweben von älteren Larven. Die UGT-Aktivitäten werden nicht stark durch BXD-Fütterung induziert und sind am höchsten im Darmgewebe und Fettkörper.

Um die relative Bedeutung der einzelnen Entgiftungsrouten im gesamten Stoffwechsel von BXDs zu berechnen und ihre Toxikodynamik zu untersuchen, führte ich kontrollierte Fütterungstests *mit S. frugiperda* Larven unter Verwendung einzelner BXDs durch und quantifiziert die vom Kot extrahierten Metabolite. Ich wertete die Anteile der finalen Metabolite relativ zu den aufgenommenen Mengen an DIMBOA, MBOA, (*2R*)-DIMBOA-Glc, und (*2R*)-HMBOA-Glc aus. Zunächst detektierte ich eine weitere Glykosylierungsreaktion in *S. frugiperda*, die Umwandlung des BXD-Lactam HMBOA zu (*2S*)-HMBOA-Glc, und charakterisierte das Produkt durch chemische Synthese und NMR-Analysen. Ich zeigte, dass DIMBOA vor allem durch die teilweise Reduktion zum Lactam HMBOA und den Abbau zu MBOA metabolisiert wird, gefolgt durch Glykosylierungsreaktionen, welche (*2S*)-DIMBOA-Glc, (*2S*)-HMBOA-Glc und MBOA-Glc ergeben. Außerdem zeigte ich, dass kurz nach der oralen Verabreichung von DIMBOA diese drei Glucoside in der Hämolymphe vorhanden sind, was auf einen Ausscheidungsmechanismus hindeutet, der diesen Transportprozess nutzt. Weiterhin demonstrierte ich, dass MBOA hauptsächlich als MBOA-Glc ausgeschieden wird, und dass die Glucoside (*2S*)-DIMBOA-Glc und (*2R*)-HMBOA-Glc teilweise von *S. frugiperda* hydrolysiert werden. Insgesamt konnte ich zwischen 30 und 60% der verabreichten BXD im Kot als seine Metabolite nachweisen, was darauf hinweist, dass ein erheblicher Anteil der verabreichten BXDs mit Komponenten der Diät reagiert, durch das Insekt als Nährstoffe absorbiert wird, und/oder zu noch unbekannten Metaboliten umgewandelt wird.

Zusammenfassend, durch den kombinierten Einsatz von Methoden aus der analytischen Chemie, organische Synthese, Molekularbiologie und Biochemie erforschte ich die Metabolisierung und Entgiftung von BXDs in *S. frugiperda* und andere lepidopteren Herbivoren. Ich identifizierte Glycosylierungen durch UGTs als einen wichtigen Entgiftungsweg und untersuchte sie auf chemischer und molekularer Ebene. Die Ergebnisse tragen zu unserem allgemeinen Verständnis zum BXD-Metabolismus durch Insekten bei, da die in dieser Dissertation beschriebenen Strategien auch von anderen Spezies verwendet werden könnten. Darüber hinaus ermöglichen die beschriebenen Erkenntnisse weitere Untersuchung zu der Verteilung, Evolution und Regulation der Enzyme, die für

die Glykosylierung verantwortlich sind, sowie vertiefendes Verständnis über die Wirkungsweisen von BXDs *in vivo*. Ganz allgemein tragen die in dieser Dissertation präsentierten Erkenntnisse zu unserem Wissen darüber bei, wie Insekten sekundäre Pflanzenverteidigungsmetabolite bewältigen, und wie sich beide Partner in ihrer evolutionären Entwicklung gegenseitig beeinflusst haben könnten. Die Erkenntnisse dieser Arbeit helfen, die Möglichkeit der Minimierung der Entgiftungsprozesse von BXDs in landwirtschaftlichen Schädlingen zu erforschen, und somit BXDs zur Kontrolle dieser Schädlinge zu nutzen.

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8. Erklärung

Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, dass ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Jena, 22.04.2016 Felipe C. Wouters

Erklärung über laufende und frühere Promotionsverfahren

Hiermit erkläre ich, dass ich keine weiteren Promotionsverfahren begonnen oder früher laufen hatte. Das Promotionsverfahren an der Biologisch-Pharmazeutischen Fakultät ist mein erstes Promotionsverfahren überhaupt.

