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Contents

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.

References

Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the *salmonella*/mammalian-microsome mutagenicity test. Mutat Res 31:347-364

Amin MU, Khurram M, Khattak B, Khan J. 2015. Antibiotic additive and synergistic action of rutin, morin and quercetin against methicillin resistant *Staphylococcus aureus*. BMC Complement Altern Med 15:59

Atanasov AG, Waltenberger B, Pferschy-Wenzig E, Linder T, Wawrosch C, et al. 2015. Discovery and resupply of pharmacologically active plant-derived natural products: A review. Biotechnol Adv 33:1582-1614

Ayres MP, Clausen TP, Maclen J, Redman AM, Reichardt PB. 1997. Diversity of structure and antiherbivore activity in condensed tannins. Ecology 78:1696-1712

Bednarek P, Osbourn A. 2009. Plant-microbe interactions: chemical diversity in plant defense. Science 324:746-748

Berenbaum M, Neal JJ. 1985. Synergism between myristicin and xanthotoxin, a naturally cooccurring plant toxicant. J Chem Ecol 11:1349-1358

Berenbaum M. 1985. Brementown revisited: interactions among allelochemicals in plants. Recent Advances in Phytochemistry Vol 19: Chemically mediated interactions between plants and other organisms (eds G.A. Cooper-Driver, T. Swain & E.E. Conn), pp.139-169. Plenum Press, New York City, New York

Berenbaum MR, Nitao JK, Zangerl AR. 1991. Adaptive significance of furanocoumarin diversity in *Pastinaca sativa* (Apicaceae). J Chem Ecol 17:207-215

Berenbaum MR. 2002. Postgenomic chemical ecology: From genetic code to ecological interactions. J Chem Ecol 28:873-896

Biavatti MW. 2009. Synergy: an old wisdom, a new paradigm for pharmacotherapy. Braz J Pharm Sci 45:371-378

Bourgaud F, Gravot A, Milesi S, Gontier E. 2001. Production of plant secondary metabolites: a historical perspective. Plant Sci 161:839-851

Bruggeman FJ, Hornberg JJ, Boogerd FC, Westerhoff HV. 2007. Introduction to systems biology. EXS 97:1-19

Caporale LH. 1995. Chemical ecology- a view from the pharmaceutical-industry. Proc Natl Acad Sci USA 92:75-82

Cardinale BJ, Harvey CT, Gross K, Ives AR. 2003. Biodiversity and biocontrol: emergent impacts of a multi-enemy assemblage on pest suppression and crop yield in an agroecosystem. Ecol Lett 6:857-865

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011a. Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. New Phytol 192:1010-1023

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011b. The relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance in F(2) hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. J Chem Ecol 10:1071-1080

Chou MW, Fu PP. 2006. Formation of DHP-derived DNA adducts in vivo from dietary supplements and Chinese herbal plant extracts containing carcinogenic pyrrolizidine alkaloids. Toxicol Ind Health 22:321-327

Cook NC, Samman S. 1996. Flavonoids - Chemistry, metabolism, cardioprotective effects, and dietary sources. J Nutr Biochem 7:66-76

Cragg GM, Newman DJ. 2013. Natural products: A continuing source of novel drug leads. Biochim Biophys Acta 1830:3670-3695

de Boer NJ. 1999. Pyrrolizidine alkaloid distribution in *Senecio jacobaea* rosettes minimises losses to generalist feeding. Entomol Exp Appl 91:169-173

de Jager CM, Butot RPT, Klinkhamer PGL, van der Meijden E. 1995. Chemical characteristics of Chrysanthemum cause resistance to *Frankliniella occidentalis* (Thysanoptera: Thripidae). J Econ Entomol 88:1746-1753

De Luca V, St Pierre B. 2000. The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci 5:168-173

Després L, David JP, Gallet C. 2007. The evolutionary ecology of insect resistance to plant chemicals. Trends Ecol Evol 22:298-307

Dias DA, Urban S, Roessner U. 2012. A historical overview of natural products in drug discovery. Metabolites 2:303-336

Diawara M, Trumble J, White K, Carson W, Martinez L. 1993. Toxicity of linear furanocoumarins to *Spodoptera exigua*: evidence for antagonistic interactions. J Chem Ecol. 19:2473-2484

Dictionary of Natural Products, 2006. version 14.1, 1982-2006; Chapman & Hall/CRC, New York.

Dixon RA, Strack D. 2003. Phytochemistry meets genome analysis, and beyond. Phytochemistry 62:815-816

Domínguez DM, Reina M, Santos-Guerra A, Santana O, Agulló T, et al. 2008. Pyrrolizidine alkaloids from *Canarian endemic* plants and their biological effects. Biochem Syst Ecol 36:153-166

Dreyer DL, Jones KC, Molyneux RJ. 1985. Feeding deterrency of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrthosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. J Chem Ecol 11:1045-1051

Dyer LA, Dodson CD, Stireman JO, Tobler MA, Smilanich AM, Fincher RM, et al. 2003. Synergistic effects of three *Piper* amides on generalist and specialist herbivores. J Chem Ecol 29:2499-2514

Edgar JA, Colegate SM, Boppré M, Molyneux RJ. 2011. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. Food Addit Contam 28:308-324

Edgar JA, Molyneux RJ, Colegate SM. 2015. Pyrrolizidine alkaloids: potential role in the etiology of cancers, pulmonary hypertension, congenital anomalies, and liver disease. Chem Res Toxicol 28: 4-20

Efferth T, Koch E. 2011. Complex interactions between phytochemicals. The multi-target therapeutic concept of phytotherapy. Curr Drug Targets 12:122-132

Ehrlich PR, Raven PH. 1964. Butterflies and plants: a study in coevolution. Evolution 18:586-608

European Food Safety Authority. 2011. Scientific opinion on pyrrolizidine alkaloids in food and feed. EFSA Journal. Parma, Italy. 9:2406

Fagerstrom T 1989. Anti-herbivory chemical defense in plants: a note on the concept of cost. Am Nat 133:281-287

Fessard V, Le Hégarat L. 2010. A strategy to study genotoxicity: application to aquatic toxins, limits and solutions. Anal Bioanal Chem 397:1715–1722.

Fraenkel GS. 1959. The raison d'être of secondary plant substances. Science 129:1466-1470

Freeman BC, Beattie GA. 2008. An overview of plant defenses against pathogens and herbivores. The Plant Health Instructor

Frei H, Luthy J, Brauchli J, Zweifel U, Wurgler FE, et al. 1992. Structure/activity relationships of the genotoxic potencies of sixteen pyrrolizidine alkaloids assayed for the induction of somatic mutation and recombination in wing cells of *Drosophila melanogaster*. Chem Biol Interact 83:1–22

Fu PP, Chou MW, Xia Q, Yang YC, Yan J, et al. 2001. Genotoxic pyrrolizidine alkaloids and pyrrolizidine alkaloid N-oxides-mechanisms leading to DNA adduct formation and tumorigenicity. Environ Carcinogen Ecotoxicol Rev C19:353

Fu PP, Xia QS, Lin G, Chou MW. 2002. Genotoxic pyrrolizidine alkaloids - mechanisms leading to DNA adduct formation and tumorigenicity. Int J Mol Sci 3:948-964

Futuyma DJ, Agrawal AA. 2009. Macroevolution and the biological diversity of plants and herbivores. Proc Natl Acad Sci USA 106:18054-18061

Gee JM, Wortley GM, Johnson IT, Price KR, Rutten AA, et al. 1996. Effects of saponins and glycoalkaloids on the permeability and viability of mammalian intestinal cells and on the integrity of tissue preparations in vitro. Toxicol In Vitro 10:117-128

Green ES, Zangerl AR, Berenbaum MR. 2001. Effects of phytic acid and xanthotoxin on growth and detoxification in caterpillars. J Chem Ecol 27:1763-1773

Guillet G, Belanger A, Arnason JT. 1998. Volatile monterpenes in *Porophyllum gracile* and *P. ruderale* (Asteraceae): identification, localization and insecticidal synergism with alpha-terthienyl. Phytochemistry 49:423-429

Hägele BF, Rowell-Rahier M. 2000. Choice, performance and heritability of performance of specialist and generalist insect herbivores towards cacalol and seneciphylline, two allelochemicals of *Adenostyles alpina* (Asteraceae). J Evol Biol 13:131-142

Hansen BG, Kerwin RE, Ober JA, Lambrix VM, Mitchell-Olds T, et al. 2008. A novel 2-oxoacid dependent dioxygenase involved in the formation of the goiterogenic 2-hydroxybut-3-enyl glucosinolate and generalist insect resistance in Arabidopsis. Plant Physiol 148:2096-2108

Harborne JB. 2001. Twenty-five years of chemical ecology. Nat Prod Rep 18:361-379

Hartmann T, Ehmke A, Eilert U, von Borstel K, Theuring C. 1989. Sites of synthesis, translocation and accumulation of pyrrolizidine alkaloid N-oxides in *Senecio vulgaris*. Planta 177:98-107

Hartmann T. 1996. Diversity and variability of plant secondary metabolism: a mechanistic view. Entomol Exp Appl 80:177-188

Hartmann T. 2007. From waste products to ecochemicals: fifty years research of plant secondary metabolism. Phytochemistry 68:2831-2846

Herrmann F, Wink M. 2011. Synergistic interactions of saponins and monoterpenes in HeLa cells, Cos7 cells and in erythrocytes. Phytomedicine 18:1191-1196

Jensen SE. 2000. Mechanisms associated with methiocarb resistance in *Frankliniella occidentalis* (Thysanoptera: Thripidae). J Econ Entomol 93:464-471

Jing J, Raaijmakers C, Kostenko O, Kos M, Mulder PPJ, Bezemer TM. 2015. Interactive effects of above- and belowground herbivory and plant competition on plant growth and defence. Basic Appl Ecol 16:500-509

Jones AC, Blum JE, Pawlik JR. 2005. Testing for defensive synergy in Caribbean sponges: Bad taste or glass spicules? J Exp Mar Biol Ecol 322:67-81

Joosten L, Cheng D, Mulder PPJ, Vrieling K, van Veen JA, et al. 2011. The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. Phytochemistry 72:214-222

Kessler A, Baldwin IT. 2002. Plant responses to insect herbivory: the emerging molecular analysis. Annu Rev Plant Biol 53:299-328

Kirk H, Choi YH, Kim HK, Verpoorte R, van der Meijden E. 2005. Comparing metabolomes: the chemical consequences of hybridization in plants. New Phytol 167:613-622

Kirk WD, Terry I. 2003. The spread of the western flower thrips *Frankliniella occidentalis* (Pergande). Agric For Entomol 5:301-310

Kliebenstein DJ. 2004. Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. Plant Cell Environ 27:675-684

Kliebenstein DJ. 2012. Plant defense compounds: Systems approaches to metabolic analysis. Annu Rev Phytopathol 50:155-173

Kostenko O, Mulder PPJ, Bezemer TM. 2013. Effects of root herbivory on pyrrolizidine alkaloid content and aboveground plant-herbivore-parasitoid interactions in *Jacobaea vulgaris*. J Chem Ecol 39:109-119

Labuschagne A, Hussein AA, Rodriguez B, Lall N. 2012. Synergistic antimycobacterial actions of *Knowltonia vesicatoria* (L.f) Sims. J Evid Based Complementary Altern Med 2012:1-9

Landis DA, Wratten SD, Gurr GM. 2000. Habitat management to conserve natural enemies of arthropod pests in agriculture. Annu Rev Entomol 45:175-201

Lattanzio V, Lattanzio VMT, Cardinali A. 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. Phytochemistry: Advances in Research 2006:23-67

Leiss KA, Choi YH, Abdel-Farid I, Verpoorte R, Klinkhamer PGL. 2009. NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. J Chem Ecol 35:219-229

Leiss KA, Cristofori G, van Steenis R, Verpoorte R, Klinkhamer PGL. 2013. An eco-metabolomic study of host plant resistance to Western flower thrips in cultivated, biofortified and wild carrots. Phytochemistry 93:63-70

Lila MA, Raskin I. 2005. Health-related interactions of phytochemicals. J Food Sci 70:R20-R27

Lindigkeit R, Biller A, Buch M, Schiebel HM, Boppre M, et al. 1997. The two faces of pyrrolizidine alkaloids: the role of tertiary amine and its N-oxide in chemical defense of insects with acquired plant alkaloids. Eur J Biochem 245:626-636

Macel M, Bruinsma M, Dijkstra SM, Ooijendijk T, Niemeyer HM, et al. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. J Chem Ecol 31:1493-1508

Macel M. 2011. Attract and deter: a dual role for pyrrolizidine alkaloids in plant-insect interactions. Phytochem Rev 10:75-82

McKinney JD, Richard A, Waller C, Newman MC, Gerberick F. 2000. The practice of structure activity relationships (SAR) in toxicology. Toxicol Sci 56:8-17

Mirnezhad M. 2011. Host plant resistance of tomato plants to western flower thrips. PhD thesis, Leiden University, The Netherlands

Mithofer A, Boland W. 2012. Plant defense against herbivores: chemical aspects. Annu Rev Plant Biol 63:431-450

Molyneux RJ, Lee ST, Gardner DR, Panter KE, James LF. 2007. Phytochemicals: The good, the bad and the ugly? Phytochemistry 68:2973-2985

Mortelmans K, Zeiger E. 2000. The Ames *Salmonella*/microsome mutagenicity assay. Mutat Res 455:29-60.

Morton LW, Caccetta RA, Puddey IB, Croft KD. 2000. Chemistry and biological effects of dietary phenolic compounds: Relevance to cardiovascular disease. Clin Exp Pharmacol Physiol 27:152-159

Mösli Waldhauer SS, Baumann TW. 1996. Compartmentation of caffeine and related purine alkaloids depends exclusively on the physical chemistry of their vacuolar complex formation with chlorogenic acids. Phytochemistry 42:985-996

Moussaieff A, Rogachev I, Brodsky L, Malitsky S, Toal TW, et al. 2013. High-resolution metabolic mapping of cell types in plant roots. Proc Natl Acad Sci USA 3:E1232-E1241

Naczk M, Shahidi F. 2006. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. J Pharmaceut Biomed 41:1523-1542

Nelson AC, Kursar TA. 1999. Interactions among plant defense compounds: a method for analysis. Chemoecology 9:81-92

Nenaah GE. 2011. Toxic and antifeedant activities of potato glycoalkaloids against *Trogoderma granarium* (Coleoptera: Dermestidae). J Stored Prod Res 47:185-190

Newman DJ, Crag GM. 2016. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 79:629-661

Nuringtyas TR, Choi YH, Verpoorte R, Klinkhamer PGL, Leiss KA. 2012**.** Differential tissue distribution of metabolites in *Jacobaea vulgaris*, *Jacobaea aquatica* and their crosses. Phytochemistry 78:89-97

Nuringtyas TR, Verpoorte R, Klinkhamer PGL, van Oers MM, Leiss KA. 2014. Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* using insect cell lines and injection bioassays. J Chem Ecol 40:609-616

Nuringtyas TR. 2014. Pyrrolizidine alkaloid variation in *Jacobaea* plants: from plant organ to cell level. PhD thesis, Leiden University, The Netherlands

Pichersky E, Lewinsohn E. 2011. Convergent evolution in plant specialized metabolism. Annu Rev Plant Physiol 62:549-566

Raasch W, Johren O, Schwartz S, Gieselberg A, Dominiak P. 2004. Combined blockade of AT1-receptors and ACE synergistically potentiates antihypertensive effects in SHR. J Hypertens 22:611-618

Radulovic NS, Blagojevic PD, Stojanovic-Radic ZZ, Stojanovic NM. 2013. Antimicrobial plant metabolites: structural diversity and mechanism of action. Curr Med Chem 20:932-952

Rasmann S, Agrawal AA. 2009. Plant defense against herbivory: progress in identifying synergism, redundancy, and antagonism between resistance traits. Curr Opin Plant Biol 12:473-478

Ravishankar GA, Rao SR. 2000. Biotechnological production of phytopharmaceuticals. J Biochem Mol Biol Biophys 4:73-102

Ravishankar GA, Venkataraman LV. 1990. Food applications of plant cell cultures. Curr Sci 57:381-383

Reina M, González-Coloma A, Gutiérrez C, Cabrera R, Rodríguez ML, et al. 2001. Defensive chemistry of *Senecio miser*. J Nat Prod 64:6-11

Richards LA, Dyer LA, Smilanich AM, Dodson CD. 2010. Synergistic effects of amides from two *Piper* species on generalist and specialist herbivores. J Chem Ecol 36:1105-1113

Richards LA, Lampert EC, Bowers MD, Dodson CD, Smilanich AM, et al. 2012. Synergistic effects of iridoid glycosides on the survival, development and immune response of a specialist caterpillar, *Junonia coenia* (Nymphalidae). J Chem Ecol 38:1276-1284

Romero-González RR, Mirnezhad M, Leiss KA, Choi YH, Verpoorte R, et al. 2010. Metabolomic analysis of host-plant resistance to thrips in wild and domesticated tomatoes. Phytochem Anal 21:110-117

Ryabushkina NA. 2005. Synergism of metabolite action in plant responses to stresses. Russian J Plant Physiology 52:547-552

Siciliano T, Leo MD, Bader A, Tommasi ND, Vrieling K, et al. 2005. Pyrrolizidine alkaloids from *Anchusa strigosa* and their antifeedant activity. Phytochemistry 66:1593-600

Smith DB, Roddick JG, Jones LJ. 2001. Synergism between the potato glycoalkaloids α-chaconine and α-solanine in inhibition of snail feeding. Phytochemistry 57:229-234

Sneath PHA. 1966. Relations between chemical structure and biological activity in peptides. J Theoret Biol 12: 157-195

Stermitz FR, Lorenz P, Tamara JN, Zenewicz LA, Lewis K. 2000. Synergy in the medicinal plant: antimicrobial action of berberine potentiated by 5'-Methoxyhydrocarpin, a multidrug pump inhibitor. Proc Natl Acad Sci USA 97:1433-1437

Trethewey R. 2004. Metabolite profiling as an aid to metabolic engineering. Curr Opin Plant Biol 7:196-201

Trigo JR. 2011. Effects of pyrrolizidine alkaloids through different trophic levels. Phytochem Rev 10:83-98

Tsao R, Marvin CH, Broadbent AB, Friesen M, Allen WR, et al. 2005. Evidence for an isobutylamide associated with host-plant resistance to western flower thrips, *Frankliniella occidentalis*, in chrysanthemum. J Chem Ecol 31:103-110

van Dam NM, Vuister LWN, Bergshoeff C, de Vos H, van der Meijden E. 1995. The ''raison d'eˆtre'' of pyrrolizidine alkaloids in *Cynoglossum officinale*: deterrent effects against generalist herbivores. J Chem Ecol 21:507-523

Vrieling K, Soldaat LL, Smit W. 1991. The influence of pyrrolizidine alkaloids of *Senecio jacobaea* on *Tyria jacobaea*, *Brachycaudus cardii* and *Haplothrips senecionis*. Neth J Zool 41:228-239

Walencka E, Rozalska S, Wysokinska H, Rozalski M, Kuzma L, et al. 2007. Salvipisone and aethiopinone from *Salvia sclarea* hairy roots modulate staphylococcal antibiotic resistance and express anti-biofilm activity. Planta Medica 73:545-551

Wei X, Vrieling K, Mulder PPJ, Klinkhamer PGL. 2015. Testing the generalist-specialist dilemma: the role of pyrrolizidine alkaloids in resistance to invertebrate herbivores in *Jacobaea* species. J Chem Ecol 41:159-167

Whitehead SR, Bowers MD. 2014. Chemical ecology of fruit defence: synergistic and antagonistic interactions among amides from Piper. Funct Ecol 28:1094-1106

Williamson EM. 2001. Synergy and other interactions in phytomedicines. Phytomedicine 8:401-409

Wink M. 2008. Plant secondary metabolism: diversity, function and its evolution. Nat Prod Commun 3:1205-1216

Zimmermann GR, Lehar J, Keith CT. 2007. Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug Discov Today 12:34-42

Chapter 2

The effect of structure-related metabolites on insect herbivores: a case study on pyrrolizidine alkaloids and Western flower thrips

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Abstract

Plant secondary metabolites (SMs) are tremendously diverse in terms of both numbers and chemical structures. The chemical diversity of SMs can occur between different chemical classes and within a single class. In the context of plant-insect interactions, it is not well understood how the chemical diversity within a class of structurally related metabolites relates to bioactivity. In this chapter, we addressed the importance of the chemical diversity within a class of SMs with respect to insect herbivory. Firstly we tested whether individual SMs within the group of pyrrolizidine alkaloids (PAs) differ in their effects on insect herbivores (western flower thrips, *Frankliniella occidentalis*). Secondly we tested if combinations of SMs are more effective than single ones. We tested the bioactivity of six free base PAs and their corresponding N-oxides. At concentrations similar to that of plants, PAs led to a lower survival of thrips. We found that the effect on thrips differed strongly among PAs. In general, free base PAs caused a lower survival than their corresponding N-oxides. Next, among the tested free base PAs, we found that jacobine and retrorsine showed stronger effects on thrips survival than erucifoline, seneciphylline, senecionine and monocrotaline. With respect to the PA N-oxides, we only found significant effects on thrips survival for senecionine N-oxide and jacobine N-oxide, although for the first the effect was small. Combinations of PA N-oxides showed no synergistic effects. Structurally related metabolites vary in their effects on insect herbivores and studying the bioactivity of the combined group of structurally related SMs without taking the composition into account is therefore of limited use.

Key words: *Jacobaea vulgaris*, Secondary metabolites, Plant defence theory, Chemical structure, *Frankliniella occidentalis*

Introduction

Plant secondary metabolites (SMs) mediate many aspects of the interaction of plants with their environment. It is well established that they can act as defences against pathogens and herbivores (Johnson 2011; Gols 2014). One of the unique features of SMs is their high diversity in terms of both numbers and chemical structures (Hartmann 1996). The chemical diversity is generated by a wide variety of different structural classes of SMs, including e.g. glucosinolates, saponins, alkaloids, essential oils, flavonoids and organic acids (Scott et al. 2002; Mithofer and Boland 2012). Also, within a class of structurally related metabolites a large variation in composition is found. For instance, at least 34 structurally different glucosinolates are known from *Arabidopsis thaliana* (Kliebenstein et al. 2001) and more than 20 indole alkaloids are produced by *Rauvolfia serpintina* (Sheludko et al. 2002).

The chemical diversity of SMs suggests a great variety in the underlying biological activities. Indeed, relatively small changes in structure may result in changes of bioactivity towards the attacking organism. For instance, a simple hydroxylation of a glucosinolate increased the plant's resistance against the lepidopteran *Trichoplusia ni* (Hansen et al. 2008; Kliebenstein 2012). One of the explanations for the diversity within a structural related group of SMs is that different herbivores are affected by different SMs (Macel 2005). Although the importance of structural variation within a class of metabolites for herbivory has been illustrated by a number of studies, biological activity has been mainly studied at the level of a total class of structurally related metabolites, e.g. phenolics (Lattanzio et al. 2006). The importance of variation within a group of structurally related PAs should be illustrated in great detail.

An alternative hypothesis for the diversity of SMs states that a mixture of related metabolites is more effective than the individual metabolites themselves (Dyer et al. 2003). If SMs act synergistically, effective protection against herbivores can be achieved at a lower concentration of the sum of the mixture than that of the single SMs. As SMs are generally assumed to be costly (Strauss et al. 2002) natural selection would favour mixtures of SMs showing synergistic effects. While it is more and more realized that interactions between SMs are important only few studies demonstrated this in the context of plant-insect relationships. When interactions have been studied it mostly concerns those between SMs within a structurally related class. Examples include the antagonistic effects of two linear furanocoumarins on the beet armyworm *Spodoptera exigua* (Diawara et al. 1993), the synergistic effects of two and three way interactions of amides on several insects and fruit-associated fungi (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 the synergistic effects of two iridoid glycosides on a specialist caterpillar *Junonia coenia* (Richards et al. 2012).

In this paper, we studied the effects of structurally related PAs from *Jacobaea vulgaris* (synonym: *Senecio jacobaea*) on a generalist herbivore: the western flower thrips (*Frankliniella occidentalis*). *J. vulgaris* is a common plant in Europe and contains more than 37 different PAs (Cheng et al. 2011a). In *Senecio* species, all PAs except senecivernine are derived from senecionine N-oxide (Hartmann 2007; Langel et al. 2011). In *Senecio* spp, including the species that are now part of the genus *Jacobaea*, senecionine N-oxide is synthesized in the roots and transported via the phloem to the shoots, where it is metabolized into other PAs (Hartmann and Toppel 1987; Hartmann and Dierich 1998; Hartmann et al. 1989). On the basis of the biosynthetic pathway, PAs in *J. vulgaris* are divided into four groups: senecionine-, erucifoline-, jacobine-, and otosenine-like PAs (Cheng et al. 2011a).

Most studies on the effects of specific PAs on herbivores, e.g. the thrips *F. occidentalis*, the slug *Deroceras invadens* and the wireworms *Agriotes lineatus*, used regression analysis and whole plants with different chemotypes or genotypes (Leiss et al. 2009; Cheng et al. 2011b; Kostenko et al. 2013; Wei et al. 2015). Especially jacobine-like PAs and their N-oxides were negatively correlated with the feeding damage of thrips (Leiss et al. 2009; Cheng et al. 2011b). As such, correlative studies do not show causation. PAs are always present as a mixture, varying in relative and absolute amounts, so direct evidence of a protective role of individual PAs is still equivocal. In addition, correlative studies are not always sensitive enough to clarify the potential bioactivity of all PAs. So, even PAs that are not detected with correlative studies still might show bioactivity.

The most straight forward way to get around this problem is to test individual PAs by using bioassays with artificial diets where single metabolites can be added. Macel et al. (2005) performed such bioassays, in which they tested different PAs on several herbivores. They were able to show that PAs differed in their effects on herbivores. In addition, different herbivores were differentially affected by the same PA (Macel et al. 2005). However, their study was restricted to commercially available PAs, which were mainly senecionine-like PAs and predominantly in the free base form and not the corresponding N-oxides that predominantly occur in plants. Therefore, they were not able to test differences between different groups of PAs occurring in the same species (*J. vulgaris*). More specifically, the prominent jacobine- and erucifoline-like PAs of *J. vulgaris* were not tested. For this study we isolated and synthesized jacobine and erucifoline and their corresponding N-oxides to include them in thrips bioassays.

Most PAs stored in plants are PA N-oxides (Hartmann et al. 1989). Some jacobine-like PAs are reported to occur upto 50% as free base in *J. vulgaris* (Joosten et al. 2011). There is still a lack of substantial experimental data comparing the effects of PA N-oxides versus free-base PAs on insects. The limited data available seem to suggest that free base PAs are more active than their corresponding N-oxides (see Nuringtyas et al. 2014). In the case of

vertebrates e.g. livestock, it is thought that the corresponding N-oxides show similar toxicity as free base PAs because they are reduced in the gut to the free base PAs (Fu et al. 2004; Wiedenfeld 2011).

The purpose of the present study is to first, examine the effects of individual free base PAs and their corresponding N-oxides on the generalist thrips *F. occidentalis*. Secondly we assessed whether PA N-oxides act synergistically in their effect on thrips. We concentrated on synergism between N-oxides because these represent the majority of PAs in *J. vulgaris.*

Material and methods

Pyrrolizidine alkaloids

We tested 10 PAs occurring in *Jacobaea* species representing the three most abundant groups of PAs within *Jacobaea* species: senecionine-like PAs (senecionine, senecionine N-oxide, seneciphylline, seneciphylline N-oxide, retrorsine and retrorsine N-oxide), erucifoline-like PAs (erucifoline and erucifoline N-oxide) and jacobine-like PAs (jacobine and jacobine N-oxide) (Cheng et al. 2011a). As an outgroup we included the structurally different PAs monocrotaline and monocrotaline N-oxide, that occur in *Crotolaria* species. Monocrotaline has been identified to be involved in plant defence against insect herbivores, showing toxic post-ingestive effects on *Spodoptera littoralis* larvae (Dominguez et al. 2008). Also, monocrotaline was toxic and deterrent to honey bees at high concentrations (Reinhard et al. 2009).

All tested PAs are macrocyclic diesters with a retronecine/retronecine N-oxide base. Senecionine, seneciphylline, retrorsine, erucifoline and jacobine and their corresponding N-oxides have a 12-membered macrocyclic ring, while monocrotaline and its corresponding N-oxide have an 11-membered ring (Figure 1).

Retrorsine (R-0382, Lot 70K3450) and retrorsine N-oxide (R-0507, Lot 31K1407) were purchased from Sigma Aldrich (St. Louis, MO, USA). Senecionine (Batch NO. 12100121) was purchased from PhytoPlan (Heidelberg, Germany). Seneciphylline, jacobine and erucifoline were obtained from EXPLANT, Leiden, The Netherlands, with a purity of 91%, 99% and 97% respectively. Monocrotaline (No. 3418.1) was purchased from Carl Roth (Karlsruhe, Germany). PA N-oxides were obtained from EXPLANT, Leiden, The Netherlands as white powders with a purity > 96%.

PAs were dissolved in MeOH to a concentration of 233 mM and from these stock solutions dilutions of 13.3, 23.3, 46.7 and 133 mM were prepared in methanol. These solutions were used to prepare the test solutions for the thrips bioassay.

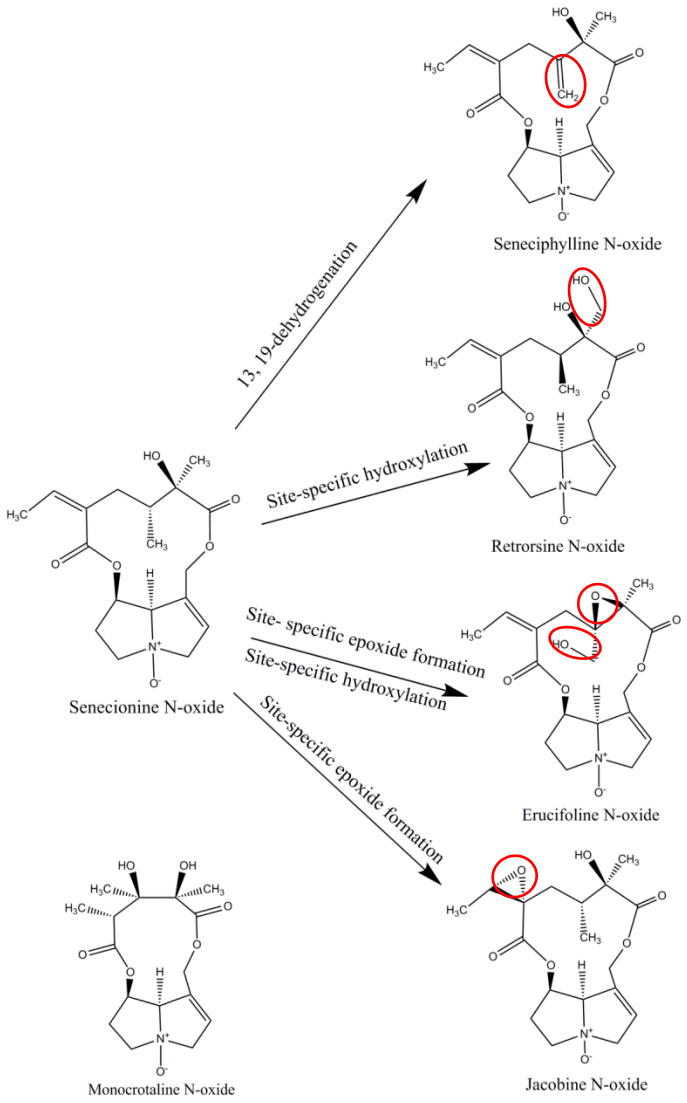


Figure 1. Chemical structures of pyrrolizidine alkaloids (PAs) and a simplified bio-synthetic pathway of PAs in *Jacobaea* species (after Hartmann 1989).

Thrips bioassay

The thrips bioassay with *F. occidentalis* larvae was adapted from Leiss et al. (2013). Wells of adapted 96-well plates were filled with 55 µL test solution (Figure S1). 1.94 mL of the test solution consisted of 10% fructose in 40 mM phosphate buffer pH 7, to which 60 µL of PA solution in methanol had been added. The 40 mM phosphate buffer was prepared from 1M phosphate buffer (pH 7), which in turn had been prepared by mixing 57.7 mL of 1M

NaH_2PO_4 and 42.3 mL of 1M Na_2HPO_4. The concentration of MeOH in the test solution was always 3%. The test solution without PAs but containing 3% MeOH was used as a negative control. There were two positive controls for each group: a solution with the insecticide abamectine (50 µg/mL) and empty wells (without solution) to verify that thrips larvae could not survive without feeding. Each bioassay was carried out using four 96-well plates at the same time. In one 96-well plate all the treatments (i.e. the negative control, 5 treatment groups (5 concentrations of a single PA), and two positive control groups) were placed in one column and therefore consisted of 8 replicates. Using four 96 wells plates for one experiment we obtained 32 replicates for all treatments. Single second instar larvae of thrips were placed into each cup of an 8 cup strip. Each cup was sealed with parafilm and placed on top of the 96 well plates and plates were put upside down. Thrips were then able to feed from the offered test solutions through the parafilm (Figure S1). The plates were randomly placed in a growth chamber with standard thrips rearing conditions (L:D, 12:12, 23°C).

Experiments with single PAs were conducted once (32 replicates per treatment). Experiments that included also combinations of PAs were conducted twice (2 x 32 replicates). The latter was to have two independent estimates of thrips survival for analysis of variance. After five days, the numbers of surviving larvae were recorded with a stereo microscope.

Second instar larvae of *F. occidentalis* were obtained from a lab culture reared on chrysanthemum flowers in a growth chamber with standard thrips rearing conditions (L:D, 12:12, 23°C).

Experiments with single PAs

Free base PAs and their corresponding N-oxides were tested at a series of concentrations. PAs were added to the test solution to reach concentrations of: 0.4, 0.7, 1.4, 4 and 7 mM. Due to their low solubility, the free bases of senecionine and seneciphylline could not be dissolved in the test solution at higher concentrations. For this reason seneciphylline could not be tested at 7 mM and senecionine could not be tested at 4 and 7 mM. Except for monocrotaline and its N-oxide, the concentrations of 0.4, 0.7, 1.4, 4 and 7 mM were equivalent to 0.25x, 0.5x, 1.0x, 2.5x and 5.0x the average total PA concentration in fresh *J. vulgaris* Gaertn. plants.

Experiments with combinations of PA N-oxides

Senecionine N-oxide, jacobine N-oxide and erucifoline N-oxide are the dominant PA N-oxides in *J. vulgaris* (Joosten et al. 2011). These three N-oxides were paired with each

other at four doses, i.e. 0.4, 0.7, 1.4 and 4 mM. These doses were the same as used for testing the individual PAs, except that the highest concentration (7 mM) was omitted.

