Phytochemical background matters for bioactivity of plant metabolites : a case study with pyrrolizidine alkaloids

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Chapter 1

General introduction

1. Plant metabolites and metabolism

Plants manufacture a myriad of metabolites of which many are known to date while many more have to be discovered yet. An estimate of the number of metabolites within the plant kingdom is in excess of 500,000 (Dixon and Strack 2003) though this may be an underestimation of the true number (Pichersky and Lewinsohn 2011). These metabolites can be divided into two major categories: primary and secondary metabolites (PMs and SMs). PMs play a role in basic functions such as cell growth and division, respiration, storage and reproduction (Bourgaud et al. 2011). Some common examples of PMs include, but not limited to, carbohydrates, lipids, proteins and certain amino acids. Conversely, SMs are often referred to metabolites that are not necessary for cell survival but are thought to be required for plant survival in their natural environment (see a review by Kliebenstein 2004).

2. Plant secondary metabolites and their various functions

SMs have various biological properties that are of ecological relevance. The ecological functions include, but are not limited to, antiviral, anti-herbivore, anti-microbial, competition, attracting pollinates, protection against frost and drought and protection against radiation. As such, they are vital in plant-environment interactions (Hartmann 1996; Kessler and Baldwin 2002; Mithofer and Boland 2012). Their bioactivity although evolved to protect plants from adverse environmental conditions is not limited to this. SMs also play an important role in our daily life. SMs play a positive role in e.g. medicines and food (e.g. phytomedicines, food health, nutritional values) (Lila and Raskin 2005), and in e.g. crop protection (Landis et al. 2000; Cardinale et al. 2003). SMs can play a negative role because of their sometimes adverse or hazardous impacts (e.g. food contaminants, poisonous or carcinogenic properties) (Molyneux et al. 2007; Edgar et al. 2011; EFSA 2011). Overall, there is no doubt that plants are a rich source of natural products possessing interesting biological and medicinal properties (Ravishankar and Venkataraman 1990; Caporale 1995; Cook and Samman 1996; Ravishankar and Rao 2000; Morton et al. 2000; Newman et al. 2003; see reviews by Dias et al. 2012, Cragg and Newman 2013 and Atanasov et al. 2015).

Nearly all energy and nutrients supporting organisms in food webs comes from plants and it is therefore not surprising that one of the most prominent adaptations of plants is defence against natural enemies (Harborne 2001; Ralphs et al. 2004). Basically, the proposed functions include: defence against micro-organisms, including bacteria, fungi and viruses, against grazing mammal and insect herbivores, and competition with other plants. In addition, plants have to protect themselves against physical stresses such as temperature and drought stress, and the damaging effects of ultraviolet radiation (Bednarek and Osbourn 2009; Saito and Matsuda 2010; Pichersky and Lewinsohn 2011; Wink 2011). To protect themselves against these threats, plants have developed an array of defensive strategies (Freeman and Beattie 2008). Among them, chemical defences covering many classes of (secondary) metabolites, represent a major barrier to these threats. This is especially true for herbivory (Mithofer and Boland 2012). Plants can have two ways to avoid being eaten.

First, they can avoid being selected for oviposition or herbivory, in other words, to send them to neighbouring plants. Second, they can increase the mortality of herbivores that do eat from them. In this thesis, we focused on mortality because relatively easy bioassays are available to study the activity of (combinations of) metabolites.

3. The diversity of plant secondary metabolites

SMs are tremendously diverse both in terms of numbers and chemical structures (Hartmann 1996; Wink 1999; Futuyma and Agrawal 2009; Wink 2010; Kliebenstein 2012). Approximately 200,000 SMs are known and recorded in databases (De Luca and St Pierre 2000; Mithofer and Boland 2012) including more than 12,000 alkaloids, more than 8,000 phenolics and over 25,000 terpenoids (Radulovic et al. 2013). Even at the level of a single cell chemical diversity is high: 50 metabolites were characterized in specific cells of *Arabidopsis* roots (Moussaieff et al. 2013).