Jena, 22.04.2016 Felipe C. Wouters

9. Curriculum Vitae

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Education

2012 – present	Ph.D. degree in Biochemistry, Max Planck Institute for Chemical Ecology/Friedrich-Schiller University Jena, Jena, Germany
2010 – 2011	M.Sc. degree in Organic Chemistry, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil
2006 – 2010	Bachelor degree in Chemistry, Universidade Federal de Santa Maria (UFSM), Santa Maria, Brazil

Research Experience

2012 – present MPI-CE, Jena, Germany – Doctoral Thesis
Department of Biochemistry – Max Planck Institute for Chemical Ecology
Dissertation: *"Detoxification and metabolism of maize benzoxazinoids by lepidopteran herbivores"*
Advisor: Prof. Dr. Jonathan Gershenzon
Fields of study: Chemical ecology, Insect biochemistry, Chemistry of natural products

2010 – 2011 UNICAMP, Campinas, Brazil – M.Sc. Thesis
Chemistry Institute – Universidade Estadual de Campinas (UNICAMP)
Dissertation: "*Harvestmen semiochemicals of the family Gonyleptidae (Arachnida: Opiliones)*"
Advisor: Prof. Dr. Anita Jocelyne Marsaioli
Fields of study: Chemical ecology, Chemistry of natural products, Organic synthesis

2008 – 2010 UFSM, Santa Maria, Brazil – Bachelor Thesis
Chemistry Department – Universidade Federal de Santa Maria (UFSM)
Dissertation: "*Synthesis of octakis(6-cyano-6-deoxy-2,3-di-O-ethyl)-β-cyclodextrin*"
Advisor: Prof. Dr. Ademir Farias Morel
Field of study: Organic synthesis

2006 – 2008 **UFSM, Santa Maria, Brazil – Research internship**

Chemistry Department – Universidade Federal de Santa Maria (UFSM)

Project: “*Coordination chemistry of bromomethyl-dibromo-indium(III) with diphosphinoxides: searching for linear indium (III) polymers*”

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Stipends and Awards

2015 Doctoral stipend from the International Max Planck Research School for the Exploration of Ecological Interactions with Molecular and Chemical Techniques of the Max Planck Institute for Chemical Ecology

2015 Frank and Mary Loewus Student Travel Award for oral presentation at the 54th Meeting of the Phytochemical Society of North America, Urbana-Champaign, IL, USA

2012 Doctoral stipend from the Sinergia grant of the Swiss National Science Foundation in collaboration with the Max Planck Institute for Chemical Ecology

2010 Master thesis grant awarded by the Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP

2008 Undergraduate apprenticeship in research grant awarded by the Programa Nacional de Iniciação Científica do Conselho Nacional de Pesquisa e Desenvolvimento, PIBIC – CNPq

2006 Undergraduate apprenticeship in research grant awarded by the Programa Nacional de Iniciação Científica do Conselho Nacional de Pesquisa e Desenvolvimento, PIBIC – CNPq

Language skills

Portuguese: Native speaker

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Spanish: Moderate listening, speaking, reading, and writing

German: Basic listening, speaking, reading, and writing (level A2)

10. List of Publications

Scientific articles

Wouters, F.C.; Ahn, S.-J.; Luck, K.; Vogel, H.; Erb, M.; Gershenzon, J.; Vassão, D.G. Screening of UDP-glycosyltransferases responsible for benzoxazinoid detoxification in *Spodoptera frugiperda*, in preparation

Wouters, F.C.; Paetz, C.; Erb, M.; Gershenzon, J.; Vassão, D.G. Metabolic fate of benzoxazinoids in *Spodoptera frugiperda*, in preparation

Wouters, F.C.; Gershenzon, J.; Vassão, D.G. Biological activities and metabolism of benzoxazinoids on insect herbivores, in preparation

Wouters, F.C.; Gershenzon, J.; Vassão, D.G. Benzoxazinoids: Reactivity and Modes of Action of a Versatile Class of Plant Chemical Defenses, submitted to *J. Braz. Chem. Soc.*

Wouters, F.C.; Reichelt, M.; Glauser, G.; Bauer, E.; Erb, M.; Gershenzon, J.; Vassão, D.G. Reglucosylation of the Benzoxazinoid DIMBOA with Inversion of Stereochemical Configuration is a Detoxification Strategy in Lepidopteran Herbivores. *Angew. Chem. Int. Ed.* **2014**, 53, 11320-11324

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Wouters, F. C.; Rocha, D. F. O.; Gonçalves, C. C. S.; Machado, G.; Marsaioli, A. Additional Vinyl Ketones and Their Pyranyl Ketones in Gonyleptid Harvestmen (Arachnida: Opiliones) Suggest These Metabolites Are Widespread in This Family. *Journal of Natural Products* **2013**, 76, 1559-1564

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Burrow, R. A.; Wouters, F. C.; De Castro, L. B.; Peppe, C. Redetermination of bis(diphenylphosphino) methane at 293 (2) K, *Acta Crystallographica Section E Structure Reports Online* **2007**, E63, o2559