Statistical analysis

Construction of the "null interaction" model

To evaluate interactions, one typically constructs a "null interaction" model that predicts the effect of metabolites in the absence of an interaction. We used a multiplicative model which is appropriate for studying survival because an organism cannot die twice.

In a multiplicative null model, assuming no interaction effects, the survival after the application of a combination of metabolite X and metabolite Y (S_{X+Y}) is the product of the survival after applications of X (S_X) and Y (S_Y), respectively, i.e., $S_{X+Y} = S_X * S_Y$. The underlying assumption of the multiplicative model is that the relationships between log survival and the concentrations of the tested individual metabolites are linear.

Correcting for survival in the negative control

The survival in the negative control was in all cases higher than 85%. Nevertheless, we corrected survival data for differences among the negative controls in the following way. The observed experimental survival of thrips (S_{X+NC}) results from the survival after application of the tested metabolite (S_X) and from the survival in the solvent (S_{NC}). The effect of the solvent is measured in the negative control. Under the assumption that the two effects are independent, we get the following equation:

$$\mathbf{S_{X+NC} = S_{NC} * S_X} \qquad \mathbf{(1)}$$

The effect of the metabolite S_X on survival can thus be calculated as

$$\mathbf{S_X = S_{X+NC} / S_{NC}} \qquad \mathbf{(2)}$$

Testing the interaction effects of the combination of two metabolites on survival

In the case of a combination of metabolites, the survival of thrips for the combination of metabolites X and Y (S_{X+Y}) results from the survival after application of the single metabolite X (S_X), the survival after application of the single metabolite Y (S_Y), and their interaction (S_{X*Y}).

$$\mathbf{S_{X+Y} = S_X * S_Y * S_{X*Y}} \qquad \mathbf{(3)}$$

Consequently, the effect of the interaction of two metabolites on survival can be calculated by:

$$\mathbf{S_{X*Y} = S_{X+Y} / (S_X * S_Y)} \qquad \mathbf{(4)}$$

In which $S_{X+Y,}$ S_X and S_Y are derived using equation (2). In equation 4, S_{X+Y} is the observed survival in experiments with mixtures while S_X and S_Y are the observed thrips survival in experiments with single metabolites while S_{X*Y} denotes the interaction effect. The interaction effect S_{X*Y} denotes the effect of the interactions between the two single metabolites on thrips survival. If the interaction effect S_{X*Y} is one it indicates that there is no interaction. If the interaction effect S_{X*Y} is larger than one it indicates an antagonistic interaction and if it is smaller than one it indicates a synergistic interaction.

As each experiment was repeated once, two independent estimates of the interaction effect S_{X*Y} are obtained.

To estimate if the interaction effect S_{X*Y} was significantly deviating from one, two-way ANOVAs were used with the concentration of metabolites X and Y as fixed factors. As dependent variables the estimates of S_{X*Y} -1 were used for all combinations of traits. Therefore if the intercept of the two-way ANOVA is significantly deviating from zero it indicates that S_{X*Y} -1 is deviating from zero and hence that the interaction effect S_{X*Y} is significantly deviating from one.

To obtain a visual representation of the effects of the single PA N-oxides and their combinations on thrips, we plotted fraction mortality (= 1- fraction survival) against the concentrations of the two metabolites in a 3-dimensional graph with the 2 horizontal axes (x and y) representing the individual metabolite concentrations, and the vertical axis (z) representing the mortality fraction.

Comparison of the effects of free base PAs and their corresponding N-oxides

Larval survival was log-transformed to obtain linear relationships with the tested concentrations. To determine whether there was a dose-related decrease, log survival was regressed against concentrations. We calculated the slopes of the regression lines and their 95% confidence intervals (CIs) to estimate and compare the effects of different PAs.

The slopes of log-transformed survival of the group of six free base PAs and the group of six corresponding N-oxides were compared by a paired-sample t-test.

All statistical analysis was performed using SPSS software for Windows (version 21.0; SPSS Inc., Chicago, IL).

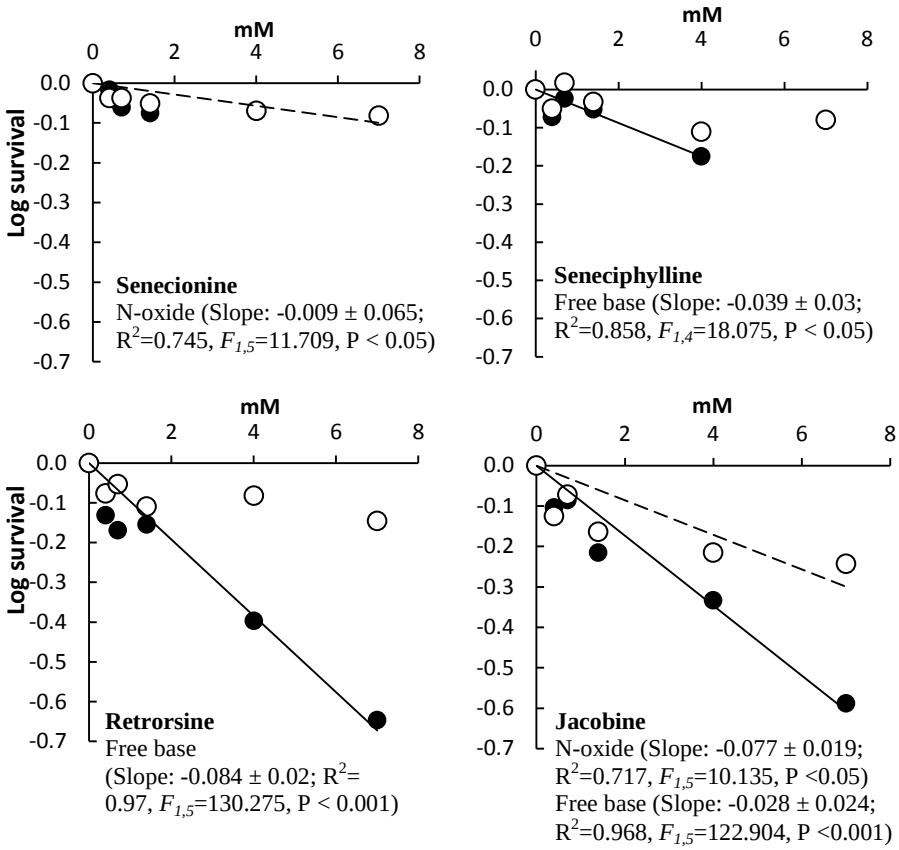
Results

Controls

The survival in the negative control was in all cases larger than 0.85. The positive control with the insecticide abamectine (50 µg/mL) solution showed an average survival of 0.11. The control with empty wells, that were included to verify that thrips cannot survive without feeding from a solution, had an average survival of 0.06.

Dose-dependent effects of individual PA N-oxides on thrips survival

We only found a significant dose-dependent effect on log-transformed thrips survival for senecionine N-oxide and jacobine N-oxide (Figure 2). Differences between the effects of the PA N-oxides were small and none of the slopes differed significantly from each other (Figure 2).



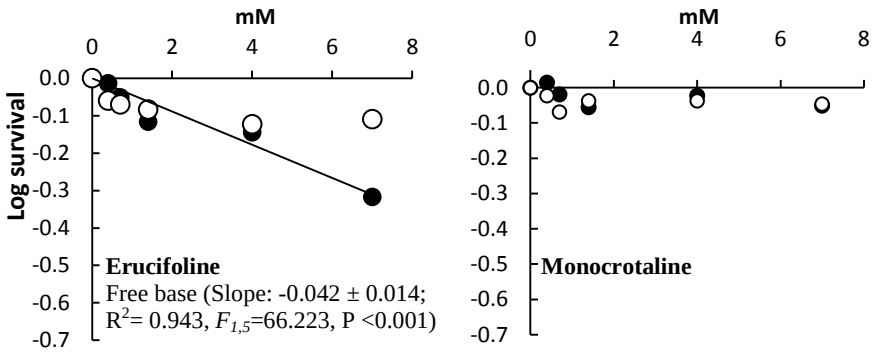


Figure 2. Log survival of 2nd instar western flower thrips (*Frankliniella occidentalis*) against the concentrations of individual pyrrolizidine alkaloids (PAs). Survival was corrected with the corresponding negative control (see M&M section). Thrips larvae were put on an artificial diet with PAs at five concentrations for 5 days. Solid dots represent the results of free base PAs while open dots represent the PA N-oxides. Only significant regression lines are shown. The slopes of log-transformed survival ± their 95% confidence intervals and there statistics are indicated in each panel.

Effects of individual free base PAs on thrips survival

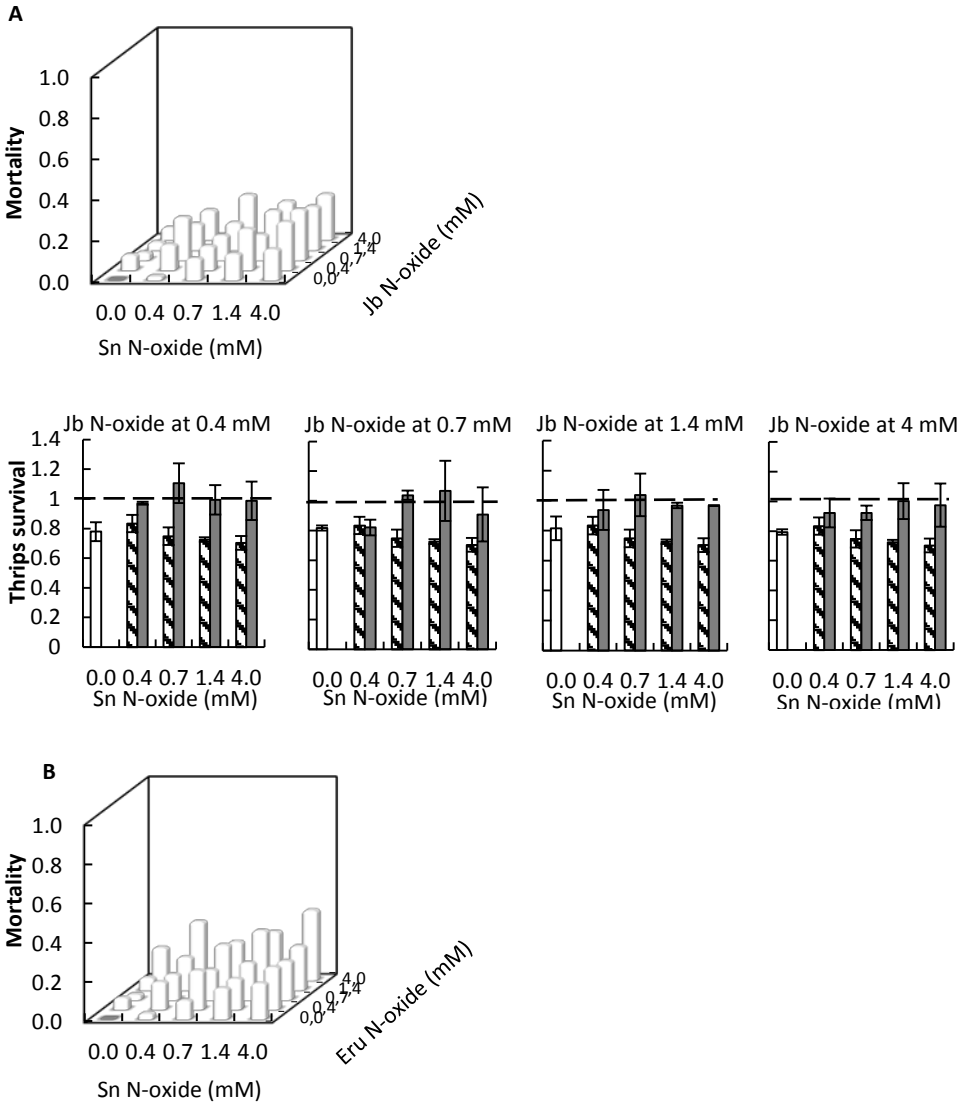
Except for monocrotaline and senecionine, all other PAs showed a dose-dependent decrease of thrips survival (Figure 2). The absence of a significant dose dependent relationship for senecionine might be due to the fact that only the lower concentrations could be tested. For the PAs occurring in *J. vulgaris*, the rank of slopes that were significant, starting with the steepest one, was retrorsine (-0.084) > jacobine (-0.077) > erucifoline (-0.042) > seneciphylline (-0.039) (Figure 2). Based on the 95% confidence intervals of the slopes, log-transformed survival differed between monocrotaline and retrorsine/erucifoline/ jacobine, and between erucifoline and retrorsine/jacobine. For the other PAs the 95% confidence intervals overlapped (Figure 2).

Comparison of the effects of free base PAs and the corresponding N-oxides

Generally, free base PAs did significantly differ in slope for the relationship between log thrips survival and concentration from the corresponding PA N-oxides (Paired-samples t-test, t = -4.0, *df* = 5, P < 0.01). The averages (± 95% CIs) were -0.051 (± 0.012) for the free base PAs and -0.013 (± 0.003) for the PA N-oxides. The 95% confidence intervals of the slopes showed that retrorsine, jacobine and erucifoline had a stronger effect on survival than the corresponding PA N-oxides (Figure 2).

The effects of combined PA N-oxides

For all three tested combinations of PA N-oxides no indications for an interaction effect on thrips survival were found (Figure 3). The intercept of the two-way ANOVAs did not significantly deviate from zero indicating that the PA N-oxides neither showed antagonistic nor synergistic effects on thrips survival (Table 1).



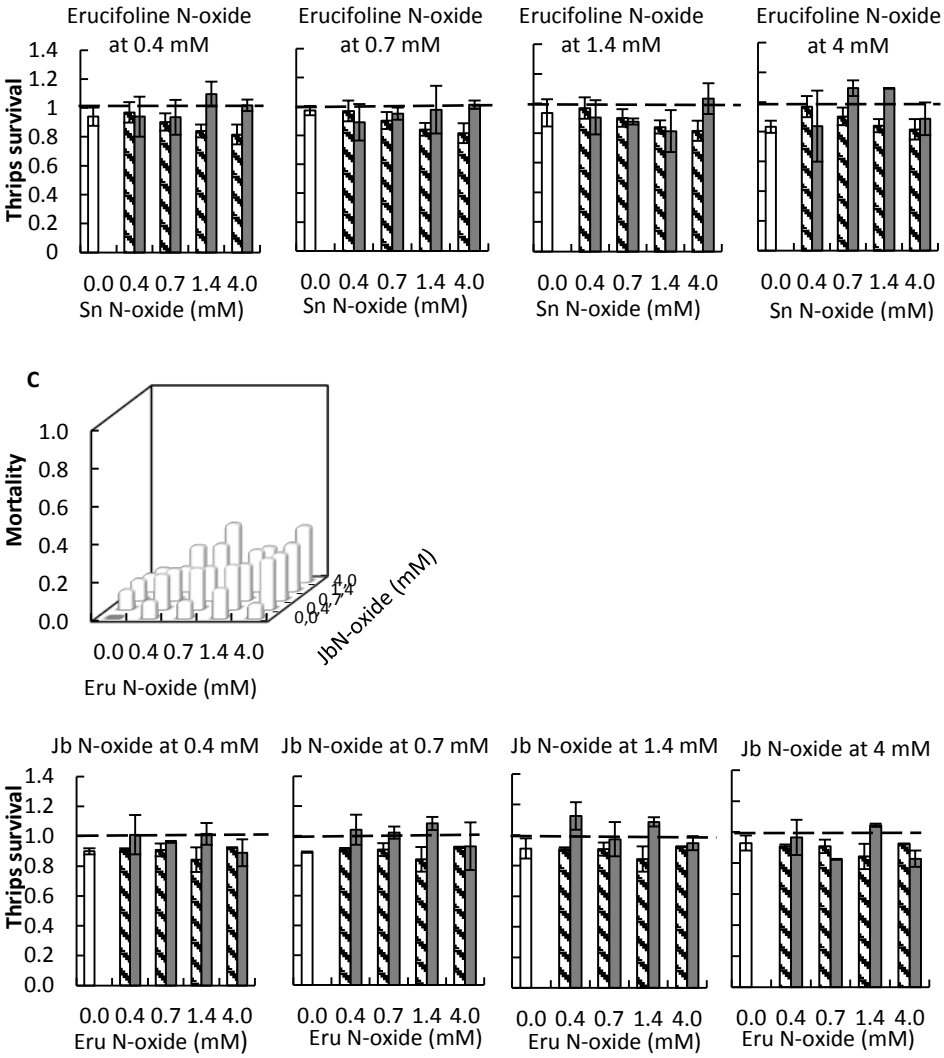


Figure 3. Top: Fraction mortality (1- fraction survival) of 2nd instar larvae of thrips (*Frankliniella occidentalis*) caused by the combinations of senecionine N-oxide and jacobine N-oxide (**A**), senecionine N-oxide and erucifoline N-oxide (**B**), erucifoline N-oxide and jacobine N-oxide (**C**). **Bottom**: Fraction survival (mean ± 95% confidence intervals) of thrips caused by PA N-oxide alone (white bars and hatched bars) at four concentrations and the interaction effects (S_{X*Y}) (Equation 2) between PA N-oxides (grey bars). 2nd instar larvae of the western flower thrips (*F. occidentalis*) were put on an artificial diet with PAs at various concentrations for 5 days. Data represent the results of two independent bioassays. In the 3D figures, the fraction mortality is plotted instead of fraction survival to increase the readability of the figure. In the bottom figures, dashed lines indicate no interactions. A significant deviation from the line would indicate synergistic or antagonistic effects between the two PAs tested however none deviated significantly from 1. Two-way ANOVAs were used to analyze if the interaction effect S_{X*Y} deviated from one.

Table 1. The interaction effects of combinations of pyrrolizidine alkaloid (PA) N-oxides on survival of 2nd instar western flower thrips (*Frankliniella occidentalis*). Two-way ANOVAs with the individual metabolite concentrations as fixed factors and the interaction effect S_{X*Y} minus one (see text) as a dependent variable. Combinations of three different PA N-oxides were tested at 16 different concentrations with two independent bioassays. The intercepts of the two-way ANOVAs are not significant. This indicates that they do not deviate significantly from zero. In turn this indicates that the interaction effect S_{X*Y} is not deviating from one and hence that there are no significant interactions between PA N-oxides.

Factors	***df***	***F***	***P***
Intercept	1, 31	1.09	0.31
Senecionine N-oxide (Sn NO)	3, 31	0.88	0.47
Jacobine N-oxide (Jb NO)	3, 31	0.25	0.86
Sn NO * Jb NO	9, 31	0.24	0.98
Intercept	1, 31	2.42	0.14
Sn NO	3, 31	0.88	0.47
Erucifoline N-oxide (Eru NO)	3, 31	0.44	0.73
Sn NO * Eru NO	9, 31	0.47	0.88
Intercept	1, 31	1.16	0.30
Eru NO	3, 31	3.15	0.54
Jb NO	3, 31	1.48	0.26
Eru NO * Jb NO	9, 31	0.21	0.99

Discussion

Are free base PAs more toxic than their corresponding N-oxides to thrips?

As expected on basis of results from previous studies on other insects we found that the free base PAs were more toxic to thrips larvae than their corresponding N-oxides. It has been reported that the total free base PA extracts of *Cynoglossum officinale* were more effective than the total PA N-oxide extracts on the caterpillar *S. exigua* (van Dam et al. 1995). The free base PA extract from *Adenostyles alliariae* leaves was deterrent for the snail *Arianta arbustorum* (Helicidae) while the N-oxide extract was not (Speiser et al. 1992). Dreyer et al. (1985) showed that riddelline and monocrotaline were more toxic to the pea aphid (*Acyrthosiphon pisum*) than their corresponding N-oxides. Macel et al. (2005) found that retrorsine was equally effective against the locust *L. migratoria* than retrorsine N-oxide. In the same article, they found that both retrorsine and retrorsine N-oxide significantly decreased the survival of the thrips *Frankliniella occidentalis* but retrorsine was more toxic than retrorsine N-oxide. Also, heliotrine, lasiocarpine and monocrotaline were more active than their corresponding N-oxides against nematodes (Thoden et al. 2009). A recent study showed that jacobine, erucifoline and seneciphylline were more toxic than their respective N-oxides towards *S. exigua* cell lines and larvae that were injected with these PAs (Nuringtyas et al. 2014). In contrast to these findings, integerrimine exhibited similar

antifeedant activity against the beetle *L. decemlineata* as integerrimine N-oxide (Reina et al. 2001) and senecionine N-oxide was more toxic than senecionine for *A. pisum* (Dreyer et al. 1985).

In vertebrates, free base PAs and PA N-oxides do not have different effects. This is because the N-oxides are reduced in the gut. Probably such a reduction does not take place in the guts of insects. The detailed knowledge about the mechanisms of PA toxicity in vertebrates (Steenkamp et al. 2000; Stewart and Steenkamp 2001; Fu et al. 2004; Wiedenfeld 2011; Langel et al. 2011) is contrasted by the paucity of studies concerning PA toxicity in insects. Lindigkeit et al. (1997) suggested that difference between toxicity of free base PAs and PA N-oxides on insects might be related to cell permeability. In general, highly hydrophobic or lipophilic metabolites pass cell membranes easier because the membrane is composed of phospholipids. Most free base PAs are rather lipophilic and able to permeate bio-membranes, while N-oxides are polar and more hydrophilic. In the context of the plant-herbivore associations, however, it is presently not yet clear why plants store PAs primarily in the least active form.

Do PAs differ in their effects on thrips?

Among the tested free base PAs, we found jacobine and retrorsine to be the most active against thrips, followed by erucifoline and seneciphylline, while senecionine and monocrotaline did not result in significant dose-dependent effects on thrips survival. For jacobine and erucifoline, thrips survival was reduced at concentrations that were similar to those in plants. Retrorsine affected thrips survival at concentration of 1.4 mM which is far higher than the concentrations that usually found in plants (20 µM according to Joosten et al. 2009). We can therefore predict that its role in plants against thrips will be negligible. For 1st instar larvae of western flower thrips, Macel et al. (2005) found significant effects only at concentrations equivalent to 10 x plant total PA concentration for senecionine, seneciphyline, retrorsine, monocrotaline while heliotrine did not affect thrips survival at all.

Do the tested PAs have effects on other herbivores?

With respect to the effects of PAs on *Spodoptera*, Dominguez et al. (2008) found that monocrotaline, when orally injected, reduced biomass gains of *S. littoralis* larvae. Macel et al. (2005) did not find any deterrent effects among (free base) monocrotaline, senecionine, retrorsine, seneciphylline and jacobine as well as retrorsine N-oxide on the feeding of *S. exigua*. However, when comparing the toxicity of PAs towards *S. exigua* cell line and injecting caterpillars, Nuringtyas et al. (2014) found jacobine was the most toxic, followed by erucifoline and seneciphylline, while senecionine and retrorsine were not toxic.

Dreyer et al. (1985) found modest feeding deterrence of free base PAs (monocrotaline, seneciphylline, and senecionine), weak effects of senecionine N-oxide and no effect of monocrotaline N-oxide towards *A. pisum*. Dominguez et al. (2008) found *M. persicae* settling behavior was affected by erucifoline while feeding behavior of *Myzus persicae* was negatively affected by monocrotaline, senecionine and seneciphylline (Macel et. al. 2005). In the same study, senecionine, integerrimine, and seneciphylline were strong antifeedants to *L. decemlineata.* In contrast, Dominguez et al. (2008) did not find any significant differences among the effects of erucifoline on *M. persicae* or *L. decemlineata*.

Altogether these studies show that individual PAs affect specific herbivores differently but also that different herbivores react differently towards the same PA. The latter may explain the chemical diversity of PAs in plants. For instance, jacobine significantly reduced thrips survival while it did not show any deterrent effect on the feeding of *S. exigua* (Macel et al. 2005). A possible explanation for the various effects of PAs on insect herbivores could be related to the insects' physiological process. For instance, the larvae of the noctuid moth *S. littoralis* prevent the toxicity of senecionine by means of efficiently excreting this compound (Lindigkeit et al. 1997). However, to date very little is known about the underlying mechanisms of PA toxicity in insects.

Does this study backup the correlative findings of studies with whole plants?

The negative effects of individual PAs on thrips we found here corroborate to some extent the results of the correlative studies conducted previously. Most studies of the effects of PAs on insect herbivores are correlative studies with whole plants, indicating the roles of PAs in plant resistance against herbivores (see review by Trigo 2011; Macel 2011; Kostenko et al. 2013). For instance, total PA concentration was negatively correlated with feeding damage of thrips (Cheng et al. 2011b) and the growth rates of the generalist aphid *Brachycaudus cardii* (Vrieling et al. 1991). Few studies demonstrated that structural groups or individual PAs were more strongly related to insect performance than others. Jacobine-like PAs had a stronger effect on *F. occidentalis* (Leiss et al. 2009; Cheng et al. 2011b).

Studies on the effects of individual PAs on insects in bioassays with artificial diets do not always present the same picture as correlative studies. For instance, in the present study we found that retrorsine strongly affected thrips survival while it did not show a significant relationship with thrips damage in Cheng et al's (2011b) study. This discrepancy can be explained by the fact that retrorsine occurs in plants at very low concentrations. Correlative studies with whole plants are not always sensitive enough to test the potential effects of all PAs. An inconsistency we found is that there is a much stronger effect of free base jacobine than jacobine N-oxide in bioassays with artificial diets while in the correlative study the negative correlations between jacobine-type N-oxides and thrips damage were slightly stronger than those of free base jacobine-type PAs. The correlation between thrips damage

and jacobine N-oxide may result from the correlation between jacobine N-oxide and jacobine. The disadvantage of correlative studies with whole plants is that correlation does not show causation. Another possible explanation is that jacobine N-oxide becomes active in concert with other metabolites, emphasizing the importance of studying the interactions between plant SMs.

Do PA N-oxides show synergistic effects on thrips survival?

One of the hypotheses for the diversity of plant SMs states that a mixture of related metabolites is more effective than individual metabolites (Nelson and Kursar 1999; Macel et al. 2005; Richards et al. 2012; Sheth and Thanker 2014). The idea that the interactions of several metabolites might be an important aspect of plant defence against herbivores is generally acknowledged. There is growing evidence that synergistic interactions affect herbivore performance, such as Piper amides on *S. frugiperda* caterpillars (Dyer et al. 2003), iridoid glycosides on the specialist buckeye caterpillar, *Junonina coenia* (Richards et al. 2010), cacalol and seneciphylline from *Adenostyles alliariae* on three specialist and three generalist insect herbivores (Hagele and Rowell-Rahier 2000). The importance of interactions among PAs has not been investigated in great detail yet. Macel et al. (2005) found a weak synergistic effect of a mixture of the free bases of senecionine, seneciphylline and senkirkine on *S. exigua* and of a mixture of the free bases of senecionine and seneciphylline on *L. migratoria*. Siciliano et al. (2005) tested the mixture of two PAs (1:1) isolated from *Anchusa strigosa*. But they did not find any synergistic effects in a feeding assay with *S. exigua*. In addition, the previous studies have only tested the effect of the combinations of free base PAs though PAs in plants occur mainly as PA N-oxides. Although in *Jacobaea* species the majority of PAs are N-oxides (Joosten et al. 2011), interacting effects among PA N-oxides have not been studied yet. Here, for the first time, we showed that the combination of the most abundant PA N-oxides of *J. vulgaris*, senecionine N-oxide, jacobine N-oxide and erucifoline N-oxide (Joosten et al. 2011) showed no synergistic (or antagonistic) effects on thrips survival. Although we tested only a limited number of PAs and restricted ourselves to two-way interactions our result seem to indicate that interactions among PA N-oxides do not provide an explanation for why they are more abundant than free base PAs.

Interactions can also occur between metabolites of different classes, which however have been even less studied in the context of plant-insect associations (Berenbaum and Neal 1985; Neal 1989; Nelson and Kursar 1999; Guillet et al. 1998; Nuringtyas, PhD thesis, 2014). As was mentioned, to reach the target sites and to be active, a metabolite has to pass through several steps in an insect, such as metabolization, detoxification, sequestration, and secretion, etc. (Berenbaum 2002; Despres et al. 2007). Many of these processes can be influenced by metabolites from different classes. As is true for many plants, besides PAs, a wide diversity of PMs and SMs has been reported to be present in *Jacobaea* species (Kirk

et al. 2005; Leiss et al. 2009), e.g. sugars (sucrose), amino acids (alanine), carboxylic acids (succinic, fumaric and malic acids), phenolic acids (chlorogenic, feruloylquinic acids), flavonoids (kaempferol), benzoquinoids (jacaranone). There is a high possibility of interactions between PAs and other SMs. Interestingly, a preliminary study with *S. exigua* cell lines presented evidence for antagonistic interactions between jacobine and chlorogenic acid (CGA) (Nuringtyas 2014). If such antagonistic interactions would not occur for PA N-oxides this might explain their higher concentrations compared to free base PAs.

Conclusions

In this study, we focused on the defence functions of individual plant SMs against a generalist insect herbivore. We found negative effects of PAs from *J. vulgaris* on larval survival of western flower thrips, which demonstrate the protective roles of PAs in plant defence against insect herbivores. Specially, four out of six free base PAs and two out of six PA N-oxides significantly decreased thrips survival in a concentration-dependent way. We also found that structurally related PAs differed in their effects on thrips. With respect to the bioactivity of the two forms of PAs, free base PAs were observed to be more toxic to thrips than their corresponding N-oxides. Among the six free base PAs tested, jacobine and retrorsine were the most toxic, followed by erucifoline and seneciphylline while senecionine and monocrotaline did not cause significant dose-dependent effects on thrips survival. We measured the effects of combinations of three different PA N-oxides that occur within the same species on insects. PA N-oxides did not interact in their effects on thrips survival. Therefore we did not find proof for the hypothesis that synergistic interactions between PA N-oxides may explain why PAs are stored in the plant mainly as N-oxides.