Classes of metabolites regarded as SMs include glucosinolates, saponins, alkaloids, essential oils, flavonoids and organic acids, and the like (Mithofer and Boland 2012). For all these broad classes, a considerable diversity is also found within a class. For instance, according to the Dictionary of Natural Products (2006), there are 147 different sesquiterpene skeletal types, and 118 different diterpene subclasses. Presence and/or absence of specific functional groups can further diversify the metabolites in the same (sub)class (Radulovic et al. 2013). An extra layer of complexity is the existence of interactions between metabolites, which can also multiply the diversity in terms of, for instance, various interaction patterns. In the context of plant defence, few studies have addressed the metabolite interactions and their effects on fending off herbivores.

Although often the diversity is not well understood, it is hypothesized that the process of coevolution between plant and herbivores is responsible for the tremendous diversification of plant SMs (Fraenkel 1959; Ehrlich and Raven 1964; Macel et al. 2005; Iason et al. 2011). Another hypothesis is that a mixture of SMs is more effective than the individual metabolites (Berenbaum et al. 1991; Rasmann and Agrawal 2009). In this thesis, I mainly focus on the 2nd hypothesis.

4. Structural diversity and the bioactivity of individual metabolites

The structural diversity of SMs suggests a great variety in bioactivities. The structure of a metabolite determines its physicochemical properties, which in interaction with bio-systems, shapes its biological activity. Accordingly, small changes in chemical structure may alter the bioactivity largely. Both the efficacy may change and the type of bioactivity can fully change (Sneath 1966). Structure-activity relationships of metabolites are well-known in the pharmaceutical and chemical industries with wide applications (McKinney et al. 2000). In an ecological context, structural variation of SMs within a single class could lead to important differences in ecological function (Kliebenstein 2012). For instance, condensed tannins with different structures differed markedly in their anti-herbivore activity (Ayres et

al. 1997). A simple hydroxylation of glucosinolates increased the resistance of *A. thaliana* against the lepidopteran *Trichoplusia ni* (Hansen et al. 2008).

Yet, we do know still little about the effects of structural variation in an ecological context because most studies to date considered ecological functions at the level of a class of metabolites (see a review by Lattanzio et al. 2006). Of equal importance is to study biological functions at the level of diversity within a class of structurally related metabolites. Comparing the activity of a group of structurally strongly related metabolites can provide a tool to determine the active chemical part of that particular group. The latter is essential determining the key factors of the activity, and in distinguishing between active and inactive molecules.

5. Interactions between plant metabolites

The co-occurrence of metabolites in plants indicates a high possibility of interactions between metabolites (Nelson and Kursar 1999; Whitehead and Bowers 2014). In line with the level of complexity of chemical diversity, interactions between SMs can occur within a structurally related class, between different classes of metabolites, and within the natural phytochemical background in which PMs occur.

Although the potential for interactions between SMs is well recognized (Gershenzon et al. 2012), interactions between SMs and the effects of such interactions on herbivore performance have not received much attention yet. It is due in part to the difficulty of detecting and analyzing metabolite interactions in a proper manner (Nelson and Kursar 1999). Previous studies mainly focused on interactions between well-characterized SMs within a single class of metabolites. Examples include the antagonistic effects of two linear furanocoumarins on the beet armyworm *Spodoptera exigua* (Diawara et al. 1993), the synergistic effects of two amides on several insects (Dyer et al. 2003; Richards et al. 2010; Whitehead and Bowers 2014), the synergistic effects of two potato glycoalkaloids on the snail *Helix aspersa* (Smith et al. 2001) and on the Khapra beetle *Trogoderma granrium* (Nenaah 2011), and synergistic effects of two iridoid glycosides on the buckeye butterfly *Junonia coenia* (Richards et al. 2012). With respect to pyrrolizidine alkaloids (PAs) (Macel et al. 2005) found synergistic effects of PAs on the beet armyworm *S. exigua* and the locust *Locusta migratoria* while no interaction was found between PAs in their effects on the thrips *Frankliniella occidentalis* and the aphid *Myzus persicae.* Meanwhile, interactions may also occur between metabolites of different classes. This has been less well studied but interesting exceptions are: the synergistic effects between myristicin (a phenylpropene) and xanthotoxin (a furanocoumarin) on the corn earworm *Heliothis zea* (Berenbaum and Neal 1985), the synergistic effects of volatile monoterpenes and α-terthienyl on the European corn borer *Ostrinia nubilalis* (Guillet et al. 1998), the antagonistic effects of potassium peroxymonosulphate, chlorogenic acid (CGA), indole and caryophyllene (a sesquiterpene) on the brine shrimp *Artemia franciscana* (Nelson and Kursar 1999), the synergistic effect of cacalol (a sesquiterpene) and seneciphylline (a pyrrolizidine alkaloid, PA) on the generalist