Oral Presentations

Wouters, F.C.; Vassão, D.G.; Reichelt, M.; Luck, K.; Erb, M.; Gershenzon, J. Detoxification of maize chemical defenses by Lepidopteran herbivores. 5th Brazilian Conference on Natural Products, **2015**, Atibaia, Brazil

Wouters, F.C.; Vassão, D.G.; Reichelt, M.; Luck, K.; Erb, M.; Gershenzon, J. Detoxification of maize chemical defenses by Lepidopteran herbivores. 54th Annual Meeting of the PSNA, **2015**, Urbana-Champaign, IL, USA

Wouters, F.C. (2015). Through the Looking-Glass, and What *Spodoptera* Found There – Detoxification of Maize Chemical Defenses. 14th IMPRS Symposium, MPI for Chemical Ecology, **2015**, Dornburg, Germany

Poster Presentations

Wouters, F.C.*; Luck, K.; Reichelt, M.; Erb, M.; Vassão, D.G.; Gershenzon, J. Detoxification of maize benzoxazinoids by lepidopteran herbivores. ICE Symposium, MPI for Chemical Ecology, **2015**, Jena, Germany

Wouters, F.C.*; Shekhov, A.; Reichelt, M.; Luck, K.; Vassão, D.G.; Gershenzon, J. Detoxification of the maize benzoxazinoid DIMBOA by lepidopteran herbivores. ICE Symposium, MPI for Chemical Ecology, **2014**, Jena, Germany

Maag, D.*; Köhler, A.; Dalvit, C.; Wouters, F.C.; Vassão, D.G.; Gershenzon, J.; Wolfender, J.; Turlings, T.; Erb, M.; Glauser, G. 3-β-D-glucopyranosyl-6-methoxy-2-benzoxazolinone - A novel detoxification product of maize benzoxazinoid derivatives in *Spodoptera* spp. 15th International Symposium on Insect-Plant Relationships, University Neuchâtel, **2014**, Neuchâtel, Switzerland

Jeschke, V.*; Wouters, F.C.*; Shekhov, A.; Luck, K.; Meyer, N.; de Melo Moreira Gomes, A.M.; Kearney, E.; Blanchette, B.; Schramm, K.; Gershenzon, J.; Vassão, D.G. Detoxification and Mode of Action of Plant Defenses in Insect Herbivores. SAB Meeting 2014, MPI for Chemical Ecology, **2014**, Jena, Germany

Wouters, F.C.*; Reichelt, M.; Erb, M.; Vassão, D.G.; Bauer, E.; Blanchette, B.; Shekhov, A.; Gershenzon, J. Detoxification of benzoxazinoids – how lepidopteran herbivores cope with maize chemical defenses. 13th IMPRS Symposium, MPI for Chemical Ecology, **2014**, Dornburg, Germany

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Wouters, F.C.*; Jeschke, V.*; Gershenzon, J.; Schramm, K.; Vassão D.G. Detoxification and Mode of Action of Plant Defenses in Insect Herbivores. SAB Meeting 2012, MPI for Chemical Ecology, **2012**, Jena, Germany

Wouters, F. C.*; Machado, G.; Marsaioli, A. J. Caracterização da secreção de defesa dos opiliões *Gonyleptes gonyleptoides* e *Progonyleptoidellus striatus* (Arachnida: Opiliones) (Characterization of defensive secretion from harvestmen *Gonyleptes gonyleptoides* and *Progonyleptoidellus striatus* (Arachnida: Opiliones)). 33th Annual Meeting of the Brazilian Chemical Society, **2011**, Florianópolis, Brazil

Wouters, F. C.*; Machado, G.; Marsaioli, A. J. Chemical investigation of harvestmen defensive secretions (Arachnida: Opiliones: Laniatores). First Latin American Meeting of Chemical Ecology, **2010**, Colonia del Sacramento-Uruguay

Wouters, F. C.*; Morel, A. F. Síntese da octaquis(6-ciano-6-deoxi-2,3-di-O-etil)-β-ciclodextrina (Synthesis of octakis(6-cyano-6-deoxy-2,3-di-O-ethyl)-β-cyclodextrin). 33th Annual Meeting of the Brazilian Chemical Society, **2010**, Águas de Lindóia, Brazil

Wouters, F. C.*; Pedroso, M.; Stüker, C.; Maldaner, G.; Morel, A. F. Atividade antimicrobiana do ácido betulínico e seus derivados (Antimicrobial activity of betulinic acid and its derivatives). 33th Annual Meeting of the Brazilian Chemical Society, **2010**, Águas de Lindóia, Brazil

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