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References

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011a. Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. New Phytol 192:1010-1023

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011b. The relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance in F(2) hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. J Chem Ecol 10:1071-1080

Cornell HV, Hawkins BA. 2003. Herbivore responses to plant secondary compounds: a test of phytochemical coevolution theory. Am Nat 161:507-522

Domínguez DM, Reina M, Santos-Guerra A, Santana O, Agulló T, et al. 2008. Pyrrolizidine alkaloids from Canarian endemic plants and their biological effects. Biochem Syst Ecol 36:153-166

Dreyer DL, Jones KC, Molyneux RJ. 1985. Feeding deterrency of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrthosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. J Chem Ecol 11:1045-1051

Dyer LA, Dodson CD, Stireman JO, Tobler MA, Smilanich AM, et al. 2003. Synergistic effects of three Piper amides on generalist and specialist herbivores. J Chem Ecol 29:2499-2514

Ehrlich PR, Raven PH. 1964. Butterflies and plants – a study in coevolution. Evolution 18:586-608

Farrell BD, Mitter C. 1993. Phylogenetic determinants of insect/plant community diversity. In: Ricklefs RE, Schluter D (eds). Species diversity in ecological communities: historical and geographic perspectives. University of Chicago Press, Chicago. pp 253-266

Fu PP, Xia Q, Lin G, Chou MW. 2004. Pyrrolizidine alkaloids-genotoxicity, metabolism enzymes, metabolic activation, and mechanisms. Drug Metab Rev 36:1-55

Gols R. 2014. Direct and indirect chemical defences against insects in a multitrophic framework. Plant Cell Environ 37:1741-1752

Gonzalez-Coloma A, Valencia F, Martín N, Hoffmann JJ, Hutter L, et al. 2002. Silphinene sesquiterpenes as model insect antifeedants. J Chem Ecol 28:117-129

Hagele BF, Rowell-Rahier M. 2000. Choice, performance and heritability of performance of specialist and generalist insect herbivores towards cacalol and seneciphylline, two allelochemicals of *Adenostyles alpine* (Asteraceae). J Evol Biol 13:131-142

Hartmann T, Dierich B. 1998. Chemical diversity, variation of pyrrolizidine alkaloids of the senecionine type, biological need or coincidence? Planta 206:443-451

Hartmann T, Ehmke A, Eilert U, von Borstel K, Theuring C. 1989. Sites of synthesis, translocation and accumulation of pyrrolizidine alkaloid N-oxides in *Senecio vulgaris*. Planta 177:98-107

Hartmann T, Toppel G. 1987. Senecionine N-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris*. Phytochemistry 26:1639-1643

Hartmann T, Witte L. 1995. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. In: Pelletier SW (ed) Alkaloids. Chemical and biological perspectives, vol 9. Pergamon Press, Oxford, pp 155-233

Hartmann T. 1996. Diversity and variability of plant secondary metabolism: a mechanistic view. Entomol Exp Appl 80:177-188

Hartmann T. 2007. From waste products to ecochemicals: fifty years research of plant secondary metabolism. Phytochemistry 68:2831-2846

Johnson MTJ. 2011. Evolutionary ecology of plant defences against herbivores. Funct Ecol 25:305-311

Joosten L, Mulder PPJ, Klinkhamer PGL, van Veen JA. Soil-borne microorganisms and soil-type effect pyrrolizidine alkaloids in *Jacobaea vulgaris*. Plant Soil 325:133-143

Joosten L, Cheng D, Mulder PPJ, Vrieling K, van Veen JA, et al. 2011. The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. Phytochemistry 72:214-222

Kliebenstein DJ, Kroymann J, Brown P, Figuth A, Pedersen D, et al. 2001. Genetic control of natural variation in Arabidopsis glucosinolate accumulation. Plant Physiol 126:811-825

Kostenko O, Mulder PPJ, Bezemer TM. 2013. Effects of root herbivory on pyrrolizidine alkaloid content and aboveground plant-herbivore-parasitoid interactions in *Jacobaea vulgaris*. J Chem Ecol 39:109-119

Langel D, Ober D, Pelser PB. 2011. The evolution of pyrrolizidine alkaloid biosynthesis and diversity in the *Senecioneae.* Phytochem Rev 10:3-74

Leiss KA, Choi YH, Abdel-Farid I, Verpoorte R, Klinkhamer PGL. 2009. NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. J Chem Ecol 35:219-229

Leiss KA, Cristofori G, van Steenis R, Verpoorte R, Klinkhamer PGL. 2013. An eco-metabolomic study of host plant resistance to western flower thrips in cultivated, biofortified and wild carrots. Phytochemistry 93:63-70

Lindigkeit R, Biller A, Buch M, Schiebel HM, Boppre M, et al. 1997. The two faces of pyrrolizidine alkaloids: the role of tertiary amine and its N-oxide in chemical defense of insects with acquired plant alkaloids. Eur J Biochem 245:626-636

Macel M, Bruinsma M, Dijkstra SM, Ooijendijk T, Niemeyer HM, et al. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. J Chem Ecol 31:1493-1508

Macel M. 2011. Attract and deter: a dual role for pyrrolizidine alkaloids in plant-insect interactions. Phytochem Rev 10:75-82

Mithofer A, Boland W. 2012. Plant defense against herbivores: chemical aspects. Annu Rev Plant Biol 63:431-50

Nelson AC, Kursar TA. 1999. Interactions among plant defense compounds: a method for analysis. Chemoecology 9:81-92

Nuringtyas TR, Verpoorte R, Klinkhamer PGL, van Oers MM, Leiss KA. 2014. Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* using insect cell lines and injection bioassays. J Chem Ecol 40:609-616

Pelser PB, de Vos H, Theuring C, Beuerle T, Vrieling K, et al. 2005. Frequent gain and loss of pyrrolizidine alkaloids in the evolution of *Senecio* section *Jacobaea* (Asteraceae). Phytochemistry 66:1285-1295

Reina M, González-Coloma A, Gutiérrez C, Cabrera R, Rodríguez ML, et al. 2001. Defensive chemistry of *Senecio miser*. J Nat Prod 64:6-11

Reinhard A, Janke M, von der Ohe W, Kempf M, Theuring C, et al. 2009. Feeding deterrence and detrimental effects of pyrrolizidine alkaloids fed to honey bees (*Apis mellifera*). J Chem Ecol 35:1086-1095

Richards LA, Lampert EC, Bowers MD, Dodson CD, Smilanich AM, et al. 2012. Synergistic effects of iridoid glycosides on the survival, development and immune response of a specialist caterpillar, *Junonia coenia* (Nymphalidae). J Chem Ecol 38:1276-1284

Sheludko Y, Gerasimenko I, Kolshorn H, Stockigt J. 2002. Isolation and structure elucidation of a new indole alkaloid from *Rauvolfia serpentina* hairy root culture: The first naturally occurring alkaloid of the of the raumacline group. Planta Med 68:435-439

Sheth BP, Thaker VS. 2014. Plant systems biology: insights, advances and challenges. Planta 240:33-54

Siciliano T, Leo MD, Bader A, Tommasi ND, Vrieling K, et al. 2005. Pyrrolizidine alkaloids from *Anchusa strigosa* and their antifeedant activity. Phytochemistry 66:1593-600

Speiser B, Harmatha J, Rowell-Rahier M. 1992. Effects of pyrrolizidine alkaloids and sesquiterpenes on snail feeding. Oecologia 92:257-265

Steenkamp V, Stewart MJ, Zuckerman M. 2000. Clinical and analytical aspects of pyrrolizidine poisoning caused by South African traditional medicines. Ther Drug Monit 22:302-306

Stewart MJ, Steenkamp V. 2001. Pyrrolizidine poisoning: A neglected area in human toxicology. Ther Drug Monit 23:698-708

Strauss SY, Rudgers JA, Lau JA, Irwin RE. 2002. Direct and ecological costs of resistance to herbivory. Trends Ecol Evol 17:278-285

Thoden TC, Boppre M, Hallmann J. 2009. Effects of pyrrolizidine alkaloids on the performance of plant-parasitic and free-living nematodes. Pest Manage Sci 65:823-830

Trigo JR. 2011. Effects of pyrrolizidine alkaloids through different trophic levels. Phytochem Rev 10:83-98

van Dam NM, Vuister LWN, Bergshoeff C, de Vos H, van der Meijden E. 1995. The ‘‘raison d'être’’ of pyrrolizidine alkaloids in *Cynoglossum officinale*: deterrent effects against generalist herbivores. J Chem Ecol 21:507-523

Vrieling K, Soldaat LL, Smit W. 1991. The influence of pyrrolizidine alkaloids of *Senecio jacobaea* on *Tyria jacobaea*, *Brachycaudus cardii* and *Haplothrips senecionis*. Neth J Zool 41:228-239

Wei X, Vrieling K, Mulder PPJ, Klinkhamer PGL. 2015. Testing the generalist-specialist dilemma: the role of pyrrolizidine alkaloids in resistance to invertebrate herbivores in *Jacobaea* species. J Chem Ecol 41:159-167

Wiedenfeld H. 2011. Plants containing pyrrolizidine alkaloids: toxicity and problems. Food Addit Contam 28:282-292

Supplementary Material

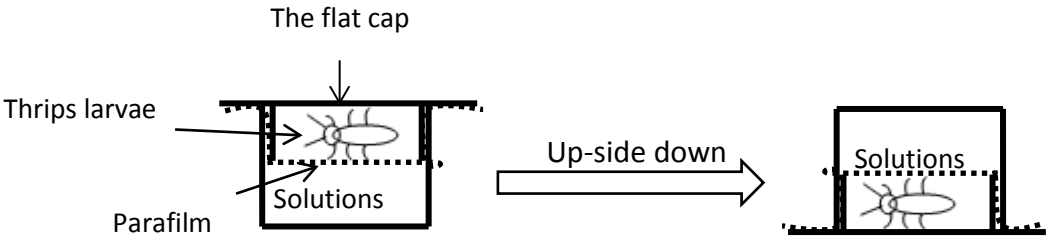


Figure S1. Experimental design of the *in vitro* thrips bioassay.

Chapter 3

Antagonistic interactions among plant metabolites in plant-insect interactions: a case study of pyrrolizidine alkaloids and chlorogenic acid on thrips

Xiaojie Liu, Klaas Vrieling, Peter G.L. Klinkhamer

Abstract

Plant metabolites play a crucial role in shaping the interactions between plants and herbivores. Plant metabolites are characterized by a high structural diversity leading to a high probability of interactions among plant metabolites. We investigated the effects of single and combinations of plant metabolites on a generalist herbivore, the thrips *Franklinella occidentalis* and detected antagonistic effects between chlorogenic acid (CGA) and free base pyrrolizidine alkaloids (PAs) on thrips survival. The magnitude of the antagonistic interaction of CGA differed for the 5 tested free base PAs. We hypothesize that antagonistic interactions represent a constraint on the accumulation of plant metabolites. We further investigated the roles of functional groups of the CGA molecule and found that the quinic acid part of the CGA molecule showed an antagonistic interaction with the PA retrorsine while the remaining caffeoyl group did not. Further experiments revealed that the carboxyl group at position C1 is essential for the antagonistic interactions with retrorsine while the hydroxyl groups were not. The results show that combinations of plant metabolites affect the bioactivity of individual metabolites on insects. Such interactions deserve more attention and the possibility of interactions among plant metabolites should be taken into account when designing experiments on their bioactivity.

Keywords: Antagonistic interactions, Chlorogenic acid, Functional groups, Pyrrolizidine alkaloids, Plant defence

Introduction

In nature plants are challenged by a multitude of herbivores and pathogens and they protect themselves against these attackers (Hartmann 2008; Gols 2014), with a highly diverse array of repellent, deterrent and/or toxic secondary and primary metabolites (SMs and PMs) (Johnson 2011; Mithofer and Boland 2012). The wide diversity of metabolites is estimated to include over 200,000 PMs and SMs in plants (Dixon and Strack 2003; Efferth and Koch 2011). This large diversity is not only due to differences among species but also comes from differences within species. For instance more than 170 SMs were recorded for *Arabidopsis thaliana* (D'Auria and Gershenzon 2005). The great variation of metabolites extends to different plant organs and tissues (Cheng et al. 2011) and even single cells (Kuhlisch and Pohnert 2015). In a single cell from *Arabidopsis* roots, more than 50 metabolites were recorded (Moussaieff et al. 2013). The natural phytochemical background in which plant metabolites occur is therefore highly variable and determined by the large number of other metabolites that are present in the same cell, tissue or organism. The co-occurrence of large numbers of metabolites potentially allows for an almost infinite number of interactions among these metabolites (Biavatti 2009). Such interactions can positively or negatively affect their bioactivity. However, although interactions between metabolites are expected to be common they seldomly are studied in an ecological context.

In this paper we focused on how interactions among plant metabolites can affect their activity against an insect herbivore. In the case of positive interactions, it can be argued that the combinations of metabolites with a greater efficiency against herbivores are selected for over the accumulation of a single defence metabolite at a high concentration that can be more costly (Nelson and Kursar 1999; Ryabushkina 2005). It is therefore expected that natural selection leads to an increased number of synergistic interactions between metabolites to increase overall plant fitness. Indeed, synergistic interactions between metabolites are reported, e.g. the effects of a combination of amides on several insects and fungi (Dyer et al. 2003; Richards et al. 2010; Whitehead and Bowers 2014), the effects of a combination of potato glycoalkaloids on the snail *Helix aspersa* (Smith et al. 2001), and the effects of a combination of iridoid glycosides on a specialist caterpillar *Junonia coenia* (Nymphalidae) (Richards et al. 2012).

Another example that may be indicative of synergistic interactions is the often reported loss of bioactivity upon fractionation of plant metabolite mixtures, particular in phytomedicine studies (see a review by Williamson 2001). The loss of bioactivity can be caused by the loss of synergistic interactions between metabolites (Williamson 2001; Herrera and Amor 2011; Labuschagne et al. 2012; Inui et al. 2012). For example, through fractionation and recombining fractions, Van Vuuren and Viljoen (2009) showed that the combination of volatile and non-volatile fractions of camphor bush *Tarchonanthus camphoratus* showed a higher anti-microbial activity than the two fractions alone.

Although the loss of activity upon fractionation of plant extracts is well known among pharmacologists, interactions between plant metabolites thus far received little attention from ecologists (but see above references). While the number of studies on synergistic effects is already very limited, potential antagonistic effects are even addressed less (but see Diawara et al. 1993; Nelson and Kursar 1999; Nuringtyas 2014). The reason for this may be that antagonistic effects are less common. If antagonistic interactions are common, one would expect that further fractionation would increase the activity because antagonistically interacting metabolites would be separated into different fractions (but see Kondoh et al. 1999). However, such a phenomenon is rarely found in phytochemical studies, suggesting that in general synergistic interactions between plant metabolites are more frequent than antagonistic interactions.

To understand antagonistic interaction from an evolutionary perspective is less straightforward than synergistic effects. In case a plant produces defence metabolites that are phytotoxic, an antagonistic interaction can be a way to reduce potential self-toxicity but at the same time that would reduce the effectiveness against herbivores. If essential PMs interact antagonistically with SMs, compartmentalization is a solution to avoid antagonistic interactions (Nelson and Kursar 1999). Indeed, metabolite content may differ largely between plant organs (Kuhlisch and Pohnert 2015) and even between different cell layers (Nuringtyas et al. 2012; Moussaieff et al. 2013). For example, the mesophyll of *Jacobaea vulagris* contained high amounts of the pyrrolizidine alkaloids (PAs) while CGA was accumulated in the epidermis (Nuringtyas et al. 2012). Yet, experimental evidence is needed to demonstrate the ecological significance of such compartmentalization.

To study the occurrence of interactions between metabolites we chose two groups of well characterized SMs, the free base PAs and organic acids (Hartmann and Ober 2000; Pelser et al. 2005). More than 400 PAs have been identified from about 6000 plant species (Chou and Fu 2006). PAs are well known for their deterrent and/or toxic effects towards insect herbivores, like the western flower thrips *Frankliniella occidentalis* (Macel et al. 2005; Leiss et al. 2009) and the beet armyworm (*S. exigua*) (de Boer 1999; Siciliano et al. 2005; Nuringtyas et al. 2014; Jing et al. 2015), and the lepidopteran *Heliothis virescens* (Cogni and Trigo 2016). Because of their negative effects on insects, it has been suggested that they can play an important role in plant-herbivore interactions.

CGA has a widespread occurrence in the plant kingdom. CGA may have a mixed effect on herbivores. Bi et al. (1997) found no effect of CGA on caterpillars of the generalist tobacco budworm *Heliothis virescens* and the specialist tobacco hornworm *Manduca sexta* feeding on tobacco, while CGA was found to be involved in resistance to the apple aphid *Aphis pomi* (Miles and Oertli 1993). Nuessly et al. (2007) also reported that maize plant resistance to corn earworm (*Helicoverpa zea*) was partly due to the presence of CGA. On the same insects, CGA showed bioactivity in a number of species while it did not show this in others.

For instance, CGA in *Chrysanthemum* was negatively correlated with the feeding damage of thrips *F. occidentalis* (Leiss et al. 2009), while no effect of CGA on thrips was detected in tomato, *Solanum lycopersicum* (Mirnezhad 2011), carrot *Daucus carota* L. (Leiss et al. 2013), and *Senecio jacobaea* (Leiss et al. 2009). The potential explanation for this difference could be that the effect of CGA depends on the phytochemical background in which it does occur through interactions with other plant metabolites.

In this study, we evaluated whether interactions exist between plant metabolites, and how these interactions affect the bioactivity against the western flower thrips. First, we studied the interactions between CGA and 5 free base PAs on the performance of thrips larvae. Second, to study if a ratio is present at which the combination may exhibit the strongest interaction effect, we tested a free base PA, retrorsine, in combination with CGA at various ratios. Finally, we used retrosine in combination with various acids for a detailed analysis of which functional groups of the organic acid molecule are involved in the interaction.

Materials and Methods

PAs and other metabolites

The five tested free base PAs are macrocyclic diesters with a retronecine base. Senecionine, retrorsine, erucifoline and jacobine have a 12-membered macrocyclic ring while monocrotaline has an 11-membered macrocyclic ring (Figure 1). Retrorsine was purchased from Sigma Aldrich (St. Louis, MO, USA). Monocrotaline was purchased from Carl Roth (Karlsruhe, Germany). Senecionine was purchased from Phytoplan (Heidelberg, Germany). Jacobine and erucifoline were isolated and purified by EXPLANT, Leiden,The Netherlands.

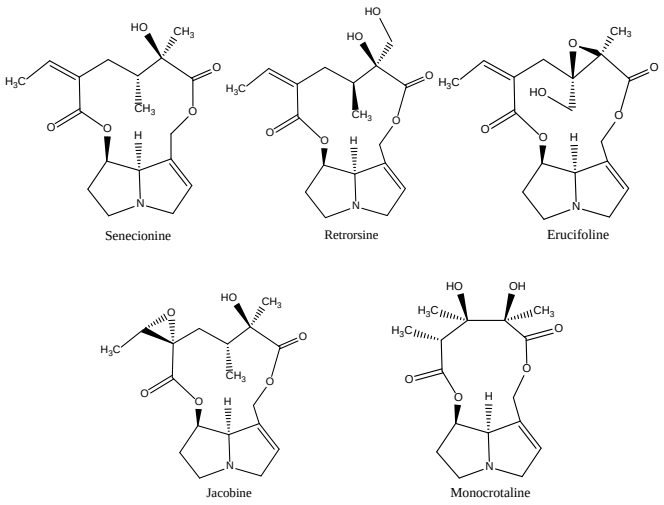


Figure 1. The chemical structures of free base pyrrolizidine alkaloids used in this study.

All organic acids used in the current study are derivatives of CGA in order to elucidate the potential involvement of functional groups of the CGA molecule in the interaction effects. CGA, caffeic acid, D-quinic acid, cyclohexanecarboxylic acid, D-glucuronic acid, *myo*-inositol, shikimic acid and malic acid were purchased from Sigma (St. Louis, MO, USA). 1-hydroxycyclohexanecarboxylic acid was purchased from MP Biomedicals, The Netherlands. Abamectin (Sigma Aldrich, USA) was used as a positive control.

Organic acids and PAs were dissolved in 30 µL methanol (MeOH) to prepare stock solutions (28 mM). From these stock solutions dilutions in MeOH were prepared with concentrations of 1.4, 2.8, 5.6 and 14 mM. These solutions were used to prepare test solutions for the thrips bioassay.

Thrips bioassay

The thrips bioassay with *F. occidentalis* larvae was conducted on adapted 96-well plates, which were filled with 55 µL test solutions (Figure S1, Chapter 2) covered with parafilm. Single second instar larvae of thrips were placed into each cup of an 8 cup flat-cup strip. Each cup was sealed with parafilm and then placed on top of the 96 well plates. Thrips were able to feed from the offered test solutions through the parafilm. The plates were randomly placed into a growth chamber with standard thrips rearing conditions (L:D, 12:12, 23°C). The test solutions consisted of 60 µL solution of the metabolites in methanol, dissolved in 1.94 mL 40 mM phosphate buffer pH 7, containing 10% fructose and 3% MeOH. The 40 mM phosphate buffer was prepared from 1M sodium phosphate buffer (pH 7), which in turn had been prepared by mixing 57.7 mL of 1 M NaH_2PO_4 and 42.3 mL of 1 M Na_2HPO_4. A solution of 10% fructose and 3% MeOH in 40 mM phosphate buffer (pH 7) was used as a negative control. There were two positive controls: the insecticide abamectin (50 µg/mL) dissolved in 40 mM phosphate buffer pH 7 containing 10% fructose and 3% MeOH and empty wells without the test solution to show that thrips cannot survive without eating and hence are feeding from the test solutions.

Each bioassay was carried out using eight 96-well plates at a time. Per two 96-well plates there are 24 columns (1-24) of 8 wells. A column received the same treatment and therefore consisted of 8 replicates. Of the 24 columns, 19 columns were filled with 19 different treatments and 5 columns were left empty. The 18 treatments included one negative control (the solvent), 16 treatment groups (3 concentrations of a PA, 5 concentrations of an organic acid and 8 concentrations of combinations of PAs with an organic acid) and two positive controls (empty wells and abamectin). Four sets of two 96 wells plates were carried out simultaneously yielding 32 replicates for all treatments. The complete bioassay was repeated at a different time in order to have two independent estimates of thrips survival/mortality for analysis of variance. After five days, the numbers of surviving larvae were recorded with a stereo microscope.

Second instar larvae of *F. occidentalis* were obtained from a lab culture reared on chrysanthemum flowers in a growth chamber with standard thrips rearing conditions (L:D, 12:12, 23°C).

Experiments of CGA in combination with five individual free base PAs

In previous experiments, antagonistic interactions between PAs and CGA were found on *Spodoptera* (Nuringtyas et al. 2014). We hypothesized that similar effects may occur on thrips. Therefore, we tested PAs at relatively high concentrations (Chapter 2). Three doses of 0, 4 and 7 mM of retrorsine, jacobine, erucifoline and monocrotaline, respectively were combined with five doses of 0, 1.4, 2.8, 5.6 and 14 mM CGA yielding 15 combinations including the tests with single metabolites. Due to its limited solubility in the test solution, senecionine was only tested at concentrations of 0, 0.8 and 1.4 mM.

One PA, retrorsine, was selected because it provided the strongest interaction with CGA. A statistical analysis showed that the magnitude of the interaction was depending on the concentration and the combination of the concentrations (see result section). A range of 30 different combinations of retrorsine and CGA was tested for their interaction on thrips (Figure S1).

Testing the active groups of the CGA molecule that are involved in the interaction

CGA showed the strongest interaction with retrorsine. Retrorsine was subsequently tested with a large set of derivatives of CGA to explore this molecule in more detail for its structure activity relationship with retrorsine (Figure 2). Structurally, CGA is the ester of caffeic acid and quinic acid. Retrorsine, at three doses, 0, 4 and 7 mM, was combined with 0, 1.4, 2.8, 5.6 and 14 mM quinic acid, caffeic acid, and derivatives of quinic acid such as shikimic acid, cyclohexanecarboxylic acid (CHCA), 1-hydroxycyclohexanecarboxylic acid (1-H-CHCA), *myo*-inositol, glucuronic acid and malic acid.

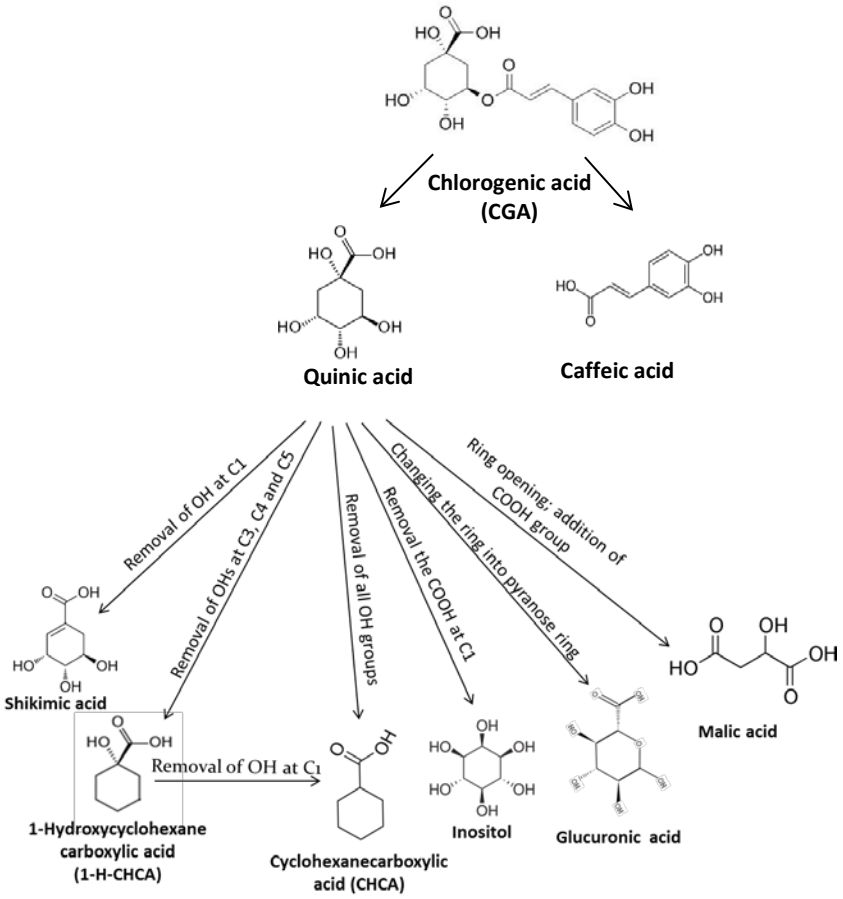


Figure 2. Chemical structures of chlorogenic acid (CGA) and related metabolites tested in combinations with retrorsine in a thrips bioassay to pinpoint the most active groups contributing to the antagonistic effect with retrorsine.

Statistical analysis

Construction of an interaction model

To evaluate the effect of the interaction between two metabolites, we first constructed a "null interaction" model that predicts the effect of metabolites in the absence of interaction. Hereby a multiplicative null model was constructed. In a multiplicative null model, assuming no interaction effects, the effect of the combination is the product of the effects of the two metabolites, i.e., $S_{X+Y} = S_X * S_Y$. The underlying assumption of the multiplicative model is that the relationship between log-transformed survival of thrips larvae against the concentration of the tested individual metabolites is linear.

Thrips survival was log-transformed to obtain linear relationships with the concentrations of CGA and retrorsine. To determine whether there was a dose-related decrease, log-transformed survival was regressed against concentrations. Based on the regression models, the effect of the combination of two metabolites in the absence of interaction was calculated.

Correcting for survival in the negative control

The survival in the negative control was in all cases high (> 86%). Nevertheless, we corrected survival data for differences among the negative controls in the following way. The observed experimental survival of larvae (S_{X+NC}) results from the survival after application of tested metabolite (S_X) and from the survival in the solvent (S_{NC}). The survival of the solvent is measured in the negative control. Under the assumption that the two effects are independent, we get the following equation:

$$\mathbf{S_{X+NC} = S_{NC} * S_X} \qquad \mathbf{(1)}$$

The survival resulting from the application of the metabolite S_X can thus be calculated as

$$\mathbf{S_X = S_{X+NC} / S_{NC}} \qquad \mathbf{(2)}$$

Testing the interaction effects of the combination of two metabolites on survival

The survival of thrips for the combination of metabolites X and Y (S_{X+Y}) results from the survival after application of the single metabolite X (S_X), the survival after application of the single metabolite Y (S_Y), and their interaction (S_{X*Y}).

$$\mathbf{S_{X+Y} = S_X * S_Y * S_{X*Y}} \qquad \mathbf{(3)}$$

Therefore, the effect of the interaction of two metabolites can be calculated by:

$$\mathbf{S_{X*Y} = S_{X+Y} / (S_X * S_Y)} \qquad \mathbf{(4)}$$

In which $S_{X+Y,}$ S_X and S_Y are derived from equation (2). In equation (4), S_{X+Y} is the observed survival in experiments with combinations while S_X and S_Y are the observed thrips survival in experiments with single metabolites while S_{X*Y} denotes the interaction effect. Note that the interaction effect thus denotes the effect of the interaction between the PA and CGA on the survival of thrips while 1-interaction effect denotes the fraction mortality caused by this interaction.

As each experiment is repeated once, two independent estimates of the interaction effect are obtained. To illustrate this effect the interaction effect S_{X*Y} was calculated for all

combinations and represented in graphs. The interaction effects were expressed as mean value ± standard error of the mean (SE).

To estimate if the interaction effect deviated significantly from one, two-way analysis of variances (ANOVAs) were used with the concentration of metabolites X and Y as factors. As dependent variables the estimates of S_{X*Y} -1 (= S_{X+Y} / (S_X * S_Y) -1 were used for all combinations of traits. Therefore, if the intercept of two-way ANOVA is significantly deviating from zero it indicates that S_{X*Y} -1 is deviating from zero and hence that S_{X*Y} is significantly deviating from one. If the interaction effect S_{X*Y} is larger than one it indicates an antagonistic interaction and if it is smaller than one it indicates a synergistic interaction. To avoid confusion with statistical interaction terms of ANOVA and multiple regression we will always refer to the value of the interaction effect between the metabolites as the "interaction effect S_{X*Y}".

The strength of the antagonistic effects were compared by three-way ANOVAs with PA, PA concentration and CGA concentration as fixed factors and the interaction effect S_{X*Y} as the dependent variable. As senecionine was tested at different concentrations than the other four PAs, it was not included in the three-way ANOVA.

In the case of the combination of retrorsine with various acids, a three-way ANOVA was performed with retrorsine concentration, different acids, and the acid's concentration as fixed factors and the interaction effect S_{X*Y} as the dependent variable.

To visually display the effects of the single metabolites and their combinations on thrips, we plotted fraction mortality (i.e. 1 – fraction survival) against the concentrations of the two metabolites in a 3-dimensionsial (3D) graph with the 2 horizontal axes (x and y) representing the two metabolite concentrations, and the vertical axis (z) representing thrips mortality.

To visually examine the most effective combination, a heat-map of the interaction effects S_{X*Y} from 30 combinations of retrorsine and CGA were plotted, with CGA concentration on the x axis and retrorsine concentration on the y axis (Figure S1). A dashed line in the heat-map indicates a ratio at 1:1 of the metabolite concentrations.

All statistical analysis were performed using SPSS software for Windows (version 21.0; SPSS Inc., Chicago, IL).

Results

Thrips larval survival in negative and positive controls

The survival in the negative control was in all cases greater than 86%. There were two positive controls for each treatment group: a solution with the insecticide abamectin (50 µg/mL) with an average survival of 6.6% and a treatment with empty wells with an average survival of 4.5% showing that thrips do feed through the parafilm from the test solution.

The effects of single free base PAs and CGA on thrips larval survival

For all the tested free base PAs we found a negative effect on thrips survival (Chapter 2). Retrorsine, jacobine and erucifoline showed the strongest effects on survival while the effects of senecionine and monocrotaline were relatively small. CGA also had a negative effect on thrips survival although the effect was relatively small compared to that of the most effective PAs. Because we studied the interaction between CGA and retrorsine in greater detail we checked for these two compounds whether or not they fitted the assumptions of a multiplicative model for the interaction effect. Both the concentrations of CGA and retrorsine showed a linear relationship with log-transformed larval survival (Figure 3).

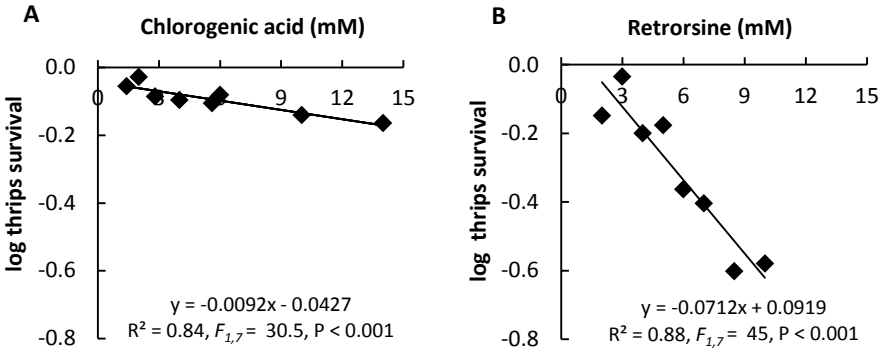
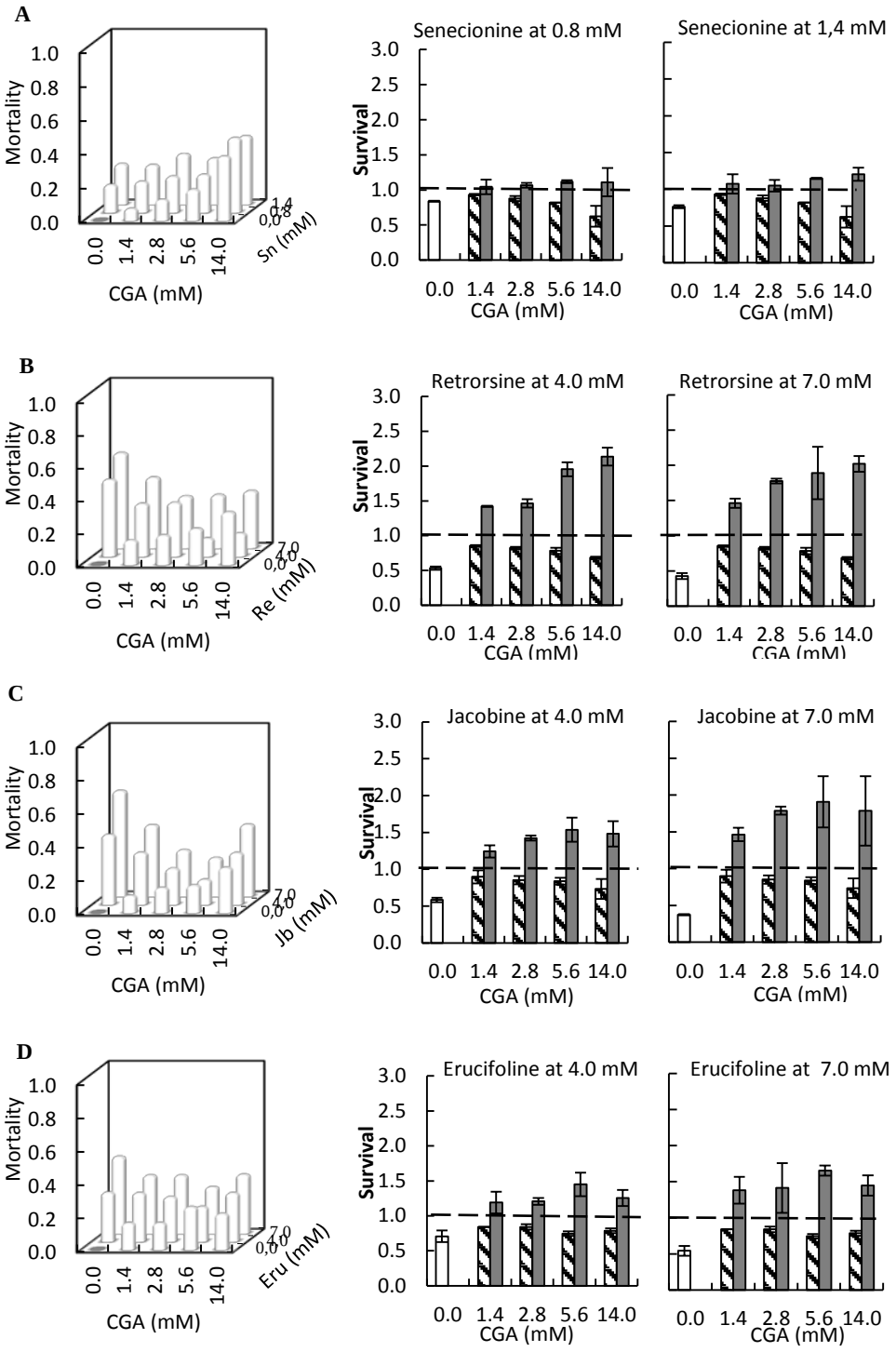


Figure 3. Log-transformed survival of 2nd instar *Frankliniella occidentalis* against the concentration of chlorogenic acid (A) and retrorsine (B). Survival was corrected for differences among negative controls.

Interactions between different free base PAs and CGA on thrips survival

We first tested for the individual PAs if they interacted with CGA in their effect on thrips survival. Compared to single metabolites, the combinations of CGA and all free base PAs showed a decreased thrips mortality indicative of an antagonistic effect (Figure 4; Table 1). For retrorsine and monocrotaline the main effect of CGA concentration on the interaction effect S_{X*Y} is significant indicating that for these two PAs the strength of the antagonistic interaction is dependent on CGA concentration.


A
Mortality
1.0
0.8
0.6
0.4
0.2
0.0
0.0 1.4 2.8 5.6 14.0
CGA (mM)
Sn (mM)
Senecionine at 0.8 mM
Senecionine at 1,4 mM
Survival
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0 1.4 2.8 5.6 14.0
CGA (mM)
B
Mortality
Re (mM)
7.0
Retrorsine at 4.0 mM
Retrorsine at 7.0 mM
Survival
C
Mortality
Jb (mM)
Jacobine at 4.0 mM
Jacobine at 7.0 mM
Survival
D
Mortality
Eru (mM)
Erucifoline at 4.0 mM
Erucifoline at 7.0 mM
Survival

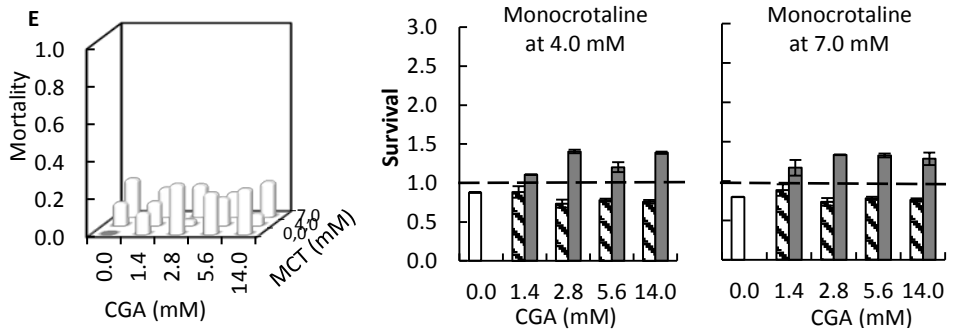


Figure 4. Left: Fraction mortality (= 1- fraction survival) of 2nd instar larvae of thrips (*Frankliniella occidentalis*) caused by the single metabolites and the combination of chlorogenic acid (CGA) with senecionine (**A**), retrorsine (**B**), jacobine (**C**), erucifoline (**D**) or monocrotaline (**E**). **Right:** Fraction survival (mean ± 95% confidence intervals) of thrips caused by the pyrrolizidine alkaloid (PA) alone (white bars), CGA alone at four concentrations (hatched bars), and the interaction effect S_{X*Y} (Equation 4) between PAs and CGA (grey bars). In the left figures, the fraction mortality was plotted to increase the readability of the figure. In the right figures, dashed line indicates a thrips survival fraction of one. Two-way ANOVAs were used to analyse whether the interaction effect S_{X*Y} deviated from one (Table 1).