Callimorpha dominula while no interaction effect on the specialist leaf beetles *Oreina cacaliae* or *O. speciosissima* was observed (Hagele and Rowell-Rahier 2000), the synergistic effects of phytic acid and xanthotoxin on two lepidopteran species *Trichoplusia ni* and *Depressaria pastinacella* (Green et al. 2001), and the antagonistic effects of CGA and jacobine (a PA) on *S. exigua* cell lines (Nuringtyas 2014).

While results from combinations of known metabolites strongly point to the importance of interactions between metabolites, investigating all possible combinations of metabolites in a single plant is simply impossible due to the tremendous number of metabolites that are present in any given plant. The situation could become even more complex if interactions occur among unidentified or unknown metabolites. It is becoming clear that unknowns account for a great part of the metabolites in plants (Kliebenstein 2012). It is thus an exceptionally challenging task to disentangle the potential interactions among SMs in complex natural conditions and to investigate their effects on relevant bioactivity in an ecological context.

The ecological and evolutionary significance of metabolite interactions

Interactions between metabolites and their biological effects are assumed to be of significance for their bioactivity e.g. protection against herbivores, from functional, ecological and evolutionary perspectives because SMs, in nature, always occur in a phytochemical background of other PMs and SMs.

Interactions may provide a more comprehensive understanding of biological functions of individual SMs. Since Fraenkel published his now-famous article in *Science* in 1959, the past six decades have witnessed a great progress in understanding plant-environment relationships. The defensive function of many plant SMs is no longer doubted. That does not mean, however, that all SMs are active as defence compounds. The ecological role of many SMs is still unknown. This raises the question how metabolites that on their own are apparently less effective or inactive contribute to plant fitness. The examples given above suggest that the bioactivity of single SMs can be greatly enhanced in concert with others. Many SMs may not be active by themselves but potentiate the function of other SMs.

Potential interactions between metabolites may also explain why some SMs show a certain bioactivity in particular species while they do not show this in others. For instance, CGA in *Chrysanthemum* was negatively correlated with the feeding damage of the western flower thrips, *Frankliniella occidentalis* (Leiss et al. 2009), while no effect of CGA on thrips was detected in tomato, *Solanum lycopersicum* (Mirnezhad 2011).

Synergism between SMs can be of selective advantage to plants by producing a greater defensive effect at a lower cost than single metabolites alone (Fagerstrom 1989; Dyer et al. 2003; Jones et al. 2005; Ryabushkina 2005; Richards et al. 2010 and 2012). Before reaching the target sites, a single metabolite has to pass counter defensive strategies employed by herbivores or pathogens, e.g. excretion, sequestration, degradation, etc.

(Berenbaum 2002; Despres et al. 2007). Working in concert with other SMs, that would protect them against these counter strategies would increase the efficacy of the bioactivity.

While synergistic interactions can provide a fitness advantage to plants, antagonistic interactions in most cases would not do so. However, given the large amount of plant metabolites, antagonistic interactions between metabolites may also occur. Currently, we know of very few studies that have reported antagonistic interactions and the effect of such interactions (but see Diawara et al. 1993; Nelson and Kursar 1999; Nuringtyas 2014). There are only few hypotheses about potentially positive effects of antagonistic interactions for plants. One of them is to avoid autotoxicity. Altogether, from an evolutionary point of view, antagonistic interactions are not easily explained and rather may represent a constraint or a trade-off caused by the accumulation of metabolites in plants (Nelson and Kursar 1999). However, experimental evidence is currently lacking to back up this hypothesis.