Table 1. Two-way ANOVAs with free base pyrrolizidine alkaloids (PAs) and chlorogenic acid (CGA) concentration as fixed factors and the interaction effect S_{X*Y} minus one as the dependent variable. Each combination was tested at 8 concentrations in two independent bioassays. Survival was corrected for differences among the negative controls (see M&M). A significant intercept indicates a synergistic or antagonistic interaction.

Factors	***df***	***F***	***P***
Intercept	1, 15	6.979	< 0.05
CGA concentration	3, 15	0.285	NS
Senecionine concentration	1, 15	0.226	NS
CGA concentration * Senecionine concentration	3, 15	0.177	NS
Intercept	1, 15	450.121	< 0.001
CGA concentration	3, 15	10.270	< 0.01
Retrorsine concentration	1, 15	1.797	NS
CGA concentration * Retrorsine concentration	3, 15	2.807	NS
Intercept	1, 15	87.780	< 0.001
CGA concentration	3, 15	1.566	NS
Jacobine concentration	1, 15	1.411	NS
CGA concentration * Jacobine concentration	3, 15	0.055	NS
Intercept	1, 15	48.814	< 0.001
CGA concentration	3, 15	1.164	NS
Erucifoline concentration	1, 15	1.231	NS
CGA concentration * Erucifoline concentration	3, 15	0.011	NS

Intercept	1, 15	244.857	< 0.001
CGA concentration	3, 15	6.964	< 0.05
Monocrotaline concentration	1, 15	0.276	NS
CGA concentration * Monocrotaline concentration	3, 15	2.595	NS

NS = Not significant

We then compared the strength of the interaction effects for different PAs in a three-way ANOVA followed by a post-hoc test. Across all PAs we found that the combination of PAs and CGA decreased thrips mortality compared to the effects of the single compounds. The strength of the antagonistic effect significantly differed between retrorsine and erucifoline/monocrotaline, and between jacobine and monocrotaline (Figure 5, Table 2). The ranking of the mean interaction effect S_{X*Y} (± S.E.) starting with the highest was as follows: CGA + retrorsine (1.93 ± 0.06) > CGA + jacobine (1.88 ± 0.06) > CGA + erucifoline (1.44 ± 0.06) > CGA + monocrotaline (1.34 ± 0.06) > CGA + senecionine (1.12 ± 0.06) (Figure 5, Table 2).

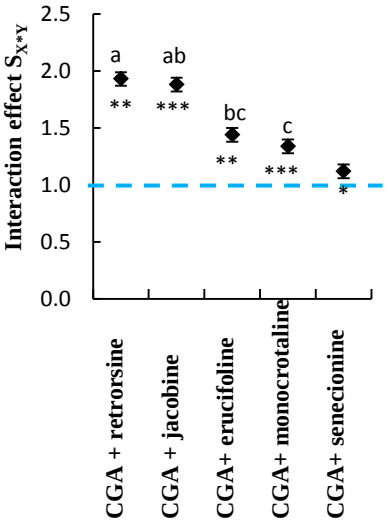


Figure 5. The interaction effect S_{X*Y} of thrips (*Frankliniella occidentalis*) larval survival (mean ± S.E.) of the combinations of chlorogenic acid (CGA) and 5 free base PAs obtained from two independent bioassays. The interaction effect S_{X*Y} were analysed by a three-way ANOVA (Table 2) with PA, PA concentration and CGA concentration as fixed factors. Different letters indicate that the interaction effect S_{X*Y} between PAs is significantly different with a Tukey post hoc test. * P < 0.05, ** P < 0.01 and ***, P < 0.001.

Table 2. Three-way ANOVA with free base pyrrolizidine alkaloid (PA), chlorogenic acid (CGA) concentration and PA concentration as factors with the interaction effect S_{X*Y} as a dependent variable. The free base PAs are retrorsine, jacobine, erucifoline and monocrotaline. Each combination was tested in two independent bioassays. Survival was corrected for differences among the negative controls (see M&M).

Factors	*df*	*F*	*P*
Intercept	1, 63	2531.6	< 0.001
PA	3, 63	13.4	< 0.001
CGA concentration	3, 63	5.8	< 0.01
PA concentration	1, 63	5.5	< 0.05
PA * CGA concentration	9, 63	1.2	NS
PA * PA concentration	3, 63	1.5	NS
CGA concentrations * PA concentration	3, 63	0.2	NS
PA * CGA concentrations * PA concentration	9, 63	0.2	NS

NS = Not significant

Is there an optimal antagonistic effect between retrorsine and CGA on thrips survival?

In the case of retrorsine and jacobine, there is a valley in the 3D plot of thrips mortality (Figure 4B, C (left)). The antagonistic effects increase with CGA concentration but become smaller as the CGA concentration exceeds 2.8 mM.

The dependence of the antagonistic interaction was explored further by testing 30 combinations of retrorsine and CGA concentrations (Figure 6). Interaction effects largely depended on the concentration of retrorsine (Y axis in the heat-map) and the ratio of the concentrations (as indicated by the valley in the heat-map), but less on the concentration of CGA (X axis in the heat-map).

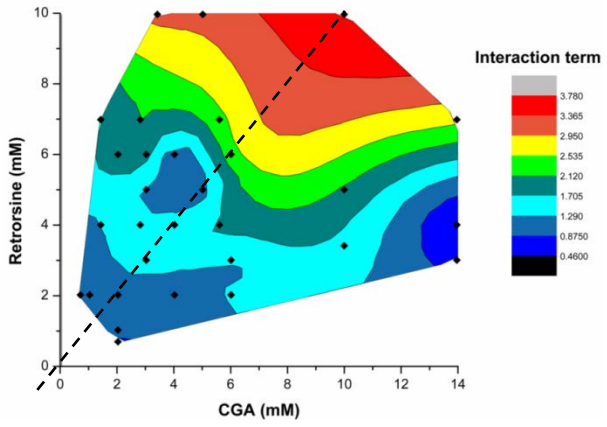


Figure 6. Heat-map of the magnitude of the interactions effect S_{X*Y} based on thrips (*Frankliniella occidentalis*) survival against retrorsine and chlorogenic acid (CGA) concentrations. Black dots indicate measured values. The dashed line represents the combined ratio of 1:1.

Interactions between retrorsine and two groups of the CGA molecule

To determine which part(s) of the CGA molecule contributed most to an antagonistic effect with retrorsine, a range of structural analogs of CGA was tested in combination with retrorsine. The CGA molecule can be split into quinic acid and caffeic acid (Figure 2). Retrorsine combined with quinic acid showed a significant antagonistic effect (Figure 7A, Table 4) while the combination of caffeic acid and retrorsine showed no significant antagonistic effects (Figure 7B, Table 4).

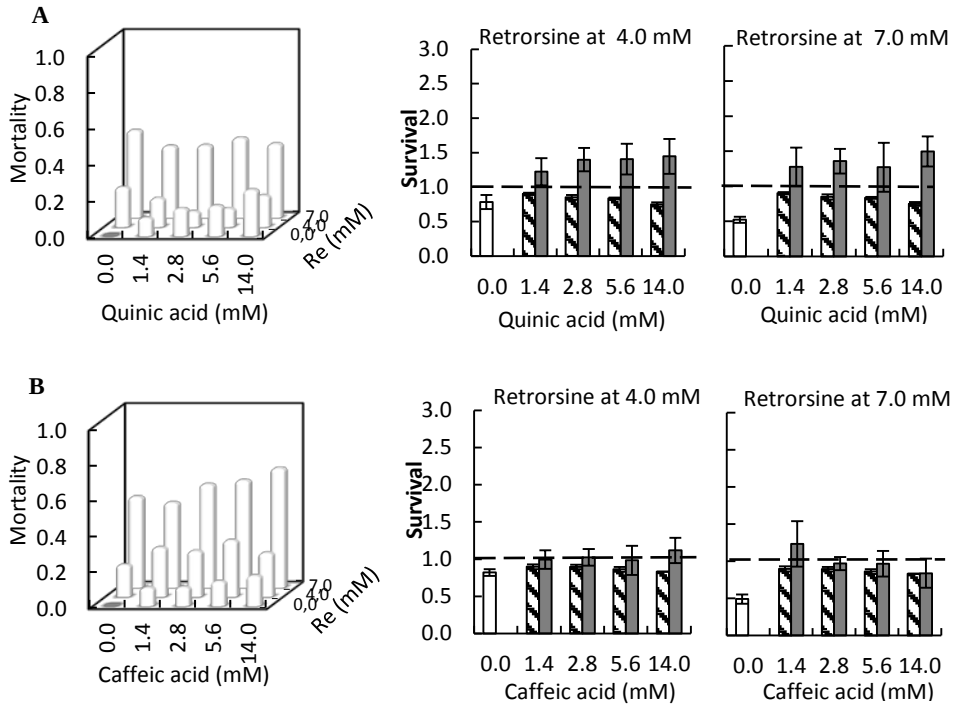


Figure 7. Left: Fraction mortality (= 1- fraction survival) of 2nd instar larvae of thrips (*Frankliniella occidentalis*) caused by the single metabolites and the combination of retrorsine with quinic acid (**A**) and with caffeic acid (**B**). **Right:** Fraction survival (mean ± 95% confidence intervals) of thrips caused by retrorsine alone (white bars), acids alone at four concentrations (hatched bars), and the interaction effect S_{X*Y} (Equation 4) between retrorsine and the acid (grey bars). In the left figures, the fraction mortality was plotted to increase the readability of the figure. In the right figures, dashed line indicates a thrips survival fraction of one.

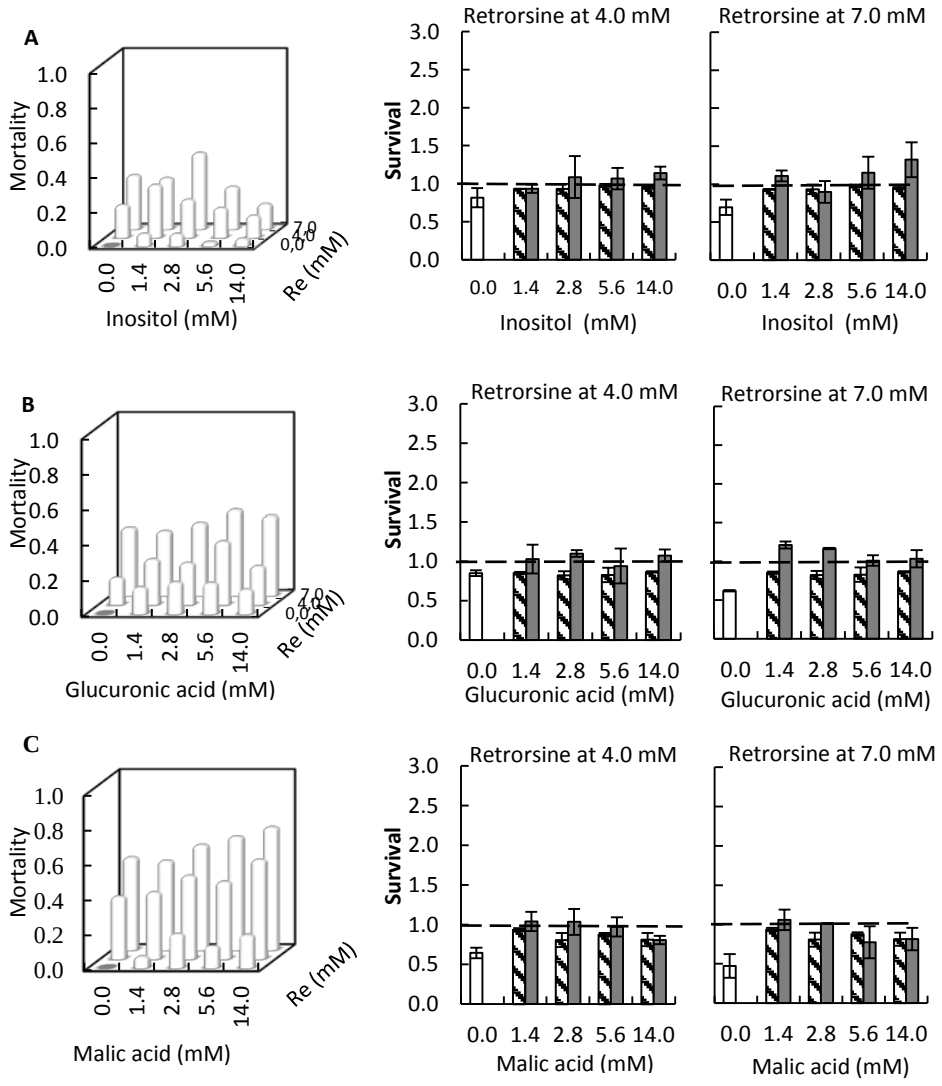
Table 4. Two-way ANOVAs with retrorsine concentration and organic acid concentration as factors and the interaction effect S_{X*Y} minus one as the dependent variable for 7 organic acids and *myo*-inositol. Each combination was tested at 8 concentrations in two independent bioassays. Survival was corrected for differences among the negative controls (see M&M). A significant intercept indicates a synergistic or antagonistic interaction.

Factors	***df***	***F***	***P***
Intercept	1, 15	20.705	< 0.01
Quinic acid concentration	3, 15	0.206	NS
Retrorsine concentration	1, 15	0.391	NS
Quinic acid concentration * Retrorsine concentration	3, 15	0.086	NS
Intercept	1, 15	0.018	NS
Caffeic acid concentration	3, 15	0.113	NS
Retrorsine concentration	1, 15	0.058	NS
Caffeic acid concentration * Retrorsine concentration	3, 15	0.481	NS
Intercept	1, 15	2.150	NS
Inositol concentration	3, 15	0.747	NS
Retrorsine concentration	1, 15	0.034	NS
Inositol concentration * Retrorsine concentration	3, 15	0.092	NS
Intercept	1, 15	2.305	NS
Glucuronic acid concentration	3, 15	0.590	NS
Retrorsine concentration	1, 15	0.399	NS
Glucuronic acid concentration * Retrorsine concentration	3, 15	0.195	NS
Intercept	1, 15	1.111	NS
Malic acid concentration	3, 15	1.433	NS
Retrorsine concentration	1, 15	8.387	NS
MA concentration * Retrorsine concentration	3, 15	0.504	NS
Intercept	1, 15	8.005	< 0.05
Shikimic acid concentration	3, 15	0.424	NS
Retrorsine concentration	1, 15	3.290	NS
Shikimic acid concentration * Retrorsine concentration	3, 15	0.587	NS
Intercept	1, 15	32.193	< 0.001
CHCA concentration	3, 15	2.790	NS
Retrorsine concentration	1, 15	0.294	NS
CHCA concentration * Retrorsine concentration	3, 15	2.144	NS
Intercept	1, 15	6.506	< 0.05
1-H-CHCA concentration	3, 15	0.206	NS
Retrorsine concentration	1, 15	0.095	NS
1-H-CHCA concentration * Retrorsine concentration	3, 15	0.071	NS

NS = Not significant;

CHCA = cyclohexanecarboxylic acid; 1-H-CHCA = 1-hydroxycyclohexanecarboxylic acid

Subsequently, all chemical functional groups of quinic acid were removed or modified and tested in combination with retrorsine to determine which functional groups contributed to the antagonistic interaction on thrips survival (Figure 2). *Myo*-inositol, the decarboxylated analogue of quinic acid, did not show an antagonistic interaction with retrorsine on thrips survival (Figure 8A). Also changing the ring into a pyranose ring (i.e. glucuronic acid) and ring opening (i.e. malic acid) resulted in the loss of the antagonistic effect with retrorsine on thrips survival (Figure 8B, C; Table 4).



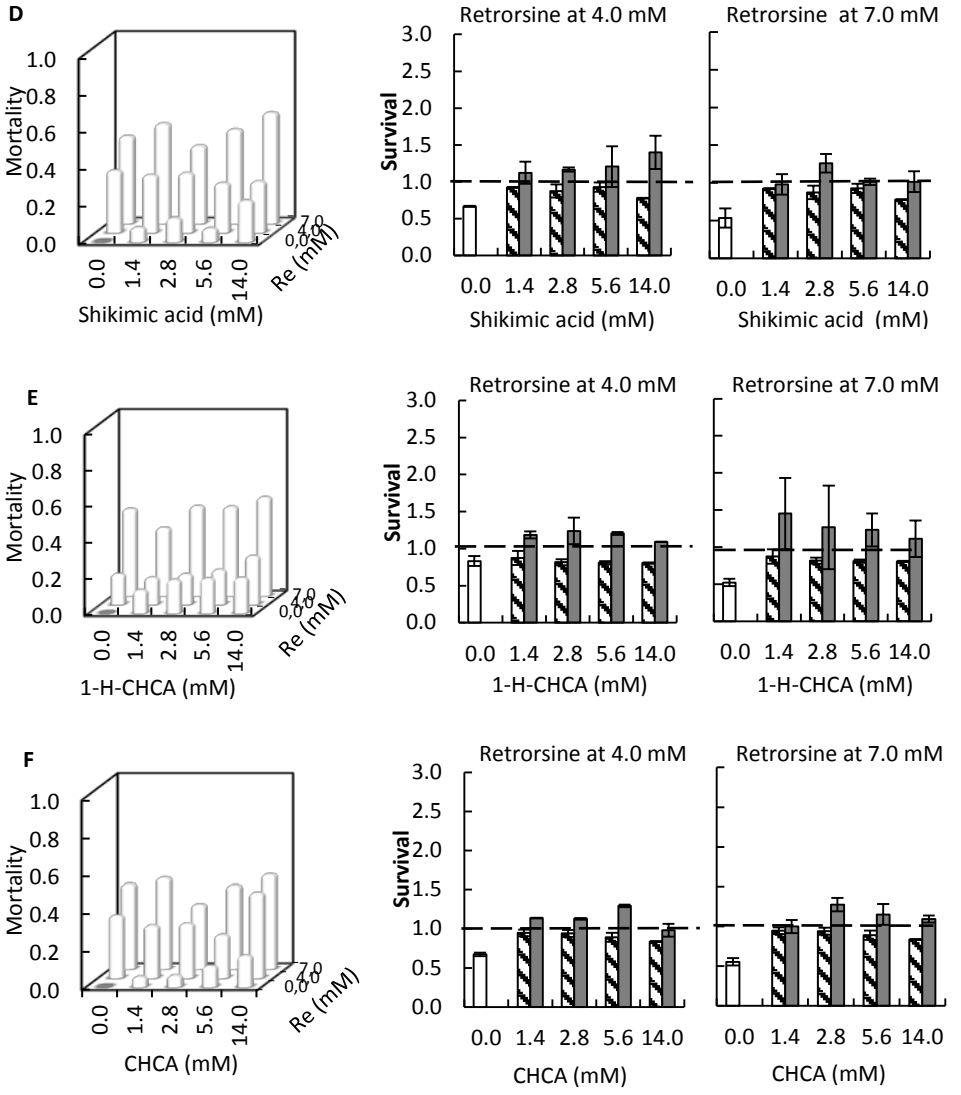


Figure 8. Left: Fraction mortality (= 1- fraction survival) of 2nd instar larvae of thrips (*Frankliniella occidentalis*) caused by the single metabolites and the combination of retrorsine (Re) with inositol (**A**), glucuronic acid (**B**), malic acid (**C**), shikimic acid (**D**), 1-H-CHCA (**E**) or cyclohexanecarboxylic acid (**F**). **Right:** Fraction survival (mean ± 95% confidence intervals) of thrips caused by retrorsine alone (white bars), organic acids alone at four concentrations (hatched bars), and the interaction effect S_{X*Y} (Equation 4) between retrorsine and each of the acids (grey bars). In the left figures, the fraction mortality was plotted to increase the readability of the figure. In the right figures, dashed line indicates a thrips survival fraction of one. CHCA = cyclohexanecarboxylic acid; 1-H-CHCA = 1-hydroxycyclohexanecarboxylic acid.

Removal of the OH groups (shikimic acid, 1-H-CHCA and CHCA) retained significant antagonistic effects with retrorsine on thrips survival (Figure 8D, E, F, Table 4). The extent of the decrease in thrips mortality was similar for the three combinations, as was the average magnitude of the antagonistic effect on thrips survival: 1.15 (± 0.07) of shikimic acid, 1.13 (± 0.07) of CHCA and 1.22 (± 0.07) of 1-H-CHCA (Figure 9). Nevertheless, a three-way ANOVA showed that the strength of the antagonistic interaction effect S_{X*Y} of these acids is smaller than quinic acid, which in turn was significantly lower than that of CGA (Figure 9; Table 5). For the other acids, the magnitude of interaction effect S_{X*Y} did not significantly differ.

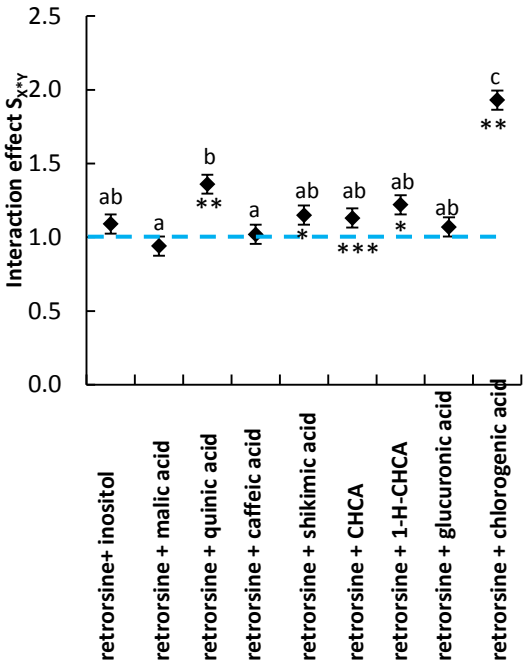


Figure 9. The interaction effect S_{X*Y} of thrips (*Frankliniella occidentalis*) (mean ± S.E.) for the combination of retrorsine with 8 organic acids and *myo*-inositol. Dashed lines indicate an interaction effect S_{X*Y} of one. Two-way ANOVAs were used to analyse whether the interaction effect S_{X*Y} deviated from one (Table 4). Stars indicate the interaction effect S_{X*Y} is significantly deviating from one. A three-way ANOVA was used to analyse whether the interaction effects S_{X*Y} of the 9 combinations of acids with retrorsine differed (Table 5). Different letters letter indicate that the interaction effects S_{X*Y} between retrorsine and each of the organic acids are significantly different with a Tukey post hoc test. CHCA = cyclohexanecarboxylic acid; 1-H-CHCA = 1-hydroxycyclohexanecarboxylic acid. * $P < 0.05$, ** $P < 0.01$ and ***, $P < 0.001$.

Table 5. Three-way ANOVA with 8 organic acids and *myo*-inositol ("Organic acids"), different concentrations of acids and different retrorsine concentrations as factors with the interaction effect S_{X*Y} as a dependent variable. The 8 organic acids and *myo*-inositol combined with retrorsine were chlorogenic, caffeic, quinic, cyclohexanecarboxylic, shikimic, glucuronic, 1-hydroxycyclohexanecarboxylic and malic acids, as well as *myo*-inositol. Each combination was tested in two independent bioassays. Survival was corrected for differences among the negative controls (see M&M).

Factors	***df***	***F***	***P***
Intercept	1, 143	3055.2	< 0.001
Organic acids	8, 143	14.5	< 0.01
Organic acid concentration	3, 143	0.3	NS
Retrorsine concentration	1, 143	0.0	NS
Organic acids * organic acid concentration	24, 143	1.1	NS
Organic acids * retrorsine concentration	8, 143	0.3	NS
Organic acid concentration * retrorsine concentration	3, 143	0.6	NS
Organic acids * organic acid concentration * retrorsine concentration	24, 143	0.3	NS

NS = Not significant

Discussion

Here, we showed that combinations of free base PAs and organic acids can have antagonistic effects on the survival of a generalist herbivore. The concentrations used are biologically relevant. The mean CGA concentration in *J. vulgaris* plants is about 1.6 mM and falls well in the tested range. For PAs, the concentrations tested were relatively high compared to the plant average. However, genotypes with these concentrations do occur (Cheng et al. 2011; Wei et al. 2015). Moreover, the concentrations in specific cell layers or cells can be much higher than the total mass concentration (Nuringtyas et al. 2012). All tested PAs showed an antagonistic interaction with CGA, though the strength of the interaction depended on the particular PA, CGA concentration and PA concentration.

Analysis of the antagonistic effect on thrips survival showed that the five tested free base PAs did not equally interact with CGA. The combinations of CGA with jacobine and with retrorsine resulted in the strongest antagonistic effect, followed by erucifoline while relatively weak antagonistic interactions with senecionine and monocrotaline were detected.

In a previous study, antagonistic effects between jacobine and CGA were observed with *Spodoptera exigua* (Nuringtyas et al. 2014), suggesting that such antagonistic effects are not specific for thrips. Facing such disadvantageous interactions, one would expect plants to have developed strategies to overcome these. One effective strategy to avoid such interaction is to store interacting metabolites in separate compartments in plants. In the case of free base PAs and CGA, Nuringtyas et al. (2012) found high amounts of CGA in the epidermis cells of *Jacobaea* leaves while palisade cells contained high levels of jacobine. In

the case of single cell feeders, e.g. thrips, this could be an effective strategy to overcome disadvantages of antagonistic effects between plant metabolites. On the other hand, such a strategy may not work for insects with other feeding types, such as leaf chewing caterpillars.

During the last decade, numerous reports in the areas of neurochemistry, pharmacology and phytotherapy have dealt with interactions between plant metabolites (see reviews by Greco et al. 1995; Williamson 2001; Zimmermann et al. 2007; Biavatti 2009). In contrast, only a few studies focused on the interactions between plant metabolites in the context of defence. Among the limited number of studies, most deal with synergistic interactions and their benefits in terms of increased cost effectiveness in the production of SMs and the resulting increased fitness (Fagerstrom 1989; Jones et al. 2005; Ryabushkina 2005). Only a few studies have shown antagonistic effects between plant metabolites on herbivore performance which could be an indication that this phenomenon might not happen very often. The examples of antagonistic effect of metabolites on herbivores include the combination of different furanocourmarins on larval mortality of *S. exigua* (Diaware et al. 1993), combinations of potassium peroxymonosulphate + CGA and of indole + caryophyllene on the mortality of brine shrimp (*Artemia franciscana*) (Nelson and Kursar 1999), and the mixture of CGA with jacobine on toxicity for *S. exigua* cell lines (Nuringtyas 2014). The reason that only few studies on interactions between metabolites were carried out probably is a lack of a theoretical framework (Nelson and Kursar 1999). Here, we describe a method to calculate the expected interaction effects of two or more components, which then allows for a proper statistical testing both of the pattern and the magnitude of interaction effects. The interaction effects then can be calculated by comparing the observed results and the expected results. The statistical framework that is put forward here can be extended to other aspects of plant-environment interactions, multi-component materials, and to the design of bioactivity studies.

Diaware et al. (1993) put forth a hypothesis that evolution of plant defences occurred under selection pressures from other factors such as abiotic stresses, pathogens, other herbivores, etc., and that these benefits compensate the negative effects of combinations of furanocoumarins on *S. exigua*. Nelson and Kursar (1999) proposed that antagonism may represent a constraint or a trade-off on the accumulation of metabolites. However, experimental evidence is currently lacking to back up these hypotheses.

The central issue in understanding the antagonistic interactions between plant metabolites is the underlying mechanism. In the case of alkaloids, the most intensively studied interaction is that of the purine alkaloid caffeine with CGA. Purine alkaloids in plants are known to form hydrophobically bound π-molecular complexes with CGA (Mösli Waldhauser and Baumann 1995) and the complex of caffeine and CGA was proposed to form at a ratio of 1:1 (Sondheimer et al. 1961; Horman and Viani 1972). The binding between CGA and caffeine is regarded as weak, demonstrated by a rapid reversibility (Chapman and Miller

1974) and two slightly different conformations (Horman and Viani 1972). We speculate that under neutral conditions the retronecine base can form a salt-like complex with the carboxylic group of CGA. This doubles the molecular weight of both molecules to 704 Da, and it likely will result in altered physico-chemical and biological properties compared to the separate molecules. If CGA and retrorsine form a complex at a ratio of 1:1, as with caffeine and CGA, we would expect that the strongest antagonistic effect between retrorsine and CGA on thrips mortality would occur at this ratio. From the experiment with the different retrosine – CGA combinations, there are indications that there is a ratio with a maximal antagonistic effect. Visual inspection of the heat-map seems to indicate that this ratio is slightly below one, however, the variation is too large to pinpoint the ratio exactly.

The current study suggests that all tested free base PAs and CGA show an antagonistic interaction with respect to thrips mortality. This indicates that the active part is the retronecine base of the PA which all tested free base PAs have in common, rather than the macrocyclic ring which differs between all tested PAs.

CGA (chlorogenic acid or caffeoylquinic acid) is a soluble ester formed between caffeic and quinic acid. Only a few studies addressed the roles of the two groups of the CGA molecule in the complex formation with alkaloids and the results are inconsistent. Martin et al. (1987) found caffeine was oriented towards the quinic group whereas D'Amelio et al. (2009) proposed that caffeine interacted with the caffeoyl group of the CGA molecule. The two studies were performed only from a chemical perspective, without taking bioactivity into account. When split into two parts, the quinic acid part of the CGA molecule showed an antagonistic interaction with retrorsine on thrips mortality while the caffeoyl group did not. However, the strength of the antagonistic interaction effect S_{X*Y} between quinic acid and retrorsine was less strong than the interaction effect S_{X*Y} between CGA and retrorsine (Figure 9). Similar to the combination of retrorsine and CGA, a valley in the 3D plot of thrips mortality is present (Figure 7) in the combination of retrorsine and quinic acid. This indicates the potential involvement of the quinic acid part of the CGA molecule into the antagonistic interaction on thrips mortality.

It is unlikely that the hydroxyl groups in the quinic acid molecule were responsible for the interaction because these metabolites significantly decreased the toxicity of retrorsine, in spite of the absence or presence of the OH group. Shikimic acid (the absence of OH group at position C1), 1-H-CHCA (the absence of OH groups at the position C3, C4 and C5) and CHCA (the absence of all OH groups of the cyclohexane ring) all antagonistically interacted with retrorsine but the strength of the antagonistic interaction effect S_{X*Y} on thrips mortality was significantly less than that of the strength of the antagonistic interaction effect S_{X*Y} between retrorsine and CGA and quinic acid (Figure 9). Modification of the cyclohexane ring rendered the antagonistic interaction with retrorsine on thrips mortality insignificant as is the case with glucuronic acid. The presence of a

COOH group at the C-1 position of the ring is critical for the antagonistic effects on thrips. Removal of the COOH group of quinic acid, e.g. *myo*-inositol, a six-fold alcohol of cyclohexane, lost the antagonistic effect in combination with retrorsine on thrips mortality.

Conclusion

We showed that 9 out of 13 tested combinations exhibited significant antagonistic effects on thrips survival. For a single cell feeder such as thrips compartmentalization of plant metabolites between cells may be an effective way for plants to reduce or control the disadvantageous antagonistic effects of the combinations of metabolites. With regard to other types of insect herbivores, the evolutionary aspects of antagonistic interactions between SMs should be further investigated.

The common interaction pattern of free base PAs and CGA may indicate that the active part is in the retronecine base rather than the macrocyclic diester ring. Testing of several analogues of CGA in combination with retrorsine showed that modification of functional groups, addition/elimination of specific groups, or changing the substitution pattern could explain the strength of the antagonistic interaction effect on thrips mortality. More specific we found that the carboxyl group at position C1 in combination with the 3-D structure of the six-membered ring was essential for the antagonistic interactions with retrorsine while the hydroxyl groups were not.

Although the exact mechanism of the antagonistic effect between CGA and free base PAs still requires further clarification, we were able to show that the bioactivity of individual metabolites is strongly influenced by other metabolites co-occurring in the same plants. This study emphasizes that the potential interactions of plant metabolites should be taken into consideration when testing for bioactivity.

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References

Bi JL, Felton GW, Murphy JB, Howles PA, Dixon RA, et al. 1997. Do plant phenolics confer resistance to specialist and generalist insect herbivores? J Agric Food Chem 45:4500-4504

Biavatti MW. 2009. Synergy: an old wisdom, a new paradigm for pharmacotherapy. Braz J Pharm Sci 45:371-378

Chapman RA, Miller DJ. 1974. The effects of caffeine on the contraction of the frog heart. J Physiol 242:89-613

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011. Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. New Phytol 192:1010-1023

Chou MW, Fu PP. 2006. Formation of DHP-derived DNA adducts in vivo from dietary supplements and Chinese herbal plant extracts containing carcinogenic pyrrolizidine alkaloids. Toxicol Ind Health 22:321-7

D'Amelio N, Fontanive L, Uggeri F, Suggi-Liverani F, Navarini L. 2009. NMR reinvestigation of the caffeine-chlorogenate complex in aqueous solution and in coffee brews. Food Biophys 4:321-330

D'Auria JC, Gershenzon J. 2005. The secondary metabolism of Arabidopsis thaliana: growing like a weed. Curr Opin Plant Biol 8:308-316

de Boer NJ. 1999. Pyrrolizidine alkaloid distribution in *Senecio jacobaea* rosettes minimises losses to generalist feeding. Entomol Exp Appl 91:169-173

Diawara MM, Trumble JT, White KK, Carson WG, Martinez LA. 1993. Toxicity of linear furanocoumarins to *Spodoptera exiga*- evidence for antagonistic interactions. J Chem Ecol 19:2473-2484

Dixon RA, Strack D. 2003. Phytochemistry meets genome analysis, and beyond. Phytochemistry 62:815-816

Dyer LA, Dodson CD, Stireman JO III, Tobler MA, Smilanich AM, Fincher RM, Letourneau DK. 2003. Synergistic effects of three Piper amides on generalist and specialist herbivores. J Chem Ecol 29:2499-2514

Efferth T, Koch E. 2011. Complex interactions between phytochemicals. The multi-target therapeutic concept of phytotherapy. Curr Drug Targets 12:122-132

Fagerstrom T. 1989. Anti-herbivory chemical defense in plants: a note on the concept of cost. The Am Nat 133:281-287

Gols R. 2014. Direct and indirect chemical defences against insects in a multitrophic framework. Plant Cell Environ 37:1741-1752

Greco WR, Bravo G, Parsons JC. 1995. The search for synergy: a critical review from a response surface perspective. Pharmacol Rev 47:331-385

Hartmann T, Ober D. 2000. Biosynthesis and metabolism of pyrrolizidine alkaloids in plants and specialized insect herbivores. Top Curr Chem 209:207-244

Hartmann T, Toppel G. 1987. Senecionine N-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris*. Phytochemistry 26:1639-1643

Hartmann T. 2008. The lost origin of chemical ecology in the late 19th century. Proc Natl Acad Sci USA 105:4541-4546

Herrera AA, Amor EC. 2011. Antiangiogenic activity of extracts and fractions from an endemic plant *Ardisia pyramidalis* (Cav.) Pers. From Bataan, Philippines using duck in ovo chorioallantoic membrane assay. J Med Plant Res 13:2637-2646

Horman I, Viani R. 1972. The nature and complexation of the caffeine–chlorogenate complex of coffee. J Food Sci 37:925-927

Inui T, Wang Y, Pro SM, Franzblau SG, Pauli GF. 2012. Unbiased evaluation of bioactive secondary metabolites in complex matrices. Fitoterapia 83:1218-1225

Jing J, Raaijmakers C, Kostenko O, Kos M, Mulder PPJ, et al. 2015. Interactive effects of above- and belowground herbivory and plant competition on plant growth and defence. Basic Appl Ecol 16:500-509

Johnson MTJ. 2011. Evolutionary ecology of plant defences against herbivores. Funct Ecol 25:305-311

Jones AC, Blum JE, Pawlik JR. 2005. Testing for defensive synergy in Caribbean sponges: Bad taste or glass spicules? J Exp Mar Bio Ecol 322:67-81

Kondoh M, Murakami A, Shigeta C, Tanimoto S. 1999. Flower-inducing and -inhibiting activities of phloem exudate from cotyledons of *Pharbitis* seedlings. Plant Growth Regul 28:107-116

Kuhlisch C, Pohnert G. 2015. Metabolomics in chemical ecology. Nat Prod Rep 32:937-955

Labuschagne A, Hussein AA, Rodriguez B, Lall N. 2012. Synergistic antimycobacterial actions of *Knowltonia vesicatoria* (L.f) Sims. Evid Based Complement Alternat Med 2012:1-9

Leiss KA, Choi YH, Abdel-Farid I, Verpoorte R, Klinkhamer PGL. 2009. NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. J Chem Ecol 35:219-229

Macel M, Bruinsma M, Dijkstra SM, Ooijendijk T, Niemeyer HM, et al. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. J Chem Ecol 31:1493-1508

Marti G, Erb M, Boccard J, Glauser G, Doyen GR, et al. 2013. Metabolomics reveals herbivore-induced metabolites of resistance and susceptibility in maize leaves and roots. Plant Cell Environ 36:621-639

Martin R, Lilley TH, Falshaw CP, Haslam E, Begley MJ, et al. 1987. The caffeine–potassium chlorogenate molecular complex. Phytochemistry 26:273-279

Miles PW, Oertli JJ. 1993. The significance of antioxidants in the aphid-plant interaction: the redox hypothesis. Entomol Exp Appl 67:275-283

Mithofer A, Boland W. 2012. Plant defense against herbivores: chemical aspects. Annu Rev Plant Physiol 63:431-450

Mösli Waldhauer SS, Baumann TW. 1996. Compartmentation of caffeine and related purine alkaloids depends exclusively on the physical chemistry of their vacuolar complex formation with chlorogenic acids. Phytochemistry 42:985-996

Moussaieff A, Rogachev I, Brodsky L, Malitsky S, Toal TW, et al. 2013. High-resolution metabolic mapping of cell types in plant roots. Proc Natl Acad Sci USA 3:E1232-E1241

Nelson AC, Kursar TA. 1999. Interactions among plant defense compounds: a method for analysis. Chemoecology 9:81-92

Nuessly GS, Scully BT, Hentz MG, Beiriger R, Snook ME, et al. 2007. Resistance to *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and *Euxesta stigmatias* (Diptera: Ulidiidae) in sweet corn derived from exogenous and endogenous genetic systems. J Econ Entomol 100:1887-1895

Nuringtyas TR, Choi YH, Verpoorte R, Klinkhamer PGL, Leiss KA. 2012. Differential tissue distribution of metabolites in *Jacobaea vulgaris*, *Jacobaea aquatica* and their crosses. Phytochemistry 78:89-97

Nuringtyas TR, Verpoorte R, Klinkhamer PGL, van Oers MM, Leiss KA. 2014. Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* using insect cell lines and injection bioassays. J Chem Ecol 40:609-616

Nuringtyas TR. 2014. Pyrrolizidine alkaloid variation in *Jacobaea* plants: from plant organ to cell level. PhD thesis, Leiden University, Leiden, The Netherlands

Pelser PB, de Vos H, Theuring C, Beuerle T, Vrieling K, Hartmann T. 2005. Frequent gain and loss of pyrrolizidine alkaloids in the evolution of *Senecio* section *Jacobaea* (Asteraceae). Phytochemistry 66:1285-1295

Richards LA, Dyer LA, Smilanich AM, Dodson CD. 2010. Synergistic effects of amides from two Piper species on generalist and specialist herbivores. J Chem Ecol 36:1105-1113

Richards LA, Lampert EC, Bowers MD, Dodson CD, Smilanich AM, et al. 2012. Synergistic effects of iridoid glycosides on the survival, development and immune response of a specialist caterpillar, *Junonia coenia* (Nymphalidae). J Chem Ecol 38:1276-1284

Ryabushkina NA. 2005. Synergism of metabolite action in plant responses to stresses. Russ J Plant Physiol 52:547-552

Siciliano T, Leo MD, Bader A, Tommasi ND, Vrieling K, et al. 2005. Pyrrolizidine alkaloids from *Anchusa strigosa* and their antifeedant activity. Phytochemistry 66:1593-600

Smith DB, Roddick JG, Jones LJ. 2001. Synergism between the potato glycoalkaloids α-chaconine and α-solanine in inhibition of snail feeding. Phytochemistry 57:229-234

Sondheimer E, Covitz F, Marquisee MJ. 1961. Association of naturally occurring compounds, the chlorogenic acid-caffeine complex. Arch Biochem Biophys 93:63-71

Van Vuuren SF, Viljoen AM. 2009. Interaction between the non-volatile and volatile fractions on the antimicrobial activity of *Tarchonanthus camphoratus*. S Afr J Bot 75:505-509

Whitehead SR, Bowers MD. 2014. Chemical ecology of fruit defence: synergistic and antagonistic interactions among amides from *Piper*. Funct Ecol 28:1094-1106

Williamson EM. 2001. Synergy and other interactions in phytomedicine. *Phytomedicine* 8:400-409

Witte L, Ernst L, Adam H, Hartmann T. 1992. Chemotypes of two pyrrolizidine alkaloid-containing *Senecio* species. Phytochemistry 31:559-565

Zimmermann GR, Lehar J, Keith CT. 2007. Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug Discov Today 12:34-42

Supplementary Material

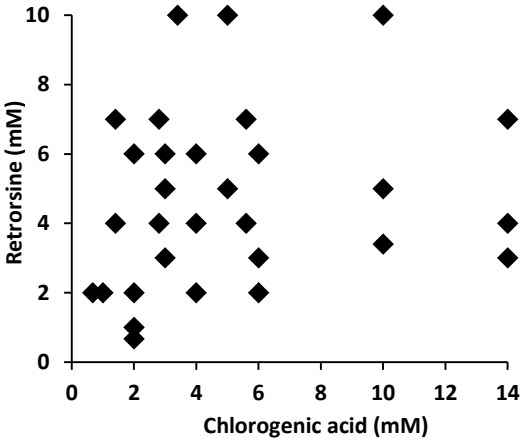


Figure S1. Combinations of retrorsine and chlorogenic acid (CGA) concentrations tested in the thrips bioassay, indicated by black dots.

Chapter 4

Synergistic effects between different classes of secondary metabolites on insect herbivores: a case study on pyrrolizidine alkaloid N-oxides and chlorogenic acid

Xiaojie Liu, Peter G. L. Klinkhamer, Klaas Vrieling

Abstract

Pyrrolizidine alkaloids (PAs) play an important role in plant-herbivore interactions. PAs can exist as free bases and N-oxides. PAs are mainly stored as N-oxides in plants, though they are less bioactive. We hypothesized that PA N-oxides may contribute to plant defence against herbivores in concert with other classes of SMs such as phenolic acids. Chlorogenic acid (CGA) is a phenolic acid also known to adversely affect insect herbivores. Therefore, we investigated the effects of the combinations of PA N-oxides and CGA on a generalist herbivore, the western flower thrips (*Franklinella occidentalis*). We found synergistic effects between CGA and all four tested PA N-oxides on thrips survival. In Chapter 3 we found antagonistic effects between free base PAs and CGA. Previous results also showed that single free base PAs were more effective than their corresponding single PA N-oxides. In this study we found that in combination with CGA, the PA N-oxides resulted in a lower thrips survival than the free base PAs. From an ecological point of view, we demonstrate that SMs which are less effective or inactive alone can positively contribute to the overall defensive efficacy of a plant because they act synergistically with other SMs. In the same manner, synergistic effects may explain the fact that PAs in plants mostly occur as N-oxides. The fact that SMs in combination can positively affect the bioactivity of individual metabolites has consequences for the way we should design bioactivity tests.

Keywords: Plant defence, Synergistic interactions, Phenolic acids, Retronecine, generalist herbivores

Introduction

Plants produce an enormous array of metabolites. The co-occurrence of metabolites in plants indicates a high possibility of interactions between metabolites to occur (Nelson and Kursar 1999; Whitehead and Bowers 2014). Although, in an ecological context, interactions between secondary metabolites (SMs) have received increasing interest (Gershenzon et al. 2012), so far only a limited number of studies on the interaction effects between plant SMs on insect herbivores have been published. These reports have mainly provided evidence for synergistic interactions, such as the synergistic effect between the phenylpropene myristicin and the furanocoumarin xanthotoxin on *Heliothis zea* (Berenbaum and Neal 1985), the synergistic effects between rutin and cholorogenic acid (CGA) on the relative growth rate of *S. exigua* (Stamp and Yang 1996), the synergistic effects of two amides on several insects and fruit-associated fungi (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 the synergistic effects of two iridoid glycosides on a specialist caterpillar *Junonia coenia* (Richards et al. 2012). Thus, the (limited) evidence suggests that synergism between plant SMs may be more common than previously been recognized, and that synergism may be the rule rather than the exception (Dyer et al. 2003; but see Chapters 2 and 3). Studies on interactions between SMs will yield additional and even novel information about the bioactivity of individual SMs, which may be overlooked when tested alone. This is of particular interest for metabolites that are weakly active or inactive alone. In concert with other metabolites, they may become potent and contribute to plant defence. For instance, Guillet et al. (1998) found synergism between volatile monoterpenes and α-terthienyl (a terthiophene), of *Porophyllum gracile* and *P. ruderale* (Asteraceae), to reduce the relative growth rate of *Ostrinia nubilalis* (Lepidoptera: Pyralidae) larvae. Monoterpenes alone had no significant effect while α-terthienyl was only slightly active.

Here, we focus on pyrrolizidine alkaloids (PAs) and their interaction with one of the most common plant phenolics, CGA. PAs are amongst the most well-known plant defence metabolites (Dreyer et al. 1985; de Boer 1999; Reina et al. 2001; Siciliano et al. 2005; Dominguez et al. 2008; Jing et al. 2015; see also the reviews by Macel 2011 and Trigo 2011 and references therein). Except for the minor PAs with an otonecine base, PAs can exist in two interchangeable forms: free base alkaloids and their N-oxides. The latter is the major storage form in plants (Hartmann et al. 1989). For instance, in *Jacobaea* species more than 90% of the PAs are present as N-oxides (Joosten et al. 2011) though jacobine-like PAs are reported to occur upto 50% as free base. With respect to fending off insect herbivores, PA N-oxides have been reported to be less active than their corresponding free bases (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005; Hartmann 2007;

Nuringtyas et al. 2014; Chapter 2). Our understanding of the function of PAs, especially of the PA N-oxides, in plant defence is still incomplete. In the first place, it is presently not yet fully clear to what extent PA N-oxides contribute to plant defence. Secondly, it is still not well explained why plants store PAs in apparently the least active form. As a potential explanation we investigated, in Chapter 2, whether or not the bioactivity of the most abundant PA N-oxides, senecionine N-oxide, jacobine N-oxide and erucifoline N-oxide, interacted synergistically in their effect on thrips. We found no synergistic (or antagonistic) effects on thrips survival. Thus, these results offered no explanation for the fact that PAs mainly occur as N-oxides. Alternatively, it has been suggested that N-oxides are better soluble in water and therefore more easily stored in the vacuoles and transported through the plant (von Borstel et al. 1986).

Much of our current knowledge about the defence role of PAs comes from studies on single PAs (Reina et al. 2001; Macel et al. 2005; Leiss et al. 2009; Cheng et al. 2011b; Kostenko et al. 2013). Interestingly, we found that free base PAs antagonistically interacted with CGA in their effects on *S. exigua* cell lines (Nuringtyas, PhD thesis, 2014) and on thrips larvae (Chapter 3). These antagonistic interactions may provide an explanation for why PAs mainly occur as N-oxides if such antagonistic interactions would not be found for PA N-oxides. Therefore, in this chapter, we studied the effects of the interactions between PA N-oxides and CGA on thrips.

CGA is known to be involved in plant defence. CGA significantly reduced the survival of thrips (Leiss et al. 2009), the growth of the cabbage looper *Trichoplusia ni* (Beninger et al. 2004), tobacco hornworm *Manduca sexta* (Stamp 1994) and the corn earworm *Heliothis zea* (Farrar and Kennedy 1987, Felton and Duffey 1990). However, effects of CGA do not seem consistent across plant species. 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 in tomato *Solanum lycopersicum* was detected on this thrips (Mirnezhad 2011). These findings suggest that the effects of an individual SM vary depending on the phytochemical contexts. More broadly speaking, interactions that potentially occur between SMs may explain the discrepancy that some SMs are bio-active in particular species while they show no activity or less activity in other species.

We investigated the interaction between PA N-oxides and CGA on the survival of western flower thrips (*F. occidentalis*), a generalist herbivore. We addressed the following research questions: Do PA N-oxide synergistically interact with CGA in their effects on thrips? Are interaction effects between PA N-oxides and CGA different from that between the free base PA and CGA? Could this explain the predominant occurrence of PA N-oxides in plants?

Material and methods

More than 37 different free base PAs and PA N-oxides have been identified from *J. vulgaris* (Cheng et al. 2011a). Except for jacobine, which also occurs as a free base, most PAs occur as N-oxides in *J. vulgaris* (Joosten et al. 2011). Senecionine N-oxide is synthesized in the roots and transported via the phloem to the shoots, where it is metabolized into other PA structures (Hartmann and Toppel 1987) in *Senecio vulgaris*. All four PA N-oxides tested in the current study have a 12-membered macrocyclic diester ring and a retronecine N-oxide base (Figure 1).

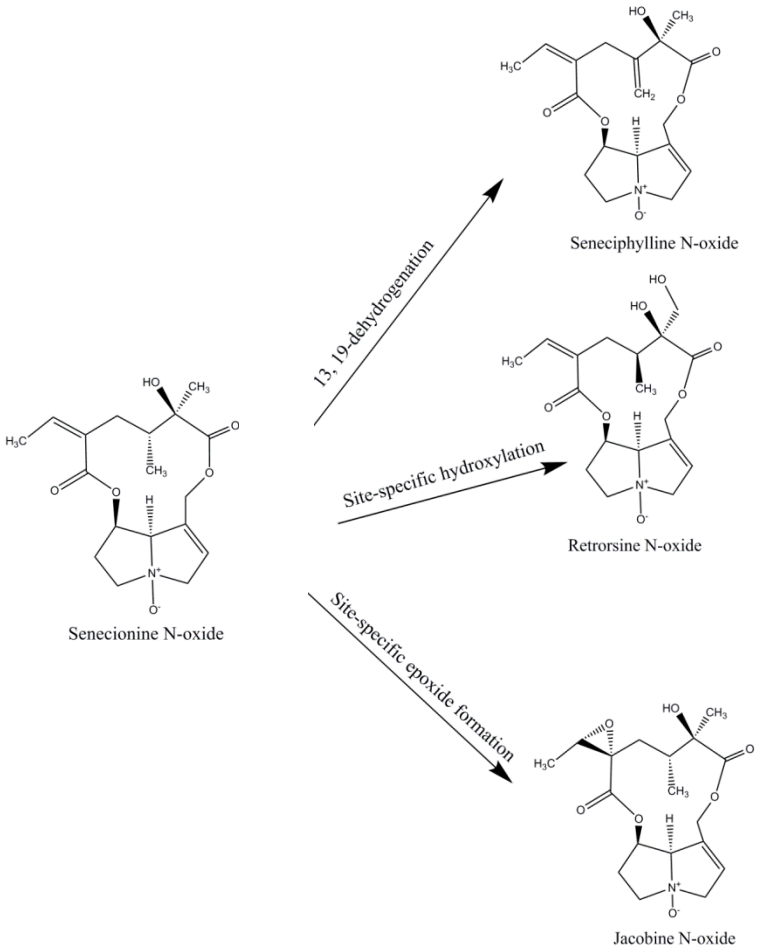


Figure 1. Chemical structures of the pyrrolizidine alkaloid (PA) N-oxides used and a simplified biosynthetic pathway of PA N-oxides in *Jacobaea* species (after Hartmann 1989).

Pyrrolizidine alkaloids N-oxides

Retrorsine N-oxide was purchased from Sigma Aldrich (St. Louis, MO, USA). The other three N-oxides, senecionine N-oxide, seneciphylline N-oxide and jacobine N-oxide were

obtained by N-oxidation of the corresponding free base PAs by EXPLANT, Leiden, The Netherlands. Seneciphylline and jacobine were isolated by EXPLANT, Leiden, The Netherlands. Senecionine was purchased from PhytoPlan (Heidelberg, Germany).

Experiments with combinations of PA N-oxides and CGA

CGA and PA N-oxides were dissolved in 30 µL methanol (MeOH) to prepare stock solutions (28 mM and 14 mM respectively). From these stock solutions dilutions in MeOH were prepared with concentrations of 2.8, 5.6 and 11.2 mM for CGA, 7 mM for PA N-oxides. These solutions were used to prepare test solutions for the thrips bioassay.

Three doses of 0, 4 and 7 mM of senecionine N-oxide, seneciphylline N-oxide, retrorsine N-oxide and jacobine N-oxide, respectively, were combined with CGA at five concentrations of 0, 1.4, 2.8, 5.6 and 14 mM. These particular concentrations were chosen because these levels were tested in the combinations of free base PAs with CGA (Chapter 3 of this thesis). Thus, we can compare the effects of the free base PAs and those of PA N-oxides in combination with CGA.

Thrips bioassay

Full details about the bioassay and the rearing conditions of thrips have been given in Chapters 2 and 3 of this thesis. Each bioassay was carried out using eight 96-well plates at a time. Per two 96-well plates there are 24 columns (1-24) of 8 wells. A column received the same treatment and therefore consisted of 8 replicates. Of the 24 columns, 19 columns were filled with 19 different treatments and 5 columns were left empty. The 19 treatments included one negative control (only the solvent), 16 treatment groups (3 concentrations of a single PA N-oxide, 5 concentrations of CGA and 8 combinations of PA N-oxide and CGA) and two positive controls (empty wells and solvent with the insecticide abamectin (50 µg/mL)). Four sets of two 96 wells plates were carried out simultaneously yielding 32 replicates for all treatments. The complete bioassay with eight 96 wells plate was repeated at a different time so that we obtained two independent estimates of another 32 replicates of thrips survival for analysis of variance. After five days, the numbers of surviving larvae were recorded with a stereo microscope.

The test solutions consisted of 30 µL solution of the PA N-oxide in methanol and 30 µL solution of CGA in methanol, dissolved in 1.94 mL 40 mM phosphate buffer pH 7, containing 10% fructose and 3% MeOH. The 40 mM sodium phosphate buffer was prepared from 1 M phosphate buffer (pH 7), which in turn had been prepared by mixing 57.7 mL of 1 M NaH_2PO_4 and 42.3 mL of 1 M Na_2HPO_4. A solution of 10% fructose and 3% MeOH in 40 mM phosphate buffer (pH 7) was used as a negative control. There were two

positive controls: the insecticide abamectin (50 µg/mL) dissolved in 40 mM phosphate buffer pH 7 containing 10% fructose and 3% MeOH.

Statistical analysis

To evaluate the effects of the interactions between two metabolites, we first constructed a multiplicative "null interaction" model that predicts the effect of metabolites in the absence of an interaction. In the multiplicative null model, the survival after the application of a combination of metabolite X and metabolite Y (S_{X+Y}) is the product of the survival after applications of X (S_X) and Y (S_Y), assuming no interaction effects, i.e. $S_{X+Y} = S_X * S_Y$. Multiplicative models assume that the relationship between log survival and the tested concentration is linear.

Correcting for survival in the negative control

The survival for all treatments was first corrected for the negative control. The observed experimental survival of larvae (S_{X+NC}) results from the survival after application of tested metabolite (S_X) and from the survival in the solvent (S_{NC}). The survival of the solvent is measured in the negative control. Under the assumption that the two effects are independent, we get the following equation:

$$\mathbf{S_{X+NC} = S_{NC} * S_X} \qquad \mathbf{(1)}$$

where S_{X+NC} is the observed experimental survival while S_{NC} is the survival when the control solvent is added to the artificial medium.

Consequently, the survival resulting from the application of the metabolite S_X can thus be calculated as

$$\mathbf{S_X = S_{X+NC} / S_{NC}} \qquad \mathbf{(2)}$$

Testing the interaction effects of the combination of PA N-oxides and CGA on survival

The survival of thrips for the combination of PA N-oxides and CGA ($S_{PANO+CGA}$) results from the survival after application of PA N-oxides (S_{PANO}), the survival after application of CGA (S_{CGA}), and their interaction ($S_{PANO*CGA}$). Consequently, the effect of the interaction of two metabolites on survival can be calculated by:

$$\mathbf{S_{PANO*CGA} = S_{PANO+CGA} / (S_{PANO} * S_{CGA})} \qquad \mathbf{(3)}$$

In which $S_{PANO+CGA}$ is the observed survival in experiments with combinations while S_{PANO} and S_{CGA} are the observed thrips survival in experiments with single PA N-oxides and CGA

respectively while $S_{PANO*CGA}$ denotes the interaction effect. The interaction effect indicates the effect of the interaction between the two metabolites on the survival of thrips. Note that the interaction effect can be synergistic ($S_{PANO*CGA} < 1$) or antagonistic ($S_{PANO*CGA} > 1$). As each experiment is repeated twice two independent estimates of the interaction effect are obtained. The interaction effects $S_{PANO*CGA}$ were calculated for all combinations and were expressed as mean value ± standard error of the mean (SE).

Two-way analysis of variances (ANOVAs) were used to analyze if the interaction effect $S_{PANO*CGA}$ was significantly deviating from one and thus if $S_{PANO*CGA}$ -1 was significantly different from zero. For all combinations of traits, CGA concentration with 4 levels and PA N-oxide concentration with 2 levels were fixed factors while the estimate of $S_{PANO*CGA}$ -1 (= $S_{PANO+CGA} / (S_{PANO} * S_{CGA})$ -1) was the dependent variable. Therefore, if the intercept of two-way ANOVA is significantly deviating from zero it indicates that $S_{PANO* CGA}$ -1 is deviating from zero and hence that interaction effect $S_{PANO*CGA}$ is significantly deviating from one. To avoid confusion with statistical interaction terms of ANOVAs, we will always refer to the value of the interaction between the metabolites as the "interaction effect $S_{PANO*CGA}$".

To increase the readability of the figure, we plotted fraction mortality (i.e. 1 – fraction survival) instead of fraction survival itself against the concentrations of the two metabolites in a 3-dimensionsial (3D) graph with the 2 horizontal axes representing the two metabolite concentrations, and the vertical axis representing thrips mortality.

The strength of the interaction effects was compared by a three-way ANOVA with PA N-oxide type (senecionine N-oxide, seneciphylline N-oxide, retrorsine N-oxide and jacobine N-oxide), PA N-oxide concentration and CGA concentration as fixed factors and the interaction effect $S_{PANO* CGA}$ as the dependent variable.

Comparison of the interaction effects of free base PAs and the corresponding N-oxides with CGA

To visually compare the interaction effects of free base PAs and PA N-oxides combined with CGA on thrips survival, we plotted CGA concentrations against the value of Δ thrips survival (y axis), i.e. the average survival of the combination of free base PAs and CGA from two independent bioassays minus the average survival of the combination of the N-oxides and CGA from two independent bioassays. Data for the (free base) PAs and CGA were taken from Chapter 3. The standard deviation (σ) of the Δ thrips survival was calculated by equation (4), where σ (PA N-oxide) and σ (free base PA) are the standard deviation of the thrips survival from the experiment with PA N-oxides combined with CGA and the standard deviations of the thrips survival from the experiment with free base PAs combined with CGA.

$$\boldsymbol{\sigma(\Delta \text{ thrips survival}) = \sqrt{\sigma\,(\text{PA N} - \text{oxide})^2 + \sigma\,(\text{free base PA})^2}} \qquad \textbf{(4)}$$

And then, the 95% confidence intervals (CIs) of Δ survival were estimated by equation (5).

$$\boldsymbol{95\%\ \text{CIs} = \frac{\sigma(\Delta \text{ thrips survival})}{\sqrt{n}} * 1.96} \qquad \textbf{(5)}$$

With σ (Δ thrips survival) being the standard deviation from equation (4) and n being number of measurements for each combination group (n = 2). 95% CIs are used to determine if Δ thrips survival is significantly deviating from zero. A Δ thrips survival larger than 0 indicates that the average thrips survival is lower in the presence of the PA N-oxides and a Δ thrips survival smaller than 0 indicates that thrips survival is lower in the presence of the free base PAs.

All statistical analysis were performed using SPSS software for Windows (version 21.0; SPSS Inc., Chicago, IL).

Results

Thrips larval survival in negative and positive controls

The average survival in the negative control was 87%. There were two positive controls for each treatment group: a solution with the insecticide abamectin (50 µg/mL) with an average survival of 11% and a treatment with empty wells with an average survival of 6%. The latter showed that thrips in the wells did feed through the parafilm from the test solution because without feeding they would have died.

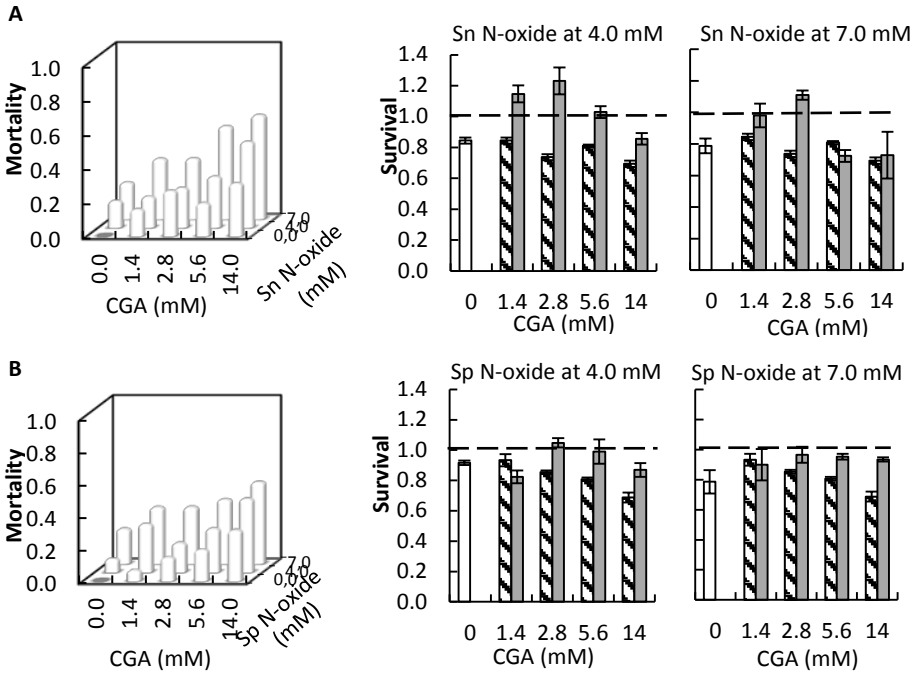
Interactions effects between PA N-oxides and CGA on thrips

Compared to PA N-oxides and CGA alone, all the combinations of CGA and PA N-oxides decreased thrips survival, indicating synergistic interactions (Table 1, Figure 2; note that here fraction mortality was plotted in the 3D figures in order to increase readability of the figure). All significant interaction effects, $S_{PANO*CGA}$ were smaller than one indicating synergistic effects except for senecionine N-oxide (Table 1, Figure 2). Although for senecionine N-oxide the intercept was not significant, both main effects, senecionine N-oxide and CGA concentration, were significant while the interaction effect $S_{PANO*CGA}$ showed synergistic interactions at the highest concentration while antagonistic effects were present at the lowest concentrations (Figure 2A).

Table 1. Two-way analyses of variance (ANOVAs) with pyrrolizidine alkaloid N-oxide concentration and chlorogenic acid (CGA) concentration as fixed factors and the interaction effect $S_{PANO*CGA}$ (Equation 2) minus one as a dependent variable. Survival was corrected for differences among the negative controls. A significant intercept or main effects indicate that synergistic or antagonistic interactions occur. For all significant intercepts, the interaction effects $S_{PANO*CGA}$ were smaller than one indicating synergistic interactions (see also Figure 2).

Factors	*df*	*F*	*P*
Intercept	1, 15	0.8	NS
CGA concentration	3, 15	10.4	< 0.01
Senecionine N-oxide concentration	1, 15	11.4	< 0.01
CGA concentration * Senecionine N-oxide concentration	3, 15	0.6	NS
Intercept	1, 15	6.2	< 0.05
CGA concentration	3, 15	1.4	NS
Seneciphylline N-oxide concentration	1, 15	0.2	NS
CGA concentration * Seneciphylline N-oxide concentration	3, 15	0.6	NS
Intercept	1, 15	90.1	< 0.001
CGA concentration	3, 15	42.7	< 0.001
Retrorsine N-oxide concentration	1, 15	80.0	< 0.001
CGA concentration * Retrorsine N-oxide concentration	3, 15	20.5	< 0.001
Intercept	1, 15	93.3	< 0.001
CGA concentration	3, 15	2.2	NS
Jacobine N-oxide concentration	1, 15	1.2	NS
CGA concentration * Jacobine N-oxide concentration	3, 15	4.1	< 0.05

NS = Not significant



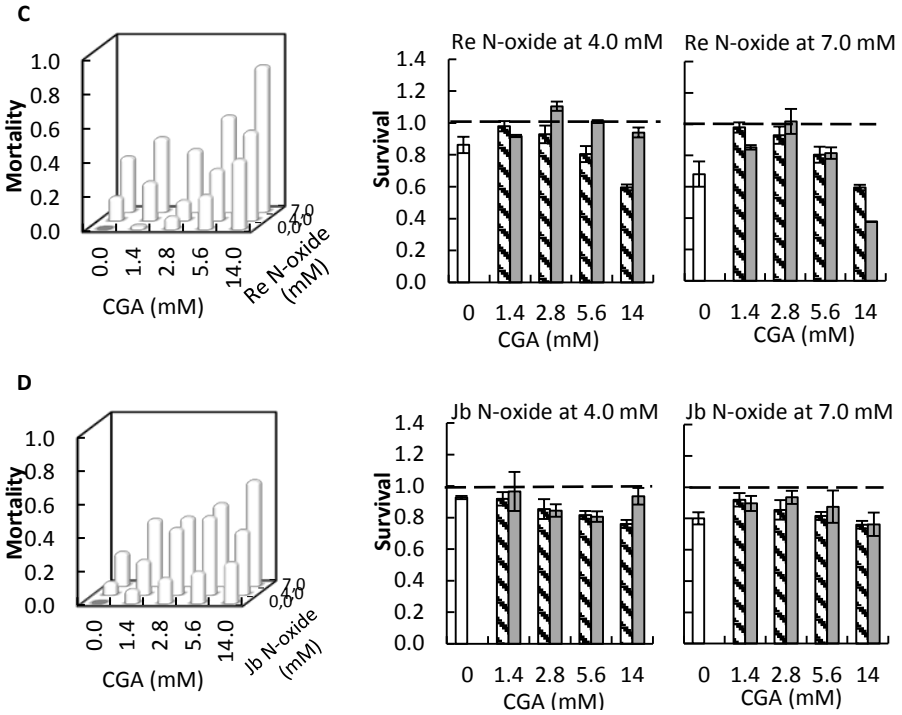


Figure 2. Left: Fraction mortality (= 1- fraction survival) of 2nd instar larvae of thrips (*Frankliniella occidentalis*) caused by the single metabolites and the combination of chlorogenic acid (CGA) with senecionine N-oxide (**A**), seneciphylline N-oxide (**B**), retrorsine N-oxide (**C**) or jacobine N-oxide (**D**). **Right:** Fraction survival (mean ± 95% confidence intervals) of thrips caused by pyrrolizidine alkaloid (PA) N-oxides alone (S_{PANO}) (white bars), chlorogenic acid (CGA) alone (S_{CGA}) at four concentrations (hatched bars), and the interaction effect $S_{PANO*CGA}$ (Equation 3) between PA N-oxides and CGA (grey bars). In the left figures, the fraction mortality was plotted to increase the readability of the figure. In the right figures, the dashed line indicates a thrips survival fraction of one. A significant deviation from one would indicate a synergistic (< 1) or antagonistic effect (> 1). Two-way ANOVAs were used to analyze whether the overall interaction effect $S_{PANO*CGA}$ deviated significantly from one (Table 1).

For seneciphylline N-oxide, the strength of the synergistic effect was independent from the concentrations of the two metabolites. In the case of retrorsine N-oxide the strength of the synergistic effect also depended on the concentrations of both metabolites and the concentrations at which they were combined (Table 1, Figure 2C). For jacobine N-oxide, the strength of the synergistic effect did not depend on the concentrations of the two metabolites while it did depend on the combinations of the concentrations (Table 1, Figure 2D).

Comparison of the interaction effects and the bioactivity of individual metabolites

The interaction effects are relatively small and vary between 0.7 and 1.1. For all PA N-oxides only two out of 16 combinations showed interaction effects larger than one, indicating that mostly synergistic interactions between the PA N-oxides and CGA occur. The strongest interaction effect was found for the interaction between retrorsine N-oxide (7 mM) and CGA at 14 mM ($S_{PANO*CGA}$ = 0.377 ± 0.001). The survival of retrorsine N-oxide alone (S_{PANO}) was 0.68 ± 0.08 and that of CGA alone (S_{CGA}) was 0.60 ± 0.02 (Figure 2C). This means that the expected survival if there is no interaction is 0.682 * 0.596 = 0.406. The observed survival was much lower ($S_{PANO+CGA}$ = 0.153) than the expected survival (0.406). From this, we calculated that the interaction effect ($S_{PANO*CGA}$) was 0.377.