Mechanisms underlying the interaction effects

As mentioned above, to be active, individual metabolites have to pass several steps of the pests' defensive system. All these steps can be supported or influenced by other metabolites, accordingly resulting in interaction effects. For insect herbivores the underlying mechanisms of synergistic or antagonistic interactions between SMs are not well understood. Still, we can borrow ideas from other research fields, e.g. pharmacology, that learns that an interaction may occur in the kinetic phase (i.e. processes of uptake, distribution, metabolism and excretion) or in the dynamic phase (i.e. effects on the receptor, cellular target or organ) (Williamson 2001; Zimmermann et al. 2007; Biavatti 2009; Efferth and Koch 2011; Labuschagne et al. 2012).

In the kinetic phase, possible interactions may be due to changing cell surface hydrophobicity, cell wall permeability (Walencka et al. 2007), and/or cytoplasmic membrane permeability (Campos et al. 2009; Amin et al. 2015). For instance, saponins are well known to modify the cell membrane and thus facilitate the uptake of glycoalkaloids of rat and human intestinal cells (Gee et al. 1996; Wink 2008; Herrmann and Wink 2011). Mechanisms of interaction may also involve the ability of one component of a mixture to interfere or inhibit the detoxification of others. For instance, phytic acid inhibits insect cytochromes P450 monoxygenases, thereby reducing the detoxification of xanthotoxin, a defensive furanocoumarin (Green et al. 2001).

In the dynamic phase, metabolites may interact by means of blocking or disturbing membrane-bound receptor function. For instance, 5'-methoxyhydnocarpin (a flavonolignan) blocked the Nor A efflux pump of bacteria and thus potentiated the antimicrobial effect of berberine (Stermite et al. 2000). Ramipril inhibits the angiotensin receptor, thereby facilitating the antihypertensive effect of candesartan-cilexetil on spontaneously hypertensive rats (Raasch et al. 2004).

6. Approaches to bioactivity research

It is a great challenge to evaluate interactions between plant metabolites given the enormous number of metabolites in plants and the even greater number of possible interactions. Additionally, there is a large number of unidentified or even unknown metabolites in a plant (Trethewey 2004), among which interactions may also occur. We can use a bottom-up or a top-down approach, both of which integrate various scales of research objectives. These are central approaches of systems biology (Bruggeman et al. 2007), however, the application of the two approaches in plant-insect context are still in infancy. In this thesis, I use both approaches to understand the importance of the interactions between plant metabolites in the context of the plant-insect associations.

A bottom-up approach usually starts with combining specific metabolites. Prederably, this should be done on the basis of the existing knowledge of the metabolites. For instance, saponins are well known to modify the cell membrane and thus facilitate the uptake of other compounds (Berenbaum 1985; Raymond 2013). In this thesis, I studied the interaction between pyrrolizidine alkaloids (PAs) and chlorogenic acid (CGA) knowing that they are differently distributed over plant cell layers (Nuringtyas et al. 2012) and that CGA is known to interact with the alkaloid caffeine (Mösli Waldhauser and Baumann 1995). Prior information of individual metabolites not only forms a starting point for the study of their interaction, but also allows us to propose or hypothesize how metabolites that are of interest may be expected to interact. This approach provides a view of the interaction effects in a metabolite-specific manner.

In the absence of prior knowledge about the metabolites that are involved, taking the metabolome into account provides an alternative starting point. I used this top-dwon approach by adding individual metabolites that are of particular interest (PAs) to plant extracts and fractions. In this thesis I only set the first step by taking the effect of fractions of a plant methanol extract into account. This approach could be continued with further sub-fractionation and recombining sub-fractions to narrow down the specific metabolites that are of particular interest.

In this thesis, I will study (i) the effects of individual metabolites, (ii) the interaction effects between metabolites within a structural related class, (ii) the interaction effects between metabolites of different classes and (iv) the influence of natural phytochemical backgrounds on the activity of individual metabolites.

7. Research systems

In this thesis, I used *Jacobaea vulgaris* as a model plant, which contains PAs, a well-known group of SMs. From a perspective of structure diversity, more than 400 PAs have been identified (Chou and Fu 2006). *J. vulgaris* contains more than 37 different PAs (Cheng et al. 2011a). PAs can occur in two forms: the free base and the N-oxide. Although some jacobine-like PAs are reported to occur upto 50% as free base in *J. vulgaris* (Joosten et al.), the N-oxide is the major storage form in plants (Hartmann et al. 1989). As to ecological

functions, PAs have been shown to play an important role in the plant-environment interactions, showing negative effects on mammalian and insect herbivores and on micro-organisms (Dreyer et al. 1985; de Boer 1999; Reina et al. 2001; Siciliano et al. 2005; Dominguez et al. 2008; also see reviews by Macel 2011 and Trigo 2011 and references therein; Jing et al. 2015). However, our understanding of the roles of PAs in plant defence is still incomplete.