Comparison of the interaction effects between the free base PAs and PA N-oxides in combination with CGA

In contrast with our current results, we found antagonistic effects for the interactions between free base PAs and CGA on thrips survival. We compared thrips survival from these two experiments for retrorsine and jacobine by calculating the difference in thrips survival between the two experiments, the Δ thrips survival. In the case of jacobine and retrorsine, the free base PAs alone resulted in a negative Δ thrips survival indicating a lower thrips survival on free bases compared to the PA N-oxides (Figure 3). Generally, we found that the Δ thrips survival for the combination between PA N-oxides and CGA are positive indicating a lower thrips survival for the combinations of PA N-oxides with CGA compared to the combination of free base PAs with CGA. For retrorsine, Δ thrips survival depends on the CGA concentration (Figure 3A). The higher the CGA concentration, the lower the thrips survival of retrorsine and jacobine N-oxide and CGA in comparison with that of free base retrorsine and jacobine and CGA (Figure 3).

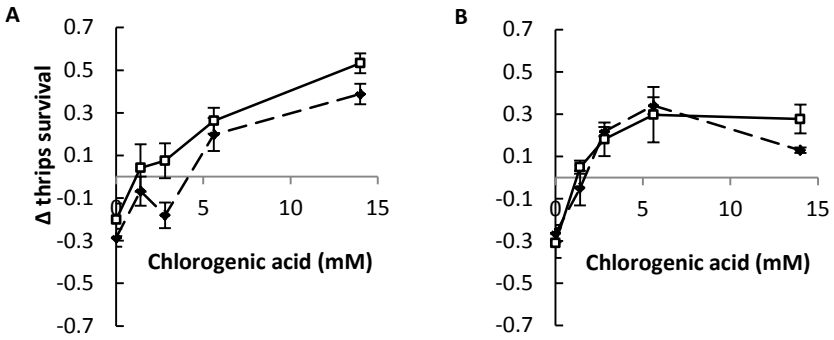


Figure 3. Δ thrips survival (= thrips survival for the combination of free base pyrrolizidine alkaloids (PAs) and chlorogenic acid (CGA) minus thrips survival for the combination of PA N-oxides and CGA (± 95% confidence interval) against CGA concentration. A. retrorsine **B.** jacobine. Dashed lines: PA concentration of 4 mM, solid lines: PA concentration of 7 mM.

Discussion

In this chapter, we showed synergistic interactions between PA N-oxides and CGA in their bioactivity against thrips. The strength of the interaction depended on the type of PA N-oxide, the PA N-oxide concentration and the CGA concentration. Moreover, we found that thrips survival when tested as a single PA were lower for the free base PAs than the PA N-oxides while thrips survival is higher for free base PAs combined with CGA compared to the PA N-oxides combined with CGA (Figure 3). The importance of synergistic or antagonistic interactions of PAs in plant defence against herbivores has so far been tested mainly for the effects of interactions among PAs only. Macel et al. (2005) found a weak synergistic effect of a mixture of senecionine, seneciphylline and senkirkine on *S. exigua* and of a mixture of senecionine and seneciphylline on *L. migratoria*. In our previous study, two-by-two combinations of three PA N-oxides (senecionine N-oxide, jacobine N-oxide and erucifoline N-oxide) showed no synergistic (or antagonistic) effects on thrips survival (Chapter 2 of this thesis).

The synergistic effects on thrips survival that were found show that PA N-oxides can be an active defence against insect herbivores through the synergistic interaction with other metabolites. The synergistic effects also supply an ecological driven alternative explanation for the predominate storage of PA N-oxides in plants. Previously, it has been reported that PA N-oxides are less bioactive than their corresponding free bases in warding off insect herbivores (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005; Nuringtyas et al. 2014; Chapter 2). This raised the question why PAs are mostly present in plants in their least active form. An alternative physiological explanation is that N-oxides are more soluble and can therefore be better stored and transported (von Borstel and Hartmann 1986; Hartmann and Toppel 1987; Lindigkeit et al. 1997; Chapter 2). Our results provide a new explanation: in combination with CGA, PA N-oxides show synergistic effects on thrips survival while free base PAs showed antagonistic effects on thrips survival (Chapter 3) and on *S. exigua* cell lines (Nuringtyas, PhD thesis, 2014).

Synergism between PA N-oxides and other SMs could also explain, to some extent, the inconsistent results from correlative studies and bioassays on individual PA N-oxides. In a correlation study, Cheng et al. (2011) found that thrips damage decreased with increasing concentration of jacobine N-oxide. In contrast, we did not find a dose-dependent effect of jacobine N-oxide on thrips survival in a bioassay with pure compounds (Chapter 2). Here we found a significantly negative dose-dependent effect of jacobine N-oxide on thrips in combination with CGA. In concert with other plant metabolites, PA N-oxide may become active, ultimately producing a greater efficiency.

Increasing evidence suggests that the importance of interactions between metabolites in plants may be even greater than is currently assumed. Our findings suggest that the

interaction between PA N-oxides and CGA may positively contribute to the plant's defence against generalist insect herbivores. Synergistic interactions may be even more interesting for SMs that are less effective or apparently lack defensive properties on their own (Harborne 1988; Ayres et al. 1997; Dyer et al. 2003).

Apart from its ecological significance, synergism between metabolites also provides promising leads for research. One main strategy in the isolation of new active compounds consists of so-called bio-guided fractionation (Queiroz et al. 2009; Sasidharan et al. 2011). This approach involves repetitive fractionation and assessment of biological activity up to the isolation of pure metabolites with the selected biological activity (Brusotti et al. 2013). One major problem of this approach is that many of the plant extracts that have been tested have yielded activities that later disappeared when the extracts were fractionated into individual metabolites (Foungbe et al. 1991; Turner 1996; Schuster 2001). As a result, a selective metabolite often fails to achieve the desired effect (Ji et al. 2009). The loss of synergistic effects between metabolites by separating metabolites by fractionation might be largely responsible for the loss of bioactivity. In phytochemical studies, very often plant metabolites work synergistically and rarely a single metabolite is fully responsible for the biological activity found (Keith et al. 2005; Brusotti et al. 2013).

Further efforts should also be made to work out the possible mechanisms underlying the synergistic effects between PA N-oxides and CGA on thrips. Several mechanisms underlying the interaction effects are reported. Insect herbivores have evolved counter defence strategies, e.g. excretion, sequestration, degradation against a particular SM. As a counter adaption to those insect strategies, plants can sabotage the defence strategy of the insects by other metabolites that interfere with the insect adaption, restoring the original activity of the SMs. For instance, monoterpenes have been proposed to increase the toxicity of α-terthienyl by inhibiting cytochrome P450 enzymes involved in α-terthienyl degradation (Guillet et al. 1998). Mechanistic understandings of the action of plant metabolites in insects have not been largely addressed. Ecologists can borrow ideas from the pharmacological studies (Williamson 2001; Zimmermann et al. 2007; Biavatti 2009; Efferth and Koch 2011; Labuschagne et al. 2012), which demonstrate that synergism can arise due to the modification of metabolites, i.e., by the conversion of inactive metabolites into active ones after their interaction with a target, by enhancing target availability, by enhancing the cell membrane permeability (Amin et al. 2015) and thus facilitating the transport, by interfering or inhibiting the enzymes that are responsible for the detoxification of other metabolites (Scott et al. 2002), by blocking the some certain ionic pump and thus potentiating the action of another metabolite (Stermitz et al. 2000), etc.

In the current chapter and Chapter 3, we studied the interactions between two known metabolites and their effects on the bioactivity of individual metabolites, based on the prior knowledge of PAs and CGA. This is a first step to study the interactions between plant

metabolites. The results obtained here are encouraging both in terms of ecological significance and of providing the approach to design bioassays. These results also demonstrate the need to further analyze the interactions between metabolites on a large-scale or at a more complex level, taking into account the complexity of the natural situation and the large part of unidentified or unknown metabolites in plants. One way forward is to add the individual metabolites that are of particular interest into the natural phytochemical background of other metabolites (Chapters 5 and 6), which will provide information in addition to what we have obtained in the specifically component-interaction analysis.

Conclusions

This study seeks to better understand the contribution of PA N-oxides to plant defence in concert with other metabolites and to provide an explanation for the predominant occurrence of PA N-oxides in plants from a perspective of plant-insect associations. Here we show for the first time the presence of synergistic interactions between PA N-oxides and CGA on thrips survival. Several conclusions emerge. Firstly, the current study indicates that the mode of action in combination with other SMs may differ significantly from that of the metabolite alone. It suggests that SMs that are less effective or inactive alone may, in concert with other metabolites, increase or even obtain biological effectiveness. In the same way, it may explain why PAs are stored in the plant as N-oxides. Secondly, synergistic effects of interactions between plant metabolites may also explain the discrepancy between correlative studies and studies using bioassays with single PA N-oxides. A given plant metabolite is likely not to be a sole agent, but rather is likely to be imbedded as a participant in a multitude of interactions that naturally occur in plants. Thus, studying the bioactivity of individual plant metabolites against a background of other metabolites can also assist in understanding plant-insect herbivore interactions.

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References

Amin MU, Khurram M, Khattak B, Khan J. 2015. Antibiotic additive and synergistic action of rutin, morin and quercetin against methicillin resistant *Staphylococcus aureus*. BMC Complement Altern Med 15:59

Ayres MP, Clausen TP, Maclen J, Redman AM, Reichardt PB. 1997. Diversity of structure and antiherbivore activity in condensed tannins. Ecology 78:1696-1712

Beninger CW, Abou-Zaid MM, Kistner ALE, Hallett RH, Iqbal MJ, Grodzinski B, Hall JC. 2004. A flavanone and two phenolic acids from *Crysanthemum morifolium* with phytotoxic and insect growth regulating activity. J Chem Ecol 30:589-606

Berenbaum M, Neal JJ. 1985. Synergism between myristicin and xanthotoxin, a naturally cooccurring plant toxicant. J Chem Ecol 11:1349-1358

Biavatti MW. 2009. Synergy: an old wisdom, a new paradigm for pharmacotherapy. Braz J Pharm Sci 45:371-378

Brusotti G. Cesari I. Dentamaro A, Caccialanza G, Massolini G. 2013. Isolation and characterization of bioactive compounds from plant resources: The role of analysis in the ethnopharmacological approach. J Pharm Biomed Anal 87:218-228

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011a. Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. New Phytol 192:1010-1023

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011b. The relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance in F(2) hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. J Chem Ecol 10:1071-1080

Craig JC, Purushothaman KK. 1970. An improved preparation of tertiary amine N-oxides. J Org Chem 35:1721

de Boer NJ. 1999. Pyrrolizidine alkaloid distribution in *Senecio jacobaea* rosettes minimises losses to generalist feeding. Entomol Exp Appl 91:169-173

Domínguez DM, Reina M, Santos-Guerra A, Santana O, Agulló T, et al. 2008. Pyrrolizidine alkaloids from *Canarian* endemic plants and their biological effects. Biochem Syst Ecol 36:153-166

Dreyer DL, Jones KC, Molyneux RJ. 1985. Feeding deterrency of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrthosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. J Chem Ecol 11:1045-1051

Dyer LA, Dodson CD, Stireman JO, Tobler MA, Smilanich AM, et al. 2003. Synergistic effects of three Piper amides on generalist and specialist herbivores. J Chem Ecol 29:2499-2514

Efferth T, Koch E. 2011. Complex interactions between phytochemicals. The multi-target therapeutic concept of phytotherapy. Curr Drug Targets 12:122-132

Farrar RR, Kennedy GG. 1987. Growth, food consumption and mortality of *Heliothis zea* larvae on foliage of the wild tomato *Lycopersicon hirsutum* f. glabratum and the cultivated tomato, *L. esculentum*. Entomol Exp Appl 44:213-219

Felton GW, Duffey SS. 1990. Inactivation of baculovirus by quinones formed in insect-damaged plant tissues. J Chem Ecol 16:1221-1236

Foungbe S, Kouassi G, Kablan JB, Marcy R. 1991. Study of Costus lucanusianus: plant juice, fraction combinations and pharmacologic estimation of natural product total activity. J Ethnopharmacol 33:221-226

Gershenzon J, Fontana A, Burow M, Wittstock U, Degenhardt J. 2012. Mixtures of plant secondary metabolites: metabolic origins and ecological benefits. The Ecology of Plant Secondary Metabolites: From Genes to Global Processes (eds G.R. Iason, M. Dicke & S.E. Hartley), pp. 56-77. Cambridge University Press, Cambridge

Guillet G, Belanger A, Arnason JT. 1998. Volatile monoterpenes in *Porophyllum gracile* and *P. ruderale* (Asteraceae): Identification, localization and insecticidal synergism with alpha-terthienyl. Phytochemistry 49:423-429

Hartmann T, Ehmke A, Eilert U, von Borstel K, Theuring C. 1989. Sites of synthesis, translocation and accumulation of pyrrolizidine alkaloid N-oxides in *Senecio vulgaris*. Planta 177:98-107

Hartmann T, Toppel G. 1987. Senecionine N-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris*. Phytochemistry 26:1639-1643

Hartmann T. 2007. From waste products to ecochemicals: fifty years research of plant secondary metabolism. Phytochemistry 68:2831-2846

Jing J, Raaijmakers C, Kostenko O, Kos M, Mulder PPJ, et al. 2015. Interactive effects of above- and below ground herbivory and plant competition on plant growth and defence. Basic Appl Ecol 16:500-509

Joosten L, Cheng D, Mulder PPJ, Vrieling K, van Veen JA, et al. 2011. The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. Phytochemistry 72:214-222

Keith CT, Borisy AA, Stockwell BR. 2005. Multicomponent therapeutics for networked systems. Nat Rev Drug Discov 4:71-78

Kostenko O, Mulder PPJ, Bezemer TM. 2013. Effects of root herbivory on pyrrolizidine alkaloid content and above ground plant-herbivore-parasitoid interactions in *Jacobaea vulgaris*. J Chem Ecol 39:109-119

Labuschagne A, Hussein AA, Rodriguez B, Lall N. 2012. Synergistic antimycobacterial actions of *Knowltonia vesicatoria* (L.f) Sims. Evid Based Complement Alternat Med 2012:1-9

Leiss KA, Choi YH, Abdel-Farid I, Verpoorte R, Klinkhamer PGL. 2009. NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. J Chem Ecol 35:219-229

Lindigkeit R, Biller A, Buch M, Schiebel HM, Boppre M,et al. 1997. The two faces of pyrrolizidine alkaloids: the role of tertiary amine and its N-oxide in chemical defense of insects with acquired plant alkaloids. Eur J Biochem 245:626-636

Macel M, Bruinsma M, Dijkstra SM, Ooijendijk T, Niemeyer HM, et al. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. J Chem Ecol 31:1493-1508

Macel M. 2011. Attract and deter: a dual role for pyrrolizidine alkaloids in plant–insect interactions. Phytochem Rev 10:75-82

Mirnezhad M. 2011. Host plant resistance of tomato plants to western flower thrips. PhD thesis, Leiden University, The Netherlands

Mithofer A, Boland W. 2012. Plant defense against herbivores: chemical aspects. Annu Rev Plant Physiol 63:431-450

Nelson AC, Kursar A. 1999. Interactions among defense compounds: A method for analysis. Chemoecology 9:81-92

Nenaah GE. 2011. Toxic and antifeedant activities of potato glycoalkaloids against Trogoderma granarium (Coleoptera: Dermestidae). J Stored Prod Res 47:185-190

Nuringtyas TR, Verpoorte R, Klinkhamer PGL, van Oers MM, Leiss KA. 2014. Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* using insect cell lines and injection bioassays. J Chem Ecol 40:609-616

Nuringtyas TR. 2014. Pyrrolizidine alkaloid variation in Jacobaea plants: from plant organ to cell level. PhD thesis, Leiden University, Leiden, The Netherlands

Queiroz EF, Wolfender JL, Hostettmann K. 2009. Modern approaches in the search for new lead antiparasitic compounds from higher plants. Curr Drug Targets 10:202-211

Reina M, González-Coloma A, Gutiérrez C, Cabrera R, Rodríguez ML, et al. 2001. Defensive chemistry of *Senecio miser*. J Nat Prod 64:6-11

Richards LA, Dyer LA, Smilanich AM, Dodson CD. 2010. Synergistic effects of amides from two Piper species on generalist and specialist herbivores. J Chem Ecol 36:1105-1113

Richards LA, Lampert EC, Bowers MD, Dodson CD, Smilanich AM, et al. 2012. Synergistic effects of iridoid glycosides on the survival, development and immune response of a specialist caterpillar, *Junonia coenia* (Nymphalidae). J Chem Ecol 38:1276-1284

Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L. 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med 8:1-10

Schuster BG. 2001. A new integrated program for natural product development and the value of an ethnomedical approach. J Altern Complement Med 7:S61-S72

Scott IM, Puniani E, Durst T, Phelps D, Merali S, et al. 2002. Insecticidal activity of *Piper tuberculatum Jacq.* extracts: synergistic interaction of piperamides. Agr Forest Entomol 4:137-144

Siciliano T, Leo MD, Bader A, Tommasi ND, Vrieling K, et al. 2005. Pyrrolizidine alkaloids from *Anchusa strigosa* and their antifeedant activity. Phytochemistry 66:1593-1600

Smith DB, Roddick JG, Jones LJ. 2001. Synergism between the potato glycoalkaloids α-chaconine and α-solanine in inhibition of snail feeding. Phytochemistry 57:229-234

Stamp NE, Yang YL. 1996. Response of insect herbivores to multiple allelochemicals under different thermal regimes. Ecology 77:1088-1102

Stamp NE. 1994. Interactive effects of rutin and constant versus alternating temperatures on performance of *Manduca sexta* caterpillars. Entomol Exp Appl 72:125-133

Stermitz FR, Lorenz P, Tamara JN, Zenewicz LA, Lewis K. 2000. Synergy in the medicinal plant: antimicrobial action of berberine potentiated by 5'-methoxyhydrocarpin, a multidrug pump inhibitor. Proc Natl Acad Sci USA 97:1433-1437

Trigo JR. 2011. Effects of pyrrolizidine alkaloids through different trophic levels. Phytochem Rev 10:83-98

Turner DM. 1996. Natural product source material use in the pharmaceutical industry: the Glaxo experience. J Ethnopharmacol 51:39-43

van Dam NM, Vuister LWN, Bergshoeff C, de Vos H, van der Meijden E. 1995. The ‘‘raison d'être’’ of pyrrolizidine alkaloids in *Cynoglossum officinale*: deterrent effects against generalist herbivores. J Chem Ecol 21:507-523

von Borstel K, Hartmann T. 1986. Selective uptake of pyrrolizidine N-oxides by cell suspension cultures from pyrrolizidine alkaloid producing plants. Plant Cell Rep 5:39-42

Whitehead SR, Bowers MD. 2014. Chemical ecology of fruit defence: synergistic and antagonistic interactions among amides from Piper. Funct Ecol 28:1094-1106

Williamson EM. 2001. Synergy and other interactions in phytomedicine. Phytomedicine 8:400-409

Zalkow LH, Glinski JA, Gelbaum LT, Fleischmann TJ, Mcgowan LS, et al. 1985. Synthesis of pyrrolizidine alkaloids indicine, intermedine, lycopsamine, and analogues and their N-oxides. Potential antitumor agents. J Med Chem 28:687-694

Zimmermann GR, Lehar J, Keith CT. 2007. Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug Discov Today 12:34-42

Chapter 5

The influence of the natural background on the bioactivity of individual plant metabolites: a case study with fractions of *Jacobaea* extracts and pyrrolizidine alkaloids

Xiaojie Liu, Klaas Vrieling, Peter G. L. Klinkhamer

Abstract

Plants produce an extremely diverse array of chemical metabolites that mediate many aspects of plant-environment interactions. In plants, metabolites co-occur with each other. This co-occurrence presents the natural background for individual metabolites. Given that herbivores always encounter a mixture of metabolites, the natural background is of ecological significance because interactions of metabolites will occur and that may positively or negatively affect the bioactivity of the metabolites. In the context of plant-herbivore interactions, it is as yet poorly understood how natural backgrounds shape the bioactivity of individual metabolites. We tested the effects of a methanol extract of *Jacobaea* plants and five fractions derived from this extract, and of two pyrrolizidine alkaloids (PAs) retrorsine and retrorsine N-oxide on survival of the western flower thrips (*Franklinella occidentalis*). We subsequently added the two PAs to the most and to the least active fraction to investigate the influence of the natural background on the bioactivity of PAs. When tested alone, retrorsine showed a stronger effect on thrips survival than retrorsine N-oxide. The methanol extract resulted in lower thrips survival than any of the five fractions derived from it. In addition, the effect of the methanol extract was higher than would be expected on basis of the combined effects of the fractions if there would be no interactions. The latter suggests that synergistic interactions between metabolites predominate in the extract. The five fractions differed in their effects on thrips survival. The *n*-BuOH fraction showed the highest effect on thrips while the $CHCl_3$ fraction was the least active against thrips. The latter fraction contained the majority of the PAs. Both PAs interacted synergistically with both these fractions in their activity against thrips. These finding strongly suggests that the activity of PAs against thrips is potentiated by other metabolites present in the plant. This study supports a commonly held notion that plant chemical defence is dependent on a variety of metabolites, which together shape the outcome of the defensive efficacy. The approach that we put forward here, to determine the associations between the bioactivity of a given plant and its metabolites, suggests a starting point for studying the effects of interactions between metabolites on their bioactivity. It also shows that the assessment of bioactivity cannot be decoupled from the natural phytochemical background in which these metabolites occur.

Keywords: Natural backgrounds, Metabolite interaction, Pyrrolizidine alkaloids, Synergy, Plant defence

Introduction

Plant metabolites play an important role in aiding the plant to survive various biotic and abiotic stresses (Fraenkel 1959; Wink 1988; Kliebenstein 2014). When attempting to identify the metabolites that are responsible for a certain bioactivity in a plant, the most common way of testing is to isolate single metabolites and test them in bioassays (Hadacek 2002). However, plants metabolites occur together with probably thousands of other metabolites with which they can interact (Williamson 2001; Wink 2003). The bioactivity of such mixtures of metabolites will be different from the sum of the effects of the individual metabolites because most likely many chemical and biological interactions will occur.

The co-occurrence of plant metabolites provides a strong likelihood of interactions between them. From a plant's perspective, metabolite interactions are of vital importance. Plants can benefit from synergism between metabolites if they increase bioactivity at a lower cost (Berenbaum and Zangerl 1993; Nelson and Kursar 1999). For a single metabolite, the mode(s) of action in concert with other metabolites may differ from that as a single compound. In the context of plant-insect associations, only few studies have demonstrated the interaction effects of plant metabolites on insects. Previous studies mostly focused on the combinations of two or more known metabolites of the same chemical class (Diawara et al. 1993; Smith et al. 2001; Dyer et al. 2003; Richards et al. 2010; Richards et al. 2012; Whitehead and Bowers 2014; Chapter 2 of this thesis). Less studied are interactions between metabolites of different classes (Berenbaum and Neal 1985; Neal 1989; Nelson and Kursar 1999; Guillet et al. 1998; Nuringtyas, PhD thesis, 2014; Chapters 3 and 4). However, it is worthwhile to put more emphasis on interactions between metabolites from different chemical classes as well. Before metabolites become bioactive they have to be taken up, pass membranes, should be protected against metabolization and secretion and reach the target site (Wu and Baldwin 2010). Many of these processes can, to different extents, be influenced by metabolites from different classes. For instance, monoterpenes inhibited cytochrome P450 enzymes involved in α-terthienyl (a terthiophene) degradation, thereby increasing the effect of α-terthienyl on the reduction of relative growth rate of the European corn borer *Ostrinia nubilalis* larvae (Guillet et al. 1998).

Interactions between plant metabolites present a great challenge to researchers in terms of the number of metabolites and even more so in terms of the number of potential combinations. Given the enormous number of metabolites in a single plant, it is impossible to evaluate all combination of metabolites. In addition, interactions may occur among unidentified or even unknown metabolites, which represent a great part of the total amount of plant metabolites (Trethewey 2004). In view of these facts, it is of concern how to measure the interactions between plant metabolites without prior knowledge about which specific metabolites are involved and what their potential mode of action is. A way forward would be to start with combinations of classes of metabolites of which we know that the

potential of interactions is high such as saponins together with metabolites that not easily pass membranes (Gee et al. 1996; Herrmann and Wink 2011).

Here we took the first step of a different approach. We studied the bioactivity of pyrrolizidine alkaloids (PAs) when they are added to (part of) their natural phytochemical background. This can be seen as a top-down approach. When the bioactivity of a compound against a particular target is increased when it occurs in extracts or fractions of extracts this would point to the importance of interactions among plant compounds for the plants defence system. We found that the mutagenicity of retrorsine, a pyryolzidine alkaloid, towards *Salmonella typhimurium* bacterial strains was significantly enhanced by the $CHCl_3$ and the EtOAc fractions of *Jacobaea* shoots while it was decreased by the *n*-BuOH and the H_2O fractions (Chapter 6). In this chapter we will study the bioactivity of retrorsine and retrorsine N-oxide against the western flower thrips (*Franklinella occidentalis*) in combination with two fractions derived from a methanol extract of shoots from *Jacobaea vulgaris*.

Jacobaea species are characterized by their PAs, which serve an important function in plant defence. Negative effects on mammalian herbivores (EFSA 2007; Fu et al. 2001 and 2002; Trigo 2011 and references therein), generalist insect herbivores (Dreyer et al. 1985; de Boer 1999; Reina et al. 2001; Siciliano et al. 2005; Dominguez et al. 2008; Macel 2011 and references therein) and pathogens (Rubiolo et al. 1992; Joosten and van Veen 2011 and references therein; Bovee et al. 2015; Jing et al. 2015) have been reported. As single metabolites, the PA N-oxides are less active against insect herbivores than the corresponding free bases (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005; Nuringtyas et al. 2014; Chapter 2). In plants, PAs occur mostly as N-oxide (Hartmann et al. 1989) with some jacobine-like PAs occurring upto 50% as free base in *J. vulgaris* (Joosten et al. 2011). However, the bioactivity of the two forms against thrips was reversed when PAs were added to artificial diets together with chlorogenic acid (CGA) (Chapters 3 and 4). Evidence for the role of PAs in the plant's defence system mostly comes from correlative studies (Vrieling et al. 1991; Leiss et al. 2009; Cheng et al. 2011; Kostenko et al. 2013; Wei et al. 2015; see review by Trigo 2011 and Macel 2011) and to a lesser extent from bioassays with single PAs (but see Macel et al. 2005). The question how the bioactivity of PAs is increased or decreased in concert with other co-occurring metabolites has as yet not been addressed (but see Chapters 3 and 4).

The purpose of this chapter is threefold. First, we tested the effect of, respectively, retrorsine and retrorsine N-oxide on western flower thrips (*Franklinella occidentalis*). Secondly, we tested the effect of the methanol extract and five fractions from *Jacobaea* shoots on thrips survival. And finally we investigated whether the combination of plant fractions and retrorsine or retrorsine N-oxide showed synergistic or antagonistic effects on thrips survival.

Materials and Methods

Chemicals and plant materials

Retrorsine and retrorsine N-oxide were purchased from Sigma (St. Louis, MO, USA). As the starting material, we used the same dried ground plant material that was kept in -80^{o}C freezer and that was used in Chapter 6. Details of extraction and fractionation have been described in Chapter 6. Briefly, a total methanol extract was obtained from dried and ground *Jacobaea* shoots. Subsequently, the methanol extract was fractionated by extraction with different polar solvents, yielding the hexane, $CHCl_3$, EtOAc, *n*-BuOH and the residual H_2O fractions (Chapter 6).

Retrorsine and retrorsine N-oxide were dissolved in 30 µL methanol (MeOH) to prepare stock solutions (14 mM). From these stock solutions dilutions in MeOH were prepared with a concentration of 2.8 mM. These solutions were used to prepare test solutions for the thrips bioassay.

Thrips bioassay

Full details about the bioassay and the rearing conditions of thrips were described in Chapters 2 and 3 of this thesis. Briefly: per two 96-well plates there are 24 columns (1-24) of 8 wells. A column received the same treatment and therefore consisted of 8 replicates. Of the 24 columns, 16 columns were filled with 16 different treatments and 8 columns were left empty. The 16 treatments included one negative control (55 µL of a phosphate buffered medium (Na_2HPO_4 and NaH_2PO_4, 40 mM, pH 7) with 10% fructose and 3% MeOH), 13 treatment groups (4 concentrations of a plant fraction, 3 concentrations of a PA and 6 concentrations of the combination of a plant fraction and a PA) and two positive controls (empty wells and abamectin). Four sets of two 96 wells plates were carried out simultaneously yielding 32 replicates for all treatments. The complete bioassay was repeated at a different time so that we obtained two independent estimates of 32 replicates each. Experiments were conducted twice to have two independent estimates of thrips survival for analysis of variance.

Experiments of *Jacobaea* extract and five fractions on thrips

A solution was made containing 10% fructose in sodium phosphate buffer (40 mM, pH 7). The total methanol extract and five fractions were dissolved in 60 µL of methanol and 1.94 mL buffer solution was added so that the final concentration of methanol was always 3%. The concentrations of the extract and fractions were expressed as the equivalent amount of dried plant shoot material from which they were derived: 0.02, 0.04, 0.06 and 0.08 g plant

shoot mass/mL. One g of plant shoot mass yielded 3.0, 5.7, 7.4, 22.8 and 128.9 mg of hexane, $CHCl_3$, EtOAc, *n*-BuOH and H_2O fraction, respectively.

Adding retrorsine and retrorsine N-oxide to fractions of *Jacobaea* shoot extract

After screening the five fractions, the most active fraction and the least active fraction were chosen to be combined with the two PAs. Three doses (0, 1.4 and 7.0 mM) of retrorsine and retrorsine N-oxide were combined with four doses (i.e. 0, 0.01, 0.05, and 0.09 g plant dry shoot mass/ml) of the $CHCl_3$ and the *n*-BuOH fractions, yielding 12 combinations including the tests with single PAs or fractions. The doses of retrosine and retrosine N-oxide represented approximately 1.0 and 5.0 times the total PA concentration of fresh mass of shoots of *J. vulgaris* plants.

For retrorsine and retrorsine N-oxide alone, we obtained four independent survival data at each concentration, from the two bioassays with two replicates each in which we tested the effect of the single compounds and fractions and their combinations.

Pyrrolizidine alkaloid content of the fractions

It was assumed that the PA content of each of the plant fractions was the same as determined for the plant fractions isolated in Chapter 6. No new analysis was therefore performed. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses of PAs of the plant fractions were conducted based on the protocol described in Cheng et al. (2011) and detailed in Chapter 6.

Statistical analysis

Construction of an interaction model and correcting for survival in the negative control

Details have been described in Chapter 3. In brief, in order to examine the effect of combinations, we constructed a multiplicative null model, in which the survival after the application of a combination is the product of the survival after application of its constituents, assuming no interaction effects. Furthermore this model assumes that the relationship between log survival and the concentration tested is linear.

In a similar manner, we corrected for survival in the negative control. The survival resulting from the application of the metabolite S_X can be calculated as:

$$S_X = S_{X+NC} / S_{NC} \qquad (1)$$

S_{X+NC} is the observed experimental survival while S_{NC} is the survival when the control solvent is added to the artificial medium.

Testing the interaction effects of the combination on survival

The survival of thrips for the combination of a PA and a fraction (S_{PA+F}) results from the survival after application of the PA (S_{PA}), the survival after application of the fraction (S_F), and their interaction (S_{PA*F}). Consequently, the effect of the interaction on survival can be calculated by:

$$S_{PA*F} = S_{PA+F} / (S_{PA} * S_F) \qquad (2)$$

In which S_{PA+F} is the observed survival in experiments with combinations while S_{PA} and S_F are the observed thrips survival in experiments with a single PA and fraction respectively while S_{PA*F} denotes the interaction effect. The interaction effect thus denotes the effect of the interaction between the PA and on survival fraction of thrips. The interaction effect can be synergistic ($S_{PA*F} < 1$) or antagonistic ($S_{PA*F} > 1$). To avoid confusion with statistical interaction terms, we will always refer to the value of the interaction between the metabolites as the "interaction effect S_{PA*F}". The interaction effect S_{PA*F} was calculated for all combinations and was expressed as mean value ± standard error of the mean (SE). As each experiment is repeated twice two independent estimates of the interaction effect are obtained.

Four-way analysis of variance (ANOVA) was performed with two PAs (retrorsine and retrorsine N-oxide), PA concentration, two fractions (the $CHCl_3$ fraction and the *n*-BuOH fraction) and fraction concentration as factors with the interaction effect S_{PA*F} as a dependent variable.

Comparison of the effects of the crude shoot extract and its five fractions

Thrips survival was log-transformed to obtain linear relationships with the tested concentrations. Log-transformed survival was regressed against concentrations to test whether there was a dose-dependent effect. Moreover, we calculated the slopes of the regression lines and their 95% confidence intervals (CIs) to estimate and compare the effects of the extract and different fractions. If two slopes have non-overlapping 95% confidence intervals they are assumed to be significantly different at the $P < 0.05$ level.

Further, we calculated the expected thrips survival when hypothetically combining the five fractions assuming no interaction, by extending formula (1) to the following formula:

$$S_{expected} = S_{hexane} * S_{chloroform} * S_{EtOAc} * S_{n\text{-}BuOH} * S_{aqueous} \qquad (3)$$

Thereafter, according to Formula (2), the interaction effect was calculated as the observed survival of the methanol extract divided by the expected survival of the combined five fractions assuming that no interactions occurred (Formula 4).

$$S_{F*F} = S_{MeOH} / S_{expected} \quad (4)$$

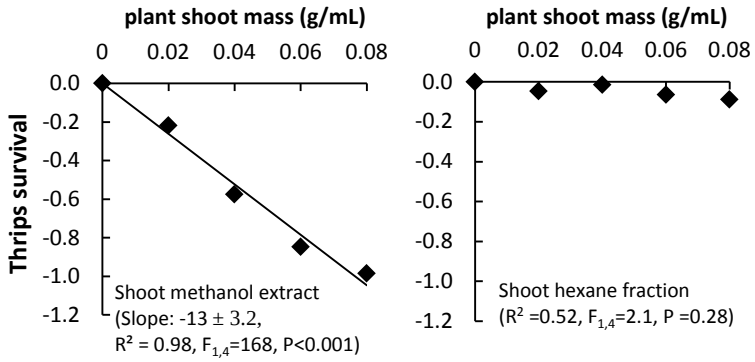
All statistical analysis were performed using SPSS software for Windows (version 21.0; SPSS Inc., Chicago, IL).