First, most of the existing evidence for the defensive effects of PAs comes from correlation studies on whole plants or genotypes (Vrieling et al. 1991; Leiss et al. 2009; Cheng et al. 2011; Kostenko et al. 2013; Wei et al. 2015) and to a lesser extent from bioassays with single PAs (but see Lindigkeit et al. 1997; Macel et al. 2005; Dominguez et al. 2008). The latter is probably due to the limited commercial availability of PAs. In this thesis, in addition to commercial PAs, I therefore isolated several PAs from their respective chemotypes of *J. vulgaris*, and the corresponding N-oxides were also obtained by N-oxidation for application in insect bioassays and bacterial tests.

Secondly, despite the fact of co-occurrence of metabolites in plants and the ecological importance of metabolite interactions, we know little about the interactions within the PA group or between PAs and other SMs, and their effects on insect herbivores. Alongside with PAs, a wide diversity of PMs and SMs is also present in *Jacobaea* species (Kirk et al. 2005; Leiss et al. 2009), including sugars (sucrose), amino acids (alanine), carboxylic acids (succinic, fumaric and malic acids), phenolic acids (chlorogenic, feruloylquinic acids), flavonoids (kaempferol) and benzoquinoids (jacaranone). The mode of actions of individual PAs and PA N-oxides in concert with other metabolites may differ from that when acting alone. Study on interactions and their effects would provide extra or even novel information on the roles of PAs and PA N-oxides in plant defence.

Another key question is the bioactivity of the two forms of PAs. Previous studies have demonstrated in general that PAs are more active than the corresponding PA N-oxides in fending off insect herbivores (Dreyer et al 1985; Hartmann et al. 1989; van Dam et al. 1995; Macel et al. 2005; Hartmann 2007; Nuringtyas et al. 2014). However, such a conclusion was built upon comparing the effects of single PAs, but not in the context of other metabolites. What we do not know is whether the two forms of PAs differ in their effects in the presence of other metabolites. Preliminary studies with *S. exigua* cell lines present evidence for antagonistic interactions between jacobine and CGA (Nuringtyas 2014). The PA N-oxides have not been studied in this respect yet.

CGA is one of the most widespread phenolics in the plant kingdom. With respect to ecological functions, CGA has been reported to be involved in defence against insect herbivores including thrips (Leiss et al. 2009), as evidenced by correlative studies and bioassays with artificial diets. From a mechanistic point of view, it is known that CGA forms a π-molecular complex with caffeine (a purine alkaloid) (Mösli Waldhauser and Baumann 1995). Furthermore, Nuringtyas et al. (2012) found that the mesophyll of *J.*

vulgaris contained large amounts of PAs while CGA was accumulated in the epidermis. It remains unclear, however, how such a differential accumulation over cell layers functions in plant defence. Overall, both viewpoints are of interest and provide a starting point for investigating the interactions between PAs and CGA and their effects.

In this thesis, a generalist herbivore, the western flower thrips, *Frankliniella occidentalis*, was used. *F. occidentalis* is a key insect pest that feeds on a wide variety of plant species, including many important crops (Kirk and Terry 2003). As a polyphagous insect, thrips has a wide range of more than 250 host plants belonging to 62 different families (Jensen 2000). Through the piercing-sucking mouthparts, they cause two types of damage on plants. Feeding on actively growing tissue leads to malformation in plant growth, and eventually yield loss, while feeding on expanded tissue results in silver damage, which affects product appearance and reduces market quality (de Jager et al. 1995). In addition, thrips can vector diseases such as tomato spotted wilt virus, which affects a wide range of plants (Tsao et al. 2005). In thrips resistance, SMs play an important role, for instance, CGA and an isobutylamide in chrysanthemum (Tsao et al. 2005; Leiss et al. 2009), PAs in *Senecio* (Macel et al. 2005), acylsugars in tomato (Romero-González et al. 2010) and flavonoids in carrots (Leiss et al. 2013).