Results

Thrips survival in the negative control was on average 0.86 ± 0.01. The positive control with the insecticide abamectine (50 µg/mL) solution showed an average thrips survival of 0.12 ± 0.02. The control with empty wells, to verify that thrips cannot survive without feeding, had an average survival of 0.06 ± 0.01.

Effect of the total extract and fractions of *Jacobaea* shoots on thrips survival

With increasing concentrations of the methanol extract, thrips survival decreased (Figure 1). After fractionation, the effect of any of the individual fractions was significantly smaller than that of the methanol extract. The methanol extract resulted in the strongest slope (average ± 95% CIs: -13 ± 3.2) between log-transformed thrips survival and concentration (Figure 1). All five fractions also reduced thrips survival but to different extents. The $CHCl_3$, EtOAc and *n*-BuOH fractions resulted in a significant concentration-dependent decrease of log-transformed thrips survival while the hexane and H_2O fractions did not (Figure 1). Of the first three fractions, the rank of the slopes (± 95% CIs) was *n*-BuOH (-4.4 ± 1.8), EtOAc (-1.4 ± 1.0) and $CHCl_3$ fraction (-0.9 ± 0.6) (Figure 1). Based on the 95% CIs of the slopes, log-transformed survival significantly differed between the *n*-BuOH fraction and EtOAc/$CHCl_3$ fractions.



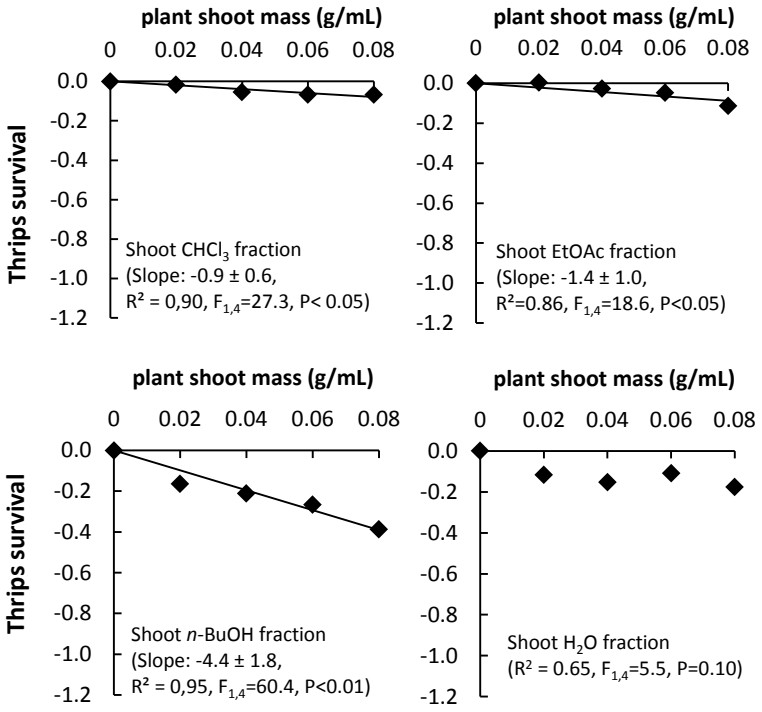


Figure 1. Log-transformed survival of 2nd instar western flower thrips (*Frankliniella occidentalis*) against the amount of *J. vulgaris* plant shoot mass of a methanol extract and five fractions of the methanol extract. Thrips larvae were put on an artificial diet at five concentrations for 5 days. Survival was corrected for differences among negative controls. For significant regressions, lines are shown.

At all four tested concentrations, the expected survival if there was no interaction between the five fractions was higher than that of the original methanol extract from which they were derived. This means that the interaction effect between the combined five fractions was lower than one (Figure 2A), indicating that overall synergistic interactions prevailed. Taking the highest concentration as an example, the individual effects of the five fractions were compared with the interaction effect among the five fractions. The fraction survival as a result from the interaction (S_{F*F}) was 0.6. The effect of the interaction on thrips survival was stronger than the effect of four of the five fractions while being only weaker than the effect of the *n*-BuOH fraction (0.4, Figure 2B).

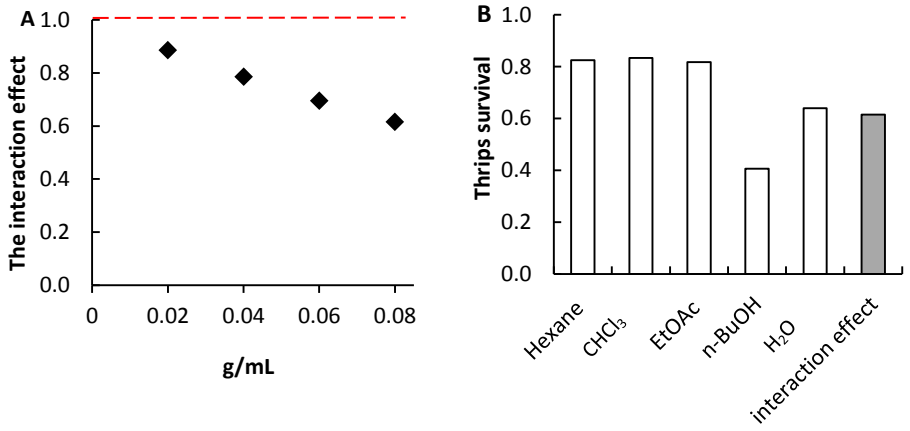


Figure 2. The effect of the interaction among the five fractions (A), and thrips survival for five fractions (S_F) and the effect of the interaction among the five fractions (S_{F*F}) at the concentration of 0.08 plant shoot mass (g/mL) (B). The expected survival for the combination of the five fractions if no interaction occurs was calculated by Equation 3. The interaction effect was calculated as the observed survival from the methanol extract divided by this expected survival for the combination of the five fractions (Equation 4).

PA levels and composition in fractions and the identification of CGA

In Chapter 6 we found that of the five shoot fractions, the majority of the PAs were present in the $CHCl_3$ fraction (67 %) (Figure 3). The *n*-BuOH fraction contained 24 %, the aqueous fraction 5 % and the ethyl acetate fraction 3 % and the hexane fraction 0.2 % of the total PA content (Figure 3). The identification of CGA was based on the analysis of NMR experiments, together with the comparison of reference compounds and previously reported data (Choi et al. 2006). In brief, the signals from the protons of CGA (H-8' at δ 6.34, H-5' at δ 6.84, H-6' at δ 7.03, H-2'8 at δ 7.13, H-7'at δ 7.58) were clearly identified in the fractions. Peak areas were used for comparative qualitative analysis of CGA in different fractions.

Effect of individual PAs on thrips survival

Retrorsine and retrorsine N-oxide showed a significant dose-dependent effect on thrips survival (Figure 4). At a concentration of 1.4 mM, the thrips survival was not significantly different for retrorsine and retrorsine N-oxide, while at 7 mM, retrorsine showed a significantly lower survival than retrorsine N-oxide (Figure 4).

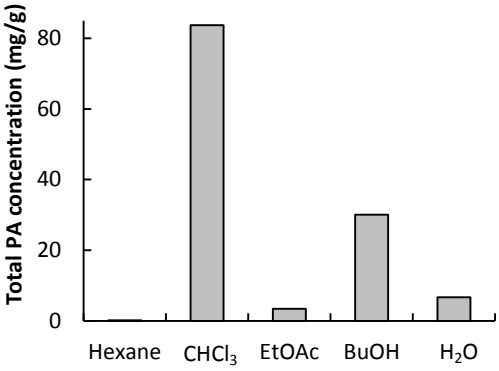


Figure 3. The total PA concentration in the five fractions of *Jacobaea* shoots derived from the methanol extract (mg/g). Data are based on the LC-MS/MS analysis of the fractions in Chapter 6.

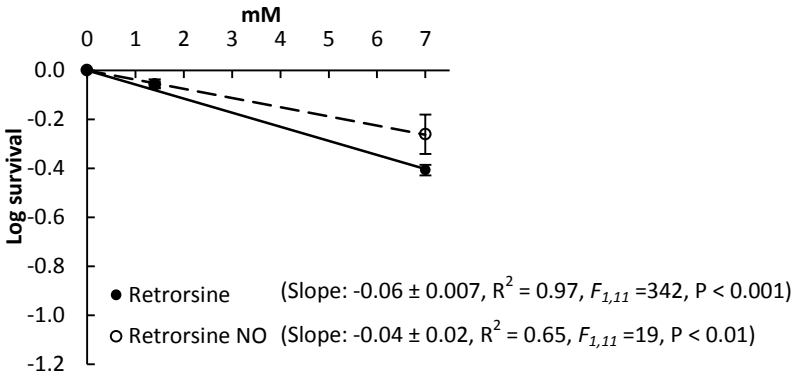
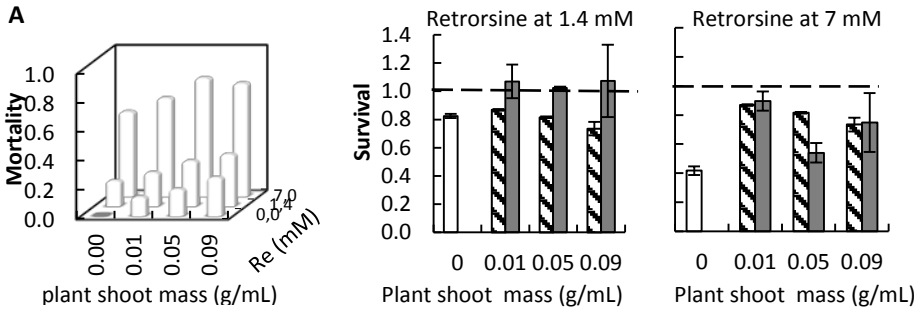


Figure 4. Log-transformed survival of 2nd instar western flower thrips (*Frankliniella occidentalis*) against the concentrations of retrorsine (solid dots) and retrorsine N-oxide (open dots). Dots in the graph are presented as average ± SE (n=4). The regression lines are shown are based on the 12 individual measurements. The slopes (± 95% confidence intervals) are based on 4 independent replicates of bioassays (see Figure 5).



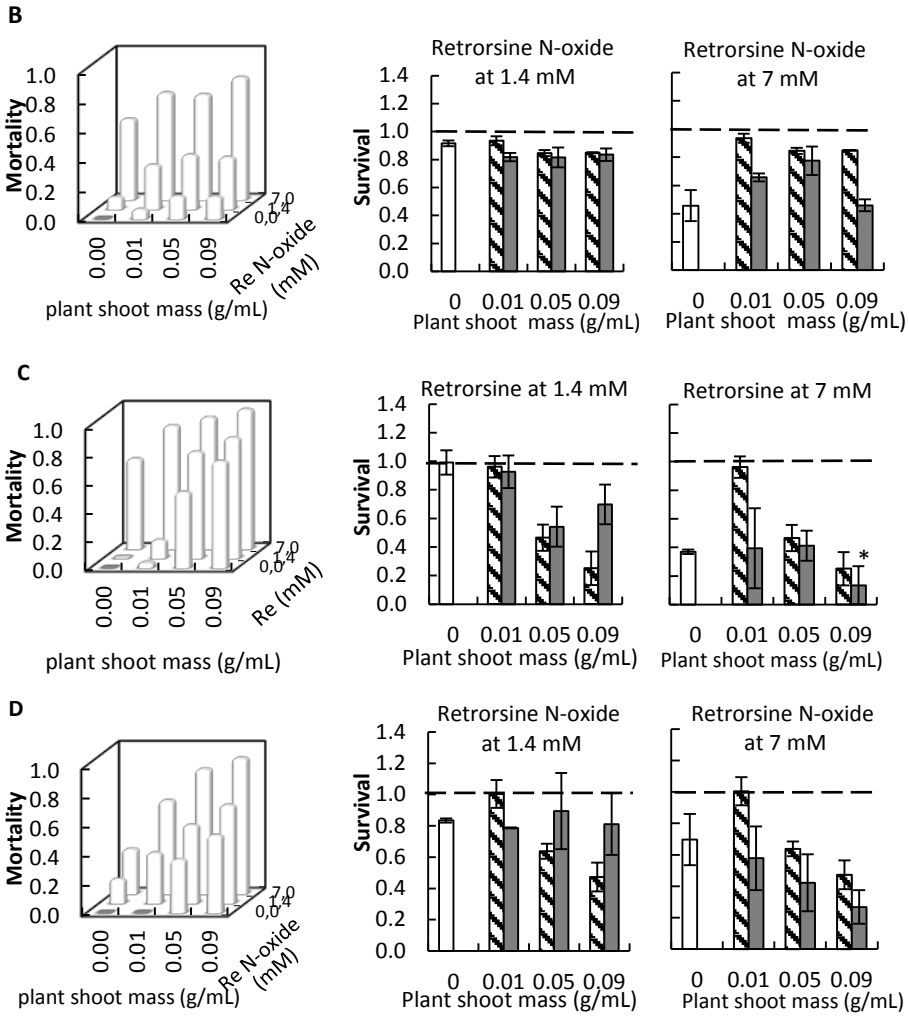


Figure 5. Left: Fraction mortality (1- fraction survival) of 2nd instar larvae of thrips (*Frankliniella occidentalis*) against the concentration of *J. vulgaris* plant shoot mass from which the $CHCl_3$ fraction was derived from the methanol extract and the concentration of retrorsine (**A**) and retrorsine N-oxide (**B**), and against the concentration of *J. vulgaris* plant shoot mass from which the *n*-BuOH fraction was derived with retrorsine (**C**) and retrorsine N-oxide (**D**). **Right:** Fraction survival (mean ± 95% confidence intervals) of thrips caused by the pyrrolizidine alkaloid alone (S_{PA}) (white bars), plant fractions alone at three concentrations (S_F, hatched bars), and the interaction effects (S_{PA*F}, see Equation 2) between PAs and the plant fraction (grey bars). In the 3D figures, fraction mortality was plotted to increase the readability of the figure. In the right figures, dashed lines represent an interaction effect on thrips survival of one. * In one replicate the fraction survival of thrips in the combination of *n*-BuOH and retrorsine in one case was zero while the other replicate had a value of 0.038.

Effect of the combination of PAs and *Jacobaea* fractions on thrips survival

Compared to the survival in the presence of retrorsine alone, the combination with the $CHCl_3$ and the *n*-BuOH fractions decreased the effect of retrorsine and retrorsine N-oxide on thrips survival (Figure 5). Thrips survival for the different combinations of the two fractions and the two PAs was lower than expected assuming there was no interaction between them (Figure 5). With the exception of the combination of the $CHCl_3$ fraction and retrorsine (Figure 5A), the other 3 combinations showed significant synergistic effects, as the interactions effects S_{PA*F} of all these combinations were significantly lower than 1 (Table 1 and Figure 5). For the combination of the $CHCl_3$ fraction and retrorsine the intercept was not significant, however the main effect, retrorsine concentration, was significant. The interaction effect S_{PA*F} showed synergism present at 7 mM while antagonistic effects were present at 1.4 mM (Figure 5A).

Table 1. Two-way analyses of variance (ANOVAs) with fraction concentration and pyrrolizidine alkaloid (PA) concentration as fixed factors and the interaction effect S_{F*PA} (Equation 2) minus one as a dependent variable. Survival was corrected for differences among the negative controls (Equation 1). A significant intercept or main effects indicate that synergistic or antagonistic interactions occur.

Factors	***df***	***F***	***P***
Intercept	1, 11	3.1	NS
$CHCl_3$ concentration	2, 11	0.9	NS
Retrorsine concentration	1, 11	7.2	< 0.05
$CHCl_3$ concentration * Retrorsine concentration	2, 11	0.5	NS
Intercept	1, 11	127.8	< 0.001
$CHCl_3$ concentration	2, 11	3.2	NS
Retrorsine N-oxide concentration	1, 11	15.8	< 0.01
$CHCl_3$ concentration * Retrorsine N-oxide concentration	2, 11	4.1	NS
Intercept	1, 11	50.7	< 0.001
n-BuOH concentration	2, 11	1.2	<0.05
Retrorsine concentration	1, 11	9.1	NS
n-BuOH concentration * Retrorsine concentration	2, 11	1.1	NS
Intercept	1, 11	21.5	< 0.01
n-BuOH concentration	2, 11	0.3	NS
Retrorsine N-oxide concentration	1, 11	6.4	< 0.05
n-BuOH concentration * Retrorsine N-oxide concentration	2, 11	0.4	NS

NS = Not significant

The synergistic effect is fraction-specific. Combined with PAs, the *n*-BuOH fraction showed a significantly stronger synergistic effect than the $CHCl_3$ fraction, irrespective of the PA type. The strength of the synergistic effect increased with PA concentration but was independent from the concentration of the fractions (Table 2). The interaction effect depended only marginally on the combination of the PA and the fraction (P = 0.06, Table 2).

The ranking of the strength of the interaction effect S_{PA*F} (mean ± S.E.) was: retrorsine + *n*-BuOH (0.57 ± 0.08), retrorsine N-oxide + *n*-BuOH (0.63 ± 0.09), retrorsine N-oxide + $CHCl_3$ (0.72 ± 0.04) and retrorsine + $CHCl_3$ (0.89 ± 0.07). The effects of the interaction on thrips survival were in all cases stronger or at least similar to that of PAs or fractions alone. As an example, the interaction effects between retrorsine N-oxide and the $CHCl_3$ fraction on thrips survival were significantly stronger than that of the $CHCl_3$ fraction at 0.01 and 0.09 g/mL (Figure 5B). For the combination of retrorsine N-oxide and the *n*-BuOH fractions, the interaction effects on thrips survival were significantly stronger than that of *n*-BuOH fractions at 0.01 and 0.09 g/mL (Table 2). The strongest effect was found for the interaction between retrorsine N-oxide and the *n*-BuOH fraction at 0.09 g/mL which lead to a survival of 0.27 (Figure 5D).

Table 2. Four-way analysis of variance (ANOVA) with PAs, fractions, PA concentration and fraction concentration as factors with the interaction effect S_{PA*F} (Equation 2) as a dependent variable. PAs tested are retrorsine and retrorsine N-oxide. The fractions are the $CHCl_3$ and the *n*-BuOH fractions of a methanol extract from *Jacobaea* shoots. Each combination was tested in two independent bioassays. Survival was corrected for differences among the negative controls.

Factors	***df***	***F***	***P***
Intercept	1, 46	479.2	< 0.001
PAs	1, 46	0.4	NS
Fractions	1, 46	12.6	< 0.01
PA concentration	1, 46	25.8	< 0.001
Fraction concentration	2, 46	1.3	NS
PAs * Fractions	1, 46	3.9	0.06
PAs * PA concentration	1, 46	0.1	NS
PAs * Fraction concentration	2, 46	1.2	NS
Fractions * PA concentration	1, 46	1.0	NS
Fractions * Fraction concentration	2, 46	0.1	NS
PA concentration * Fraction concentration	2, 46	0.5	NS
PAs * Fractions * PA concentration	1, 46	0.5	NS
PAs * Fractions * Fraction concentration	2, 46	0.1	NS
PAs * PA concentration * Fraction concentration	2, 46	0.3	NS
Fractions * PA concentration * Fraction concentration	2, 46	0.1	NS
PAs * Fractions * PA concentration * Fraction concentration	2, 46	1.7	NS

NS = Not significant

Discussion

Are plant extracts and fractions of *Jacobaea* shoots toxic to thrips?

The methanol extract of *Jacobaea* shoots significantly decreased thrips survival compared to the negative control, even at concentrations that were much lower than the original concentration in the plant. Upon fractionation, the effects of individual fractions on thrips

survival were significantly smaller than that of the total methanol extract. We also theoretically combined the fractions and calculated the expected survival assuming no interaction. The magnitude of the interaction effect between the five fractions calculated in this way was significantly smaller than one, indicating an overall synergistic interaction. We should be careful however with the interpretation of the data because the loss of activity may be due to the loss of active constituents during the fractionation. The extract and fractions that were tested for the thrips bioassays from this chapter came from the same batch of ground plant material as for the Ames test (Chapter 6). As described in Chapter 6, we lost about 10% of the material during sub-fractionation. We therefore assume that the loss of activity upon sub-fractionation in the thrips bioassay was not caused by a loss of active constituents. We suggest that fractionation disrupted or even eliminated the interactions between metabolites. Our results show that using single metabolites to study their bioactivity would often not be a fruitful strategy if interactions are present. Similar conclusions were reached in a number of phytochemical studies that also often reported a loss of bioactivity upon fractionation, however without providing specific reasons for the observed loss of bioactivity (Williamson 2001; Herrera and Amor 2011; Labuschagne et al. 2012; Inui et al. 2012).

Do plant fractions of *Jacobaea* shoots differ in their effects on thrips?

The five shoot fractions of the methanol extract differed in their effects on thrips survival. The order of the PA content of the fractions was not consistent with the order of activity against thrips, suggesting that besides PAs, other metabolites also contributed directly or indirectly, through interacting effects, to the overall activity of the fractions. By using solvents with increasing polarity in the fractionation process, the fractions will contain different types of metabolites (Sasidharan et al. 2011). For instance, metabolites with low polarity (e.g. essential oils) will be extracted by the solvent with the lowest polarity, hexane (polarity index of 0.1). Moderately polar solvents such as $CHCl_3$ (polarity index of 2.7) and EtOAc (polarity index of 4.1) mainly will extract steroids, alkaloids, etc. Polar components like phenolic compounds, e.g. flavonoids and glycosides, are concentrated in the *n*-BuOH fraction (polarity index of 6.0). Water, the most polar solvent (polarity index of 10.2) is effective in extracting the metabolites with higher molecular weights such as proteins, glycans, etc. (Cos et al. 2006; Anupam et al. 2012). PAs were mainly present in the $CHCl_3$ fraction while the *n*-BuOH fraction showed the largest peak area for chlorogenic acid (CGA). Identification and characterization of chemical components especially those of concern will be a part of further work.

How natural backgrounds influence the effects of individual PAs on thrips?

To study possible interactions and to address the importance of natural backgrounds, we investigated the effects of individual metabolites in absence and presence of their natural

background by adding individual PAs to the most active fraction (the *n*-BuOH fraction) and the least active fraction (the $CHCl_3$ fraction). When tested alone, retrorsine showed a stronger effect on thrips than retrorsine N-oxide. Both the $CHCl_3$ and the *n*-BuOH fractions increased the effect of retrorsine and retrorsine N-oxide on thrips survival, showing synergistic interactions. In Chapters 3 and 4, we studied the interacting effects of PAs and CGA on thrips. We found antagonistic effects between retrorsine and CGA on thrips survival (Chapter 3) while we found synergistic effects between retrorsine N-oxide and CGA (Chapter 4). The synergistic effect between retrorsine N-oxide and CGA on thrips is in line with the fact that we found the strongest interactions between retrosine N-oxide and the *n*-BuOH fraction, because the *n*-BuOH fraction had the highest concentration of CGA (NMR data). However, in contrast to our expectation based on Chapter 3, we also found synergistic effects between retrorsine and the *n*-BuOH fraction. Apparently, there were effects of other metabolites that masked or even override the antagonistic effects between CGA and retrorsine. Further sub-fractionation and recombining sub-fractions can be used to narrow down the candidate metabolites that are involved in these interactions.

Following the whole-mixture analysis, a component-interaction analysis will provide more detailed information. The synergistic effects are fraction-specific. Specifically, the strength of the interaction of the *n*-BuOH fraction with PAs was stronger than that of the $CHCl_3$ fraction. The PA concentration in the $CHCl_3$ fraction was about three times higher than in the *n*-BuOH fraction. These results suggest that interactions between PAs and metabolites of other classes may dominate the overall synergistic effects between fractions and PAs on thrips.

How important are natural backgrounds for the bioactivity of individual SMs?

Because insect herbivores always encounter mixtures of metabolites in nature, the natural phytochemical background is of ecological relevance to both insects and plants. Despite this, for plant-insect associations, we know little about how natural backgrounds shape the bioactivity of individual metabolites. The effect of the interactions was similar or stronger than the effects of the metabolites alone (Figure 5), demonstrating the importance of interactions between these metabolites. Potential interactions may be even more interesting for compounds that are only weakly active or inactive on their own.

The result that the strength of the interaction depended on the fractions suggests that the effects of plant metabolites may vary depending on the phytochemical background in where they are. In a broad sense, this could explain, to some extent, that a single metabolite is active in one species while it is less active or even inactive in another species. For instance, CGA in *Chrysanthemum* was negatively correlated with the feeding damage of thrips (Leiss et al. 2009), while no effect of CGA on thrips was detected in tomato *Solanum lycopersicum* (Mirnezhad 2011).

A top-down approach to interactions between plant metabolites

In this chapter, we took a first step of a top-down approach to study the effect of the interactions between plant metabolites in their bioactivity. Results from this initial step gave us a general impression of the interactions between metabolites from a whole-metabolome point of view. Further progress demands to determine which metabolites are most likely to be involved in these interactions. This can be achieved by sub-fractionation in combination with e.g. NMR. One challenge in the following component-interaction analysis is to measure the effects of the infinite number of possible combinations due to the enormous number of metabolites in a plant. This would be an impossible task with the bioassays that are now used to study plant-insect interactions. In this light, high-throughput screening is essential for assaying the bioactivity of a large number of potential candidates against a chosen target. One of potential useful approaches to screen for anti-herbivore activities is the use of insect cell lines. However, for thrips cell lines are not available yet. Still, we can use cell lines of other insects, e.g. the beet armyworm, *S. exigua*. This has been used in our group (Nuringtyas et al. 2014). We found similar results regarding the interactions between PAs and CGA. On the other hand, bioassays on cell lines does not account for the digestive track on the toxicity of the metabolites. However, the cell lines can still be employed as the first step of the screening system to select candidate compounds or combinations of interest to be used in bioassays with the living organisms.

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References

Anupam G, Nandita C, Goutam C. 2012. Plant extracts as potential mosquito larvicides. Indian J Med Res 135:581-598

Berenbaum M, Neal JJ. 1985. Synergism between myristicin and xanthotoxin, a naturally cooccurring plant toxicant. J Chem Ecol 11:1349-1358

Berenbaum MR, Zangerl AR. 1993. Furanocoumarin metabolism in *Papilio polyxenes*: biochemistry, genetic variability, and ecological significance. Oecologia 95:370-375

Bovee TF, Helsdingen RJ, Hoogenboom RL, de Nijs MW, Liu X, et al. 2015. Are effects of common ragwort in the Ames test caused by pyrrolizidine alkaloids? Mutat Res 778:1-10

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011a. Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. New Phytol 192:1010-1023

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011b. The relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance in F(2) hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. J Chem Ecol 37:1071-1080

Choi YH, Casas Tapias EC, Kim HK, Lefeber AWM, Erkelens C, et al. 2004. Metabolic discrimination of *Catharanthus roseus* leaves infected by phytoplasma using 1H-NMR spectroscopy and multivariate data analysis. Plant Physiol 135:2398-2410

Cos P, Vlietinck AJ, Vanden Berghe D, Maes L. 2006. Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. J Ethnopharmacol 106:290-302

de Boer NJ. 1999. Pyrrolizidine alkaloid distribution in *Senecio jacobaea* rosettes minimises losses to generalist feeding. Entomol Exp Appl 91:169-173

Diawara MM, Trumble JT, White KK, Carson WG, Martinez LA. 1993. Toxicity of linear furanocourmarins to *Spodoptera exiga*- evidence for antagonistic interactions. J Chem Ecol 19:2473-2484

Domínguez DM, Reina M, Santos-Guerra A, Santana O, Agulló T, et al. 2008. Pyrrolizidine alkaloids from *Canarian* endemic plants and their biological effects. Biochem Syst Ecol 36:153-166

Dreyer DL, Jones KC, Molyneux RJ. 1985. Feeding deterrency of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrthosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. J Chem Ecol 11:1045-1051

Dyer LA, Dodson CD, Stireman JO III, Tobler MA, Smilanich AM, et al. 2003. Synergistic effects of three Piper amides on generalist and specialist herbivores. J Chem Ecol 29:2499-2514

European Food Safety Authority. 2011. Scientific opinion on pyrrolizidine alkaloids in food and feed. EFSA Journal. Parma, Italy. 9:2406

Fraenkel GS. 1959. The raison d'être of secondary plant substances. Science 129:1466-1470

Fu PP, Chou MW, Xia Q, Yang YC, Yan J, Dorege DR, et al. 2001. Genotoxic pyrrolizidine alkaloids and pyrrolizidine alkaloid N-oxides-mechanisms leading to DNA adduct formation and tumorigenicity. Environ Carcinogen Ecotoxicol Rev. C19:353

Fu PP, Xia QS, Lin G, Chou MW. 2002. Genotoxic pyrrolizidine alkaloids - mechanisms leading to DNA adduct formation and tumorigenicity. Int J Mol Sci 3:948-964

Gee JM, Wortley GM, Johnson IT, Price KR, Rutten AA, et al. 1996. Effects of saponins and glycoalkaloids on the permeability and viability of mammalian intestinal cells and on the integrity of tissue preparations in vitro. Toxicol In Vitro 10:117-128

Guillet G, Belanger A, Arnason JT. 1998. Volatile monoterpenes in *Porophyllum gracile* and *P. ruderale* (Asteraceae): Identification, localization and insecticidal synergism with alpha-terthienyl. Phytochemistry 49:423-429

Hadacek F. 2002. Secondary metabolites as plant traits: current assessment and future perspectives. Crit Rev Plant Sci 21:273-322

Herrera AA, Amor EC. 2011. Antiangiogenic activity of extracts and fractions from an endemic plant *Ardisia pyramidalis* (Cav.) Pers. From Bataan, Philippines using Duck in ovo chorioallantoic membrane assay. J Med Plants Res 13:2637-2646

Herrmann F, Wink M. 2011. Synergistic interactions of saponins and monoterpenes in HeLa cells, Cos7 cells and in erythrocytes. Phytomedicine 18:1191-1196

Inui T, Wang Y, Pro SM, Franzblau SG, Pauli GF. 2012. Unbiased evaluation of bioactive secondary metabolites in complex matrices. Fitoterapia 83:1218-1225

Jing J, Raaijmakers C, Kostenko O, Kos M, Mulder PPJ, et al. 2015. Interactive effects of above- and below ground herbivory and plant competition on plant growth and defence. Basic Appl Ecol 16:500-509

Joosten L, Cheng D, Mulder PPJ, Vrieling K, van Veen JA,et al. 2011. The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. Phytochemistry 72:214-222

Joosten L, van Veen JA. 2011. Defensive properties of pyrrolizidine alkaloids against microorganisms. Phytochemistry Reviews 10:127-136

Kliebenstein DJ. 2012. Plant defense compounds: Systems approaches to metabolic analysis. Annu Rev Phytopathol 50:155-173

Kostenko O, Mulder PPJ, Bezemer TM. 2013. Effects of root herbivory on pyrrolizidine alkaloid content and above ground plant-herbivore-parasitoid interactions in *Jacobaea vulgaris*. J Chem Ecol 39:109-119

Labuschagne A, Hussein AA, Rodriguez B, Lall N. 2012. Synergistic antimycobacterial actions of *Knowltonia vesicatoria* (L.f) Sims. Evid Based Complement Alternat Med 2012:1-9

Leiss KA, Choi YH, Abdel-Farid I, Verpoorte R, Klinkhamer PGL. 2009. NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. J Chem Ecol 35:219-229

Leiss KA, Cristofori G, van Steenis R, Verpoorte R, Klinkhamer PGL. 2013. An eco-metabolomic study of host plant resistance to western flower thrips in cultivated, biofortified and wild carrots. Phytochemistry 93:63-70

Macel M, Bruinsma M, Dijkstra SM, Ooijendijk T, Niemeyer HM, et al. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. J Chem Ecol 31:1493-1508

Macel M. 2011. Attract and deter: a dual role for pyrrolizidine alkaloids in plant–insect interactions. Phytochem Rev 10:75-82

Neal JJ. 1989. Myristicin, safrole, and fagaramide as phytosynergists of xanthotoxin. J Chem Ecol 15:309-315

Nelson AC, Kursar TA. 1999. Interactions among plant defense compounds: a method for analysis. Chemoecology 9:81-92

Nuringtyas TR, Verpoorte R, Klinkhamer PGL, van Oers MM, Leiss KA. 2014. Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* using insect cell lines and injection bioassays. J Chem Ecol 40:609-616

Nuringtyas TR. 2014. Pyrrolizidine alkaloid variation in *Jacobaea* plants: from plant organ to cell level. PhD thesis, Leiden University, The Netherlands

Reina M, González-Coloma A, Gutiérrez C, Cabrera R, Rodríguez ML, et al. 2001. Defensive chemistry of *Senecio miser*. J Nat Prod 64:6-11

Richards LA, Dyer LA, Smilanich AM, Dodson CD. 2010. Synergistic effects of amides from two Piper species on generalist and specialist herbivores. J Chem Ecol 36:1105-1113

Richards LA, Lampert EC, Bowers MD, Dodson CD, Smilanich AM, et al. 2012. Synergistic effects of iridoid Glycosides on the survival, development and immune response of a specialist caterpillar, *Junonia coenia* (Nymphalidae). J Chem Ecol 38:1276-1284

Rubiolo P, Pieters L, Calomme M, Bicchi C, Vlietinck A, et al. 1992. Mutagenicity of pyrrolizidine alkaloids in the *Salmonella typhimurium*/mammalian microsome system. Mutat Res 281:143-147

Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L. 2011. Extraction, isolation and characterization of bioactive compounds from Plants' extracts. Afr J Tradit Complement Altern Med 8:1-10

Siciliano T, Leo MD, Bader A, Tommasi ND, Vrieling K, et al. 2005. Pyrrolizidine alkaloids from *Anchusa strigosa* and their antifeedant activity. Phytochemistry 66:1593-600

Smith DB, Roddick JG, Jones LJ. 2001. Synergism between the potato glycoalkaloids α-chaconine and α-solanine in inhibition of snail feeding. Phytochemistry 57:229-234

Trethewey R. 2004. Metabolite profiling as an aid to metabolic engineering. Curr Opin Plant Biol 7:196-201

Trigo JR. 2011. Effects of pyrrolizidine alkaloids through different trophic levels. Phytochem Rev 10:83-98

van Dam NM, Vuister LWN, Bergshoeff C, de Vos H, van der Meijden E. 1995. The ‘‘raison d'être’’ of pyrrolizidine alkaloids in *Cynoglossum officinale*: deterrent effects against generalist herbivores. J Chem Ecol 21:507-523

Wei X, Vrieling K, Mulder PPJ, Klinkhamer PGL. 2015. Testing the generalist-specialist dilemma: the role of pyrrolizidine alkaloids in resistance to invertebrate herbivores in *Jacobaea* species. J Chem Ecol 41:159-167

Whitehead SR, Bowers MD. 2014. Chemical ecology of fruit defence: synergistic and antagonistic interactions among amides from Piper. Funct Ecol 28:1094-1106

Williamson EM. 2001. Synergy and other interactions in phytomedicine. Phytomedicine 8:400-409

Wink M. 1988. Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. Theor Appl Genet 75:225-233

Wink M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64:3-19

Wu JQ, Baldwin IT. 2010. New insights into plant responses to the attack from insect herbivores. Annu Rev Genet 44:1-24

Chapter 6

The neglected side of interactions among plant metabolites: a case study of mutagenicity of plant extracts containing pyrrolizidine alkaloids

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Abstract

To determine the correlation between the bioactivity of a given plant and its metabolites, these are often isolated and tested as pure metabolites. If these metabolites lack the targeted bioactivity it is tempting to assume that they are not responsible for the bioactivity of the plant. However, the selected metabolites are obviously not alone in the plant. They co-exist with many other metabolites producing a chemically diverse array of bioactive metabolites. Although the mechanisms are not always known, it is highly likely that the bioactivity of a given metabolite is potentiated or reduced by other metabolites in the plant.