While the focus of this thesis is on the importance of synergistic and antagonistic effects between plant metabolites on insect herbivores, we wanted to investigate whether or not the impact of interactions between plant metabolites plays an important role on other types of bioactivity.

Next to being deterrents and toxins for insect herbivores, PAs have been shown to be carcinogenic in rats (European Food Safety Authority 2011) and genotoxic to Drosophila melanogaster (Frei et al. 1992). The genotoxicity of PAs are presumably induced by nucleoside adduct formation, such as DNA cross-linking, DNA-protein cross-linking, and DNA-alkylation (Frei et al. 1992; Fu et al. 2001 and 2002). Cross-linking with DNA can produce mutations. As important early steps in genotoxicity, mutations can occur as point mutations, deletions, rearrangements of DNA, chromosomal breaks and rearrangements and finally, as gain or loss of whole chromosomes (Mortelmans and Zeiger 2000).

The most well-known genotoxicity assay, the *Salmonella*/microsome mutagenicity test, also known as the Ames test, is a short-term *in vitro* bacterial reverse mutation assay specifically designed to detect DNA mutations, involving substitution, addition or deletion of one or a few DNA base pairs (Ames et al. 1975; McCann et al. 1975; Fessard and Le Hégarat 2010). In this thesis, therefore, testing mutagenicity of PAs, an important indicator of bioactivity, was included as a supplementary to the anti-herbivore bioactivity of PAs.

8. Research questions

The central theme of this thesis is to understand the importance of interactions between plant metabolites and their effects in the plant-insect associations. In particular, I first

studied the effects of individual PAs and their corresponding N-oxides on thrips. On the basis of the results of individual SMs, I further searched for evidence of interactions between SMs and their effects on thrips. Lastly, I investigated the influence of natural backgrounds on the activity of individual SMs. I will address the following questions.

1. How does the phytochemical background influence the bioactivity of individual PAs: resistance against thrips?

a) Do individual free base PAs and PA N-oxides have an effect on thrips mortality? (Chapter 2)

b) Do PA N-oxides show synergistic effects on thrips mortality? (Chapter 2)

c) How do PAs interact with CGA on thrips mortality?

- How does CGA combined with free base PAs affect thrips mortality? (Chapter 3)

- How does CGA combined with PA N-oxides affect thrips mortality? (Chapter 4)

d) How do plant fractions combined with free base PAs and PA N-oxides affect thrips mortality? (Chapter 5)

2. How does the phytochemical background influence the bioactivity of individual PAs: mutagenicity?

a) Are free base PAs, plant fractions and their combination mutagenic to *Salmonella typhimurium*? (Chapter 6)

9. Outline of this thesis

Chapter 2 details the effects of individual PAs and their corresponding N-oxides on thrips. I studied whether individual SMs within a structurally related group differed in their effects on thrips mortality.

Next, I evaluated whether interactions exist between SMs by testing the effects of combinations of two well-characterized SMs on thrips mortality. In **Chapter 2** I tested whether PA N-oxides act synergistically on thrips mortality in bioassays. **Chapter 3** reports on the antagonistic effects of PAs and CGA on thrips mortality in bioassays. This chapter also investigates the roles of the functional groups of the CGA molecule in the interaction with PAs by addition/elimination of specific groups, or changing the substitution pattern.

In **Chapter 4** I tested whether PA N-oxides and CGA interact in their effects on thrips mortality in bioassays. The interaction effects of PAs and PA N-oxides with CGA on thrips were compared with data obtained in Chapter 3.

In **Chapter 5** I investigated the effects of a whole extract from *Jacobaea* leaves and five fractions on thrips mortality. To the plant extract fractions, PAs were added to study the influence of natural backgrounds on the effects of individual PAs on thrips mortality. In

Chapter 6 I used a quick and simple indicator of bioactivity, the Ames test, to study the mutagenicity of 10 plant fractions and 13 sub-fractions of *Jacobaea* plant extracts. Here I also studied the metabolite interactions on mutagenicity by re-covering sub-fractions and by demonstrating the influence of natural backgrounds by adding PAs into five fractions of leaf extracts.

Chapter 7 summarizes the findings presented in this thesis.

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