We tested five different shoot fractions and five root fractions derived from a methanol extract of *Jacobaea* plants. When the chloroform shoot fraction and the *n*-butanol root fraction of *Jacobaea* plants were investigated by the Ames test TA100 strain, significant mutagenic activity was found. Upon further fractionation the mutagenicity was largely lost in the sub-fractions, while the level of mutagenicity was restored when the sub-fractions were combined again, showing that the metabolites responsible for the mutagenic activity had not been degraded or were lost in the fractionation process. This suggests the presence of synergistic interactions between metabolites that were separated in the fractionation process. We then tested the mutagenicity of individual pyrrolizidine alkaloids (retrorsine and its N-oxide). We subsequently combined retrorsine with five shoot fractions, which naturally contain small amounts of retrorsine and other PAs. Retrorsine combined with chloroform and ethyl acetate fractions showed significant synergistic effects on mutagenicity while retrorsine showed equal or even lower mutagenicity when combined with the hexane, *n*-butanol and aqueous fractions. The mutagenic index (MI) of the combination of retrorsine and chloroform fraction was 2.7 times higher than the expected MI (4.09) calculated from the MIs of the retrorsine and the chloroform fraction alone.

This study used two methods to study mixtures for synergistic or antagonistic interactions. Specifically in the case of *Jacobaea* plants, it was shown that the weak or moderate mutagenicity for individual PAs was greatly potentiated when these metabolites were combined with chloroform and ethyl acetate fractions of the plant itself, indicative of synergistic interactions. The growing body of evidence on the importance of interactions between metabolites has major implications for the design of bioactivity studies in fields as toxicity, mutagenicity, health and plant protection.

Keywords: Natural background, Synergy, Antagonism, Ames test, Fractionation

Introduction

When searching for bioactive components, many phytochemical studies focus on assessing the bioactivity of individual metabolites (Hadacek 2002). These metabolites often have less activity than the plant material from which the metabolite was isolated (Herrera and Amor 2011). Plants contain a complex mixture of chemically highly diverse metabolites. It is well known that the bioactivity and metabolism of one metabolite may be affected by the presence of other metabolites through many mechanisms. Such mechanisms include for example, an increase in membrane permeability (Keukens et al. 1995), modification of metabolic processes (Keukens et al. 1995), the formation of a new complex, like the π-molecular complex between caffeine and CGA (Mösli Waldhauer and Baumann 1996), and blocking or disturbing membrane-bound receptor functions in insects, like the binding of alkaloids to insect alpha2, serotonin and nicotinic acetylcholine receptors (Wink et al. 1998; Liu et al. 2008). Saponins are well known to modify the cell membrane and thus facilitate the uptake of chemicals from the midgut (Gee et al. 1996; Herrmann and Wink 2011). Monoterpenes also can interact with the lipophilic side chain of phospholipids or cholesterol of the membrane and thus increase the membrane fluidity and permeability (Berenbaum and Zangerl 1993). In the black swallowtail *Papilio polyxenes*, combinations of furanocoumarins significantly reduced larval growth since individual furanocoumarins interfered with cytochrome P450-mediated metabolism of other furanocoumarins and prevented further metabolism (Berenbaum and Zangerl 1993; Nelson and Kursar 1998). These examples show that the occurrence of numerous metabolites in plants can result in many possible interactions to enhance or decrease bioactivity (Houghton 2000).

In nature, plants can benefit from the interactions between various metabolites. In combating natural enemies, synergistic interactions can increase plant fitness by producing a greater bioactivity at a lower cost. Interactions also have a wide range of applications, for instance in herbal medicine (Tallarida 2001; Williamson et al. 2001; Ma et al. 2009). Few studies address the interactions between plant metabolites and the effects of such interactions on the bioactivity of individual metabolites, especially the less active or inactive metabolites. In part this is due to the difficulty of detecting and analyzing interactions between plant metabolites in a proper manner (Nelson and Kursar 1999).

There are several ways to study the importance of interactions in plant extracts. One way to find prove for such interactions is to further sub-fractionate an active fraction. If the activity is based on interactions between metabolites, separating the interacting metabolites into different sub-fractions may result in the loss of the activity which can be restored again if the sub-fractions are recombined in their original proportions. Another approach is to test the effect of plant metabolites not only individually but also when added to the fraction or sub-fraction of plant extracts. In this paper we used both methods showing how the low or

moderate mutagenic activity of individual metabolites was potentiated when they were added to fractionated plant material they were derived from.

Pyrrolizidine alkaloids (PAs) are a group of plant metabolites that occur naturally in *Jacobaea* plants. These PAs are notorious contaminants of animal fodder but also enter the human food chain e.g. via honey and tea (Mathon et al. 2014). Several PAs have been shown to be carcinogenic in rats (European Food Safety Authority 2011) and genotoxic in various assays, causing DNA binding and effects like DNA cross-linking, DNA-protein cross-linking, and mutagenicity (Fu et al. 2002). An important indicator of bioactivity is the Ames test for mutagenicity. Previous studies have measured the mutagenicity of individual PAs, demonstrating that clivorine, heliotrine, lasiocarpine, senkirkine, retrorsine, seneciphylline and riddelline were mutagenic in the Ames test using *Salmonella typhimurium* TA100 (Yamanaka et al. 1979; Frei et al. 1992; Rubiolo et al. 1992; Fu et al. 2001). However, in a recent study we showed that individual PAs were only weakly mutagenic, while extracts prepared from *S. jacobaea* were strongly mutagenic to *S. typhimurium* TA98 (Bovee et al. 2015). In this study, we investigated the mutagenicity of *Jacobaea* plant fractions, sub-fractions and the recombined sub-fractions. We also studied the effect on the mutagenicity of combining retrorsine with the different plant fractions. We specifically addressed the following questions: are fractions of *Jacobaea* plant extracts mutagenic? Is mutagenicity maintained after sub-fractionation of the active fractions? If not is mutagenicity restored if sub-fractions are put together again? Is the effect of retrorsine potentiated or mitigated when added to plant fractions?

Material and Methods

Plant materials and chemicals

One hundred *Jacobaea* genotypes were planted in a garden in Lisse (52° 25’ 12” N, 4° 54’10” E, The Netherlands) and grown for 17 months and harvested in March 2013. All plants were separated into shoots and roots, and were dried in an oven at 50°C, milled to a fine powder, then stored in an air-tight container and kept at room temperature until further use. All plants were separated into shoots and roots. The shoots of all plants were pooled and the same was done for the roots. The plant materials were extracted in April 2013 and the two most active fractions were sub-fractionated in August of 2013 (see below).

Retrorsine (R-0382, Lot 70K3450) and retrorsine N-oxide (R-0507, Lot 31K1407) were purchased from Sigma Aldrich (St. Louis, MO, USA). Quercetin, 2-amino-anthracene, biotin and L-histidine were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). $Na_2HPO_4 \cdot 2H_2O$, NaH_2PO_4, $MgSO_4 \cdot 7H_2O$, citric acid monohydrate, K_2HPO_4, $NaNH_4HPO_4 \cdot 4H_2O$ and glucose monohydrate were obtained from Merck (Darmstadt, Germany). NADP disodium salt and glucose-6-phosphate were obtained from Roche

Diagnostics (Almere, The Netherlands), S9 rat liver mix from Trinova Biochem (Giessen, Germany), nutrient broth Nr 2 from Thermo Scientific (Landsmeer, The Netherlands) and agarose from Becton Dickinson (Breda, The Netherlands). The TA 100 strain used for the mutagenicity test was obtained from Prof. Bruce Ames (Berkeley, CA, USA).

Extraction of *Jacobaea* shoots and roots and sub-fractionation

Two separate extracts were made from the same batch of dried ground shoot and root plant material that was stored at -80°C. The first methanol extract of shoots and of roots was used to obtain five shoot fractions and five root fractions. These ten fractions were used in the Ames test.

Fifty grams of powdered *Jacobaea* shoot and root material were 3 times extracted for one hour with 80% methanol containing 0.1% formic acid (3×3 L) using a speed extractor (Büchi E-916, Büchi Labortechnik, Flawil, Switzerland) at 30°C and 50 bar. The three crude methanolic extracts were combined, evaporated under reduced pressure and redissolved in 100 mL of water. The aqueous extract was successively extracted with hexane (3×100 mL), chloroform ($CHCl_3$) (3×100 mL), ethyl acetate (EtOAc) (3×100 mL) and *n*-butanol (*n*-BuOH) (3×100 mL) (Supplementary material Figure S1). Removal of the solvents under reduced pressure yielded the hexane, $CHCl_3$, EtOAc, *n*-BuOH fractions and the residual H_2O fraction. Residues of these five fractions were each re-dissolved in 3.57 mL DMSO, from which 1.5 mL was used for testing a fraction in the Ames test, 199.4 µL was used for testing the fractions with the addition of retrorsine and 0.5 mL was evaporated and stored for PA analysis. The PAs in these root and shoot fractions were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) as described below.

Sub-fractionation of the chloroform shoot fraction and of the *n*-butanol root fraction

The 2nd batch of dried ground plant material (50 g) was extracted and fractionated as indicated above. Because the $CHCl_3$ shoot fraction and the *n*-BuOH root fraction showed the strongest mutagenic effects (see below), they were used for a further sub-fractionation.

The complete $CHCl_3$ shoot fraction was dissolved in 1 mL $CHCl_3$ (384 mg corresponding to 50 g dry shoot) and was fractionated on a silica gel column (4 g, Büchi Labortechnik AG, Flawil, Switzerland), using a step-wise gradient elution of $CHCl_3$ and MeOH in the following compositions (v/v; 14 mL/increment): 100/0, 99.5/0.5, 99/1, 98.5/1.5, 97/3, 95/5, 90/10, 80/20, 70/30, 60/40, 50/50, 30/70 and 10/90. The profiles of most natural compounds e.g. phenols, sugars, steroids, and terpenes in these sub-fractions were visualized by thin-layer chromatography (TLC) on silica gel 60 F_{254} (Merck, Darmstadt, Germany) with two different mobile phases: MeOH:$CHCl_3$ (1:1) and MeOH:$CHCl_3$ (1:4).

Spots were visualized with UV (ultraviolet) light (366 nm) and by spraying with an anisaldehyde reagent. The anisaldehyde reagent was a freshly prepared solution of 0.5 mL *p*-anisaldehyde in 50 mL glacial acetic acid and 1 mL sulfuric acid. Based on the TLC results, the 13 sub-fractions were pooled into 7 sub-fractions. The amounts of these 7 sub-fractions are given in the supplementary material (Figure S2).

The complete *n*-BuOH root fraction (556 mg) was dissolved in 2 mL 5% methanol in water and was fractionated on a C-18 column (10 g, 60 mL, Phenomenex, Torrance, CA, USA) and eluted using a step-wise gradient (60 mL/increment) of MeOH and H_2O, starting at 5% MeOH with 5%-increments to 50% MeOH followed with 10%-increments to 100% MeOH. A total of 15 sub-fractions were collected and analyzed by TLC on silica gel (Si 60 F_{254}) plates as above and eluted with a mobile phase of EtOAc:HCOOH:AcOH-H_2O (100:11:11:27) and $CHCl_3$-MeOH-H_2O (6:5:1). Spots were visualized with UV light (366 nm) and by spraying with an anisaldehyde reagent. Based on the TLC results, the 15 sub-fractions were pooled into 6 sub-fractions. The amounts of these sub-fractions are given in Supplementary material Figure S2.

Each of the 7 $CHCl_3$ shoot sub-fractions were dissolved in 5.95 mL dimethylsulfoxide (DMSO), from which 1.19 mL solutions (equal to 10 g plant material) were tested for the mutagenicity of individual sub-fractions. For testing the mutagenicity of the re-combined fractions, 1.19 mL of $CHCl_3$ shoot sub-fraction was evaporated by freeze drying to dryness and re-dissolved in 170 µL DMSO (58.8 g plant material/mL). And another 0.5 mL solution was used for PA analysis by LC-MS/MS.

Each of the 6 *n*-BuOH root sub-fractions were dissolved in 5.95 mL DMSO, from which 1.19 mL solutions were tested for the mutagenicity of individual sub-fractions. For testing the mutagenicity of the re-combined fractions, 1.19 mL of *n*-BuOH root sub-fraction was evaporated to dryness and re-dissolved in 198 µL DMSO (58.8 g plant material/mL). And another 0.5 mL solution was used for PA analysis by LC-MS/MS.

Our previous results revealed that the mutagenic effects of methanol/water and acetone extracts of a mixture of *Jacobaea vulgaris* and *S. inaequidens* could be attributed to the flavonoid quercetin (Bovee et al. 2015). Therefore the shoot and root fractions of *Jacobaea* were analysed for quercetin by LC-MS/MS (see Bovee et al. 2015 for experimental details) but no quercetin was detected above the limit of detection (1 µg/g dry plant material).

Analysis of pyrrolizidine alkaloids

The LC-MS/MS analyses of PAs of the plant fractions and sub-fractions were conducted based on the protocol described in Cheng et al. (2011). Prior to analysis, 10 µL of each fraction or sub-fraction in DMSO was diluted with 1 mL of water and transferred to an

HPLC vial. Analysis was conducted on an Acquity UPLC system coupled to a Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA) operated in positive electrospray mode. Separation of the PAs was accomplished on a BEH C18 150 x 2.1 mm, 1.7 µm, UPLC column (Waters) by using an acetonitrile/water/6.5 mM ammonia gradient, from 0 to 50% acetonitrile in 12 min. Column temperature was set at 50°C and the flow at 400 µL/min. The PAs were quantified using external standard calibration prepared from a blank extract spiked with PA standards (range: 0-500 ng/mL). The limit of quantification for individual PAs in the fractions was approx. 0.5 µg/g dry plant material.

The *Salmonella* mutagenicity test (Ames test)

The *Salmonella*/microsome mutagenicity test, also known as the Ames test, is a short-term *in vitro* mutation assay designed to investigate whether chemical substances can cause gene mutations (Ames et al. 1975; McCann et al. 1975). Based on pilot studies we used *Salmonella* tester strain TA100, which is histidine dependent due to a mutation in the histidine operon (Maron and Ames 1983). The mutation is reverted to the wild-type state by mutagens that cause base-pair substitutions (point mutations) (Maron and Ames 1983).

We also measured the mutagenicity with metabolic activation using Arochlor induced rat S9 (for details of the method and the obtained results see the Supplementary File).

The mutagenicity assay was performed using a pre-incubation assay, by adding 50 µL of sample extract in DMSO to 500 µL of sodium phosphate buffer (0.2 M, pH 7.4), or to 500 µL of S9 mix, subsequently mixed with 100 µL of an overnight culture of *S. typhimurium* TA100, and then pre-incubated at 37°C for 30 min. Subsequently, 2 mL of molten top agar supplemented with 200 µL solution of *L*-histidine (0.5 mM) and *D*-biotin (0.5 mM) at 48°C was added to the mixture. After vortexing, the mixture (2.85 mL in total) was poured onto minimal glucose agar plates. The plates were incubated at 37°C for 3 days after which the revertant colonies were counted. An aqueous solution of 1.7% DMSO was applied as a negative (solvent) control and 1.0 mg/mL quercetin served as the positive control (Bjeldanes and Chang 1977; Czeczot et al. 1990; Resende et al. 2012). All fractions and sub-fractions were tested on triplicate plates.

Mutagenicity testing of *Jacobaea* plant fractions

The five root and five shoot fractions of the first batch prepared from *Jacobaea* plants were dissolved in 3.57 mL DMSO, corresponding to 14.0 g plant material/mL DMSO (See supplementary Figure S2). From these stock solutions further dilutions in DMSO with an end volume of 180 µL were prepared corresponding to 0.08, 0.14, 0.80, 1.4, 2.8, 8.4 and 14 g plant material/mL DMSO. Of each solution 50 µL was added to the plates, corresponding to: 3.99, 6.84, 39.90, 68.40, 139.65, 418.95 and 698.25 mg plant material/plate. The root

and shoot fractions were assayed on TA 100 without and with S9. Assuming an average water content of 90% in *Jacobaea* plants, this corresponds to 0.015x, 0.025x, 0.15x, 0.25x, 0.5x, 1.5x and 2.5x the average *Jacobaea* fresh weight concentration in a plant.

Mutagenicity testing of the $CHCl_3$ and *n*-BuOH sub-fractions

The sub-fractions each were dissolved in 5.95 mL DMSO (see above). The concentration of these stock solutions corresponded to 8.4 g plant material/mL (418.95 mg/plate) based on the amounts of sub-fractions (Supplementary material Figure S2). From these stock solutions further dilutions in DMSO with an end volume of 180 µL were prepared corresponding to 0.08, 0.14, 0.80, 1.4, 2.8 and 8.4 g plant material/mL DMSO. Note that the highest concentration of 14 g plant material/mL DMSO was not tested for the sub-fractions. Of each solution 50 µL was added to the plates, corresponding to: 3.99, 6.84, 39.90, 68.40, 139.65 and 418.95 mg plant material/plate.

Mutagenicity testing of recombined sub-fractions

The seven $CHCl_3$ shoot sub-fractions and six *n*-BuOH root sub-fractions were recombined in the original proportions to reconstitute the original $CHCl_3$ and *n*-BuOH fractions. These combined sub-fractions were only tested at a concentration of 8.4 g/mL plant to test whether the original observed mutagenicity could be restored. To avoid a dilution effect during the combining process, 1.19 mL of the 7 shoot $CHCl_3$ sub-fractions in DMSO were evaporated and re-dissolved in 170 µL DMSO (see above). The concentration of these solutions is exactly 7 times higher than the original concentration, so that on combining those solutions in equal amounts the final concentration is equal to the undiluted $CHCl_3$ fraction. In practice, 60 µL solution from each of the sub-fractions was added together to obtain the re-combined fraction.

To avoid a dilution effect during the combining process, 1.19 mL of the 6 root *n*-BuOH sub-fractions in DMSO were evaporated and re-dissolved in 198 µL DMSO (as explained above). The concentration of these solutions is exactly 6 times higher than the original concentration, so that on combining those solutions in equal amounts the final concentration is equal to the undiluted *n*-BuOH fraction. In practice, 60 µL solution from each of the sub-fractions was added together to obtain the re-combined fraction.

Mutagenicity testing of PA standards

Retrorsine and retrorsine N-oxide (174.5 mg) were dissolved in 1.33 mL of DMSO, respectively, to prepare two stock solutions with a concentration of 131.2 mg/mL. The stock solutions were diluted with DMSO to a series of dilutions with a concentration of 5.8, 11.6, 28.4, 79.8 and 131.2 mg/mL DMSO. Of each solution 50 µL was added to the plates, corresponding to: 0.29, 0.57, 1.42, 3.99 and 6.56 mg retrorsine or retrorsine N-oxide/plate.

The concentrations were equivalent to approximately 0.2x, 0.4x, 1.0x, 2.8x and 4.6x, respectively, of the total PA concentration in fresh weight *Jacobaea* plants.

Adding retrorsine to fractions of *Jacobaea* shoots

This test was carried out with retrorsine only because it gave a higher mutagenicity than retrorsine N-oxide (see results). To test for interactions between PAs and *Jacobaea* fractions the combination of retrorsine and five shoot fractions was used. Retrorsine (316.8 mg) was dissolved in 1.2 mL DMSO to provide a stock solution of 264 mg/mL. 199.4 µL aliquots of the stock solutions of the five fractions (50 g shoots in 3.57 mL DMSO, see above) were evaporated by freeze drying to dryness and re-dissolved in 100 µL DMSO corresponding to 27.93 g shoot/mL. From this stock solution further dilutions in DMSO with an end volume of 100 µL were prepared corresponding to 0.279, 2.79 and 27.93 g shoot material/mL DMSO. On each plate, a mixture of 25 µL retrorsine stock solution and 25 µL fraction stock solution was added. Consequently, the final concentration was 6.56 mg/plate of retrorsine and three different concentrations (6.84, 68.40 and 698.25 mg shoot/plate) of the five shoot fractions.

The five shoot fractions of the first batch prepared from *Jacobaea* plants used in testing shoot and root fractions from above were used a control. Of each solution 50 µL was added to the plates, corresponding to: 6.84, 68.40, and 698.25 mg plant material/plate. We also included again retrorsine as control using the stock as indicated above. Of the solution 50 µL was added to the plates, corresponding to 6.56 mg retrosine/plate.

Statistical analysis

Colony numbers and mutagenic index (MI)

The colony numbers counted per plate were averaged over the three replicates and expressed as the mean number of revertant colonies per plate ± standard error of the mean (SE). To determine whether there was a dose-related increase or decrease in the colony numbers, the colony numbers per plate were regressed against PA concentrations.

A commonly used measure is the mutagenic index (MI) (Bjeldanes and Chang 1977; Maron and Ames 1983; Czeczot et al. 1990; Resende et al. 2012). For all treatments, the mutagenic index (MI) was calculated as the average number of revertant colonies per plate divided by the average number of revertants per plate of the negative (solvent) control. The 95% confidence intervals (CIs) of MIs were estimated by equation (1) and were used to compare the effects of different PAs. MI therefore represents the mean ± 95% CIs of three replicates (n = 3 in each group).

$$\mathbf{95\%\ CIs} = \frac{\mathbf{Var}}{\sqrt{\mathbf{n}}} * \mathbf{1.96} \qquad \textbf{(1)}$$

With **Var** being the variance of the MIs of the three replicates in each group and n being the sample size of each group. If two MIs have non-overlapping 95% CIs they are assumed to be significantly different at the $p < 0.05$ level.

The mutagenic effects of retrorsine and retrorsine N-oxide were compared by a two-way analysis of variance (ANOVA) with PAs and PA concentrations as fixed factors and MI as dependent variable.

Testing on mutagenicity on two or more metabolites or fractions

To evaluate interactions, one typically constructs an expected "null interaction" model that predicts the effect of metabolites in the absence of an interaction. We constructed a null model under the assumption that there is no interaction between two metabolites. Since the probability of a revertant colony (circa 300) is very small compared to the initial number of cells (circa 10^5~10^6) the effects of different metabolites can be considered additive if there is no interaction.

The expected combined effect of metabolites A and B ($N_{A,B}$) is the sum of the effect of metabolite A (N_A) and the effect of metabolite B (N_B) if these metabolites do not interact.

$$\mathbf{N_{A,B} = N_A + N_B} \qquad \textbf{(2)}$$

In the Ames test, the final colony number (**N)** on plates is counted. The observed number of colonies results from spontaneous mutations and from the mutagenic effect of the tested metabolite. Therefore the total colony number ($N_{obs,X}$) found after testing metabolite X can be written as:

$$\mathbf{N_{obs,X} = N_X + N_{NCX}} \quad \text{and hence:} \quad \mathbf{N_X = N_{obs,X} - N_{NCX}} \qquad \textbf{(3)}$$

With N_X being the number of colonies due to metabolite X and N_{NCX} being the number of spontaneous colonies in the negative control.

Assuming there is not interaction, for a mixture of two metabolites equations (2) and (3) can be combined to obtain:

$$\mathbf{(N_{obs,A,B} - N_{NCA,B}) = (N_{obs,A} - N_{NCA}) + (N_{obs,B} - N_{NCB})} \qquad \textbf{(4)}$$

With $N_{obs,A,B}$ being the observed colony number in the treatment with metabolites A and B together, $\mathbf{N_{NCA,B}}$, $\mathbf{N_{NCA}}$ and $\mathbf{N_{NCB}}$ being the number of spontaneous colonies in the negative control (DMSO only). $\mathbf{N_{obs,A}}$ being the observed number of colonies for the treatment with

metabolite A and $\mathbf{N_{obs,B}}$ being the observed number of colonies for the treatment with metabolite B. As each treatment has its own negative control, we weighed the above equation by dividing the colony numbers by their own negative controls, because the number of the revertant colonies of negative controls reflects the density of the bacterial suspension in the experiment:

$$\mathbf{(N_{obs,A,B} - N_{NCA,B}) / N_{NCA,B} = (N_{obs,A} - N_{NCA}) / N_{NCA} + (N_{obs,B} - N_{NCB}) / N_{NCB}} \quad \mathbf{(5)}$$

$$\mathbf{N_{obs,A,B} / N_{NCA,B} - 1 = N_{obs,A} / N_{NCA} - 1 + N_{obs,B} / N_{NCB} - 1} \quad \mathbf{(6)}$$

Because the colony number of a treatment group divided by the colony number of its negative control is the mutagenic index (MI), assuming no interaction between metabolites A and B equation (6) can be expressed as follows:

$$\mathbf{MI_{obs,A,B} = MI_{obsA} + MI_{obsB} - 1} \quad \mathbf{(7)}$$

With $MI_{obs,A,B}$ being the observed MI in treatment with metabolites A and B, $MIobs_A$ and $MIobs_B$ being the MI of the treatment of metabolite A and B, respectively. When the equation of formula 7 is not met i.e. $\mathbf{MI_{obsA} + MI_{obsB} - MI_{obs,A,B} \neq 1}$ it can concluded that an interaction is present.

For the combination of n metabolites/fractions we get:

$$\mathbf{MI_{obs,\ 1,2...n} = MI_{obs1} + MI_{obs2} + MI_{obs3} + \cdots + MI_{obsn} - (n-1)} \quad \mathbf{(8)}$$

Again, when $\mathbf{MI_{obs1} + MI_{obs2} + MI_{obs3} + \cdots + MI_{obsn} - MI_{obs,\ 1,2...n} \neq n-1,}$ it can concluded that an interaction is present.

The variance of $\mathbf{MI_{obs1} + MI_{obs2} + MI_{obs3} + \cdots + MI_{obsn} - MI_{obs,\ 1,2...n}}$ can be estimated with:

$$\mathbf{Var\ (MI_{obs1}) + Var\ (MI_{obs2}) + \cdots + Var\ (MI_{obsn}) + Var\ (MI_{obs,\ 1,2...n})} \quad \mathbf{(9)}$$

The 95% CIs of $\mathbf{MI_{obs1} + MI_{obs2} + MI_{obs3} + \cdots + MI_{obsn} - MI_{obs,\ 1,2...n}}$ is then estimated by equation (9) multiplied by 1.96. This 95% confidence limit was used to estimate if $\mathbf{MI_{obs1} + MI_{obs2} + MI_{obs3} + \cdots + MI_{obsn} - MI_{obs,\ 1,2...n}}$ is significantly deviating from n-1.

Synergistic interactions are present if the experimentally observed MI is significantly higher than n-1 and antagonistic interactions are present if the observed MI is significantly lower than n-1.

All statistical analysis were performed using SPSS software for Windows (version 21.0; SPSS Inc., Chicago, IL).

Results

Mutagenicity of *Jacobaea* plant fractions

In both cases of shoots and roots, with the exception of the hexane fraction, all the other fractions resulted in a concentration-dependent increase in revertant colony numbers of TA100 (Figure 1). Of the ten fractions, only the $CHCl_3$ fraction of shoots and the *n*-BuOH fraction of roots exhibited MIs above 2.

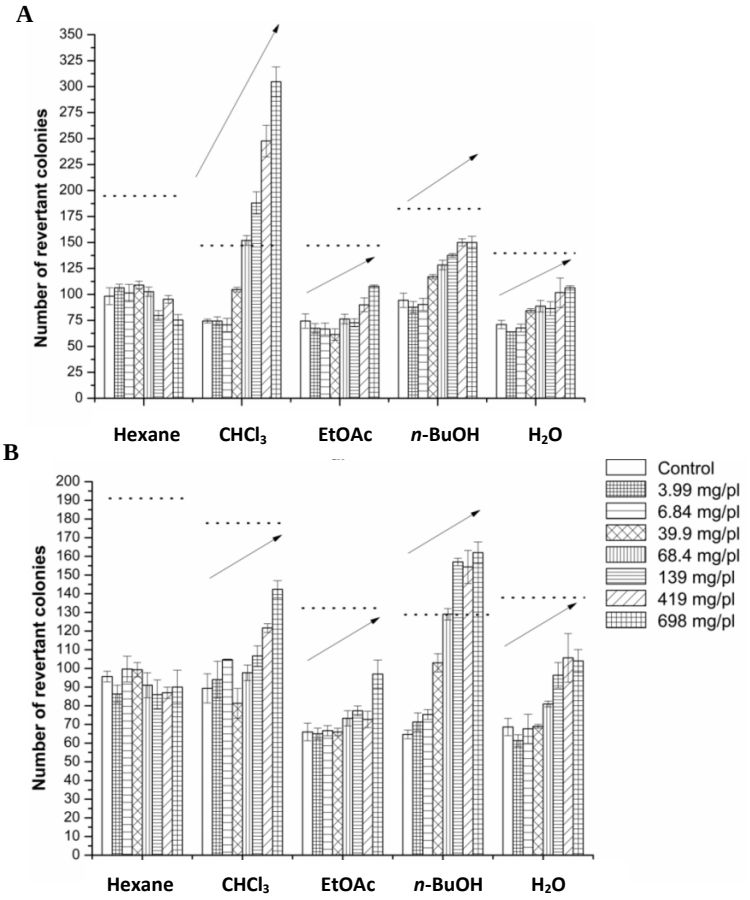


Figure 1. Number of revertant colonies of *Salmonella typhimurium* TA100 exposed to 7 different concentrations of five fractions of *Jacobaea* shoots (A) and roots (B) and a control (without metabolic activation). Data are presented as the mean number of revertant colonies per plate ± standard error of the mean (SE) of three replicates. The dashed lines shows MI = 2. Arrows indicate a significant positive correlation between plant shoot/root concentration and colony number. Levels indicated are the amount of original plant material per plate.

With metabolic activator S9, the hexane and the EtOAc fractions of shoots had no significant effects on TA100 strain while the $CHCl_3$, *n*-BuOH and H_2O fractions of shoots

induced a concentration-dependent increase in revertant colony numbers in TA100 (Figure S3A). All root fractions, except the hexane fraction induced a concentration dependent increase of revertant colony numbers of TA100 with S9 (Figure S3B). With S9, the MIs of the $CHCl_3$ shoot fraction and the *n*-BuOH root fraction were above 2 (Figure S3).

Of the five shoot fractions, the highest content of the total PA was detected in the H_2O fraction (0.60 mg/plate), followed by the *n*-BuOH fraction (0.48 mg/plate) and the $CHCl_3$ fraction (0.34 mg/plate) (Figure 2A). Although the total PA content in the *n*-BuOH fraction was 1.5 times higher than that in the $CHCl_3$ fraction, the latter caused the highest mutagenicity (MI = 4.10) among the five shoot fractions. For retrorsine N-oxide at a concentration similar to that of the total PA's in the $CHCl_3$ fraction we found MI=1.21. Among the five root fractions, the *n*-BuOH fraction contained the highest content of the total PA content (1.06 mg/plate), followed by the H_2O fraction (0.64 mg/plate) and the $CHCl_3$ fraction (0.59 mg/plate) (Figure 2B). Containing the highest total PA content, the *n*-BuOH root fraction also caused the strongest mutagenicity (MI = 2.51). For retrorsine N-oxide at a concentration similar to that of the total PA's in the *n*-BuOH fraction we found MI = 1.46. Altogether, the results of the PA analysis of the fractions suggest that natural occurring PA's were not the main cause of mutagenicity.

The two most active fractions, the shoot $CHCl_3$ fraction and the root *n*-BuOH fraction, were subjected to further fractionation.

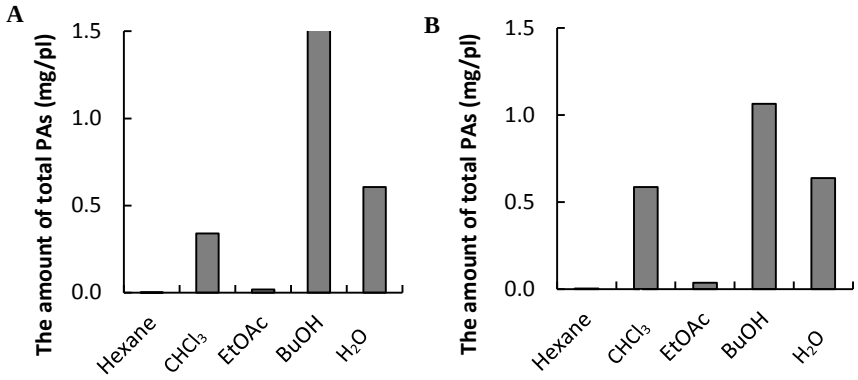


Figure 2. The total amount of naturally occurring PAs per Ames test plate for the five shoot fractions (A) and the five root fractions (B), for the highest tested dose of *Jacobaea* plant shoot (698 mg/plate).

Synergistic effects between the sub-fractions of the $CHCl_3$ shoot and *n*-BuOH root fractions

The 1st, 2nd, 4th, and 7th sub-fractions of the $CHCl_3$ shoot fraction induced a concentration-related increase of revertant colony numbers (Figure 3A). However, the MIs of all sub-

fractions were below 2 (Figure 3A), despite the fact that the original $CHCl_3$ fraction resulted in MI values above 3.3 at the same concentration. Similar results were observed for the sub-fractions of the *n*-BuOH fraction. The MIs of all sub-fractions were below 2 (Figure 3B). Thus, in both cases, sub-fractionation led to a loss of most of the mutagenic activity. In spite of this, PAs are still present in some of the sub-fractions, and to a different extent. Of the seven sub-fractions of the $CHCl_3$ shoot fraction, SFr.S7 contained the highest amount of PAs (0.08 mg/plate) while SFr.S6 contained 0.02 mg/plate (Figure 4A). For sub-fractions of the *n*-BuOH root fraction, the total PA content in SFr.R2, SFr.R3 and SFr.R4 on one plate was 0.04, 0.17, and 0.19 mg (Figure 4B). This suggests that PAs themselves may not be related to the observed MIs but they may play a role in combination with other compounds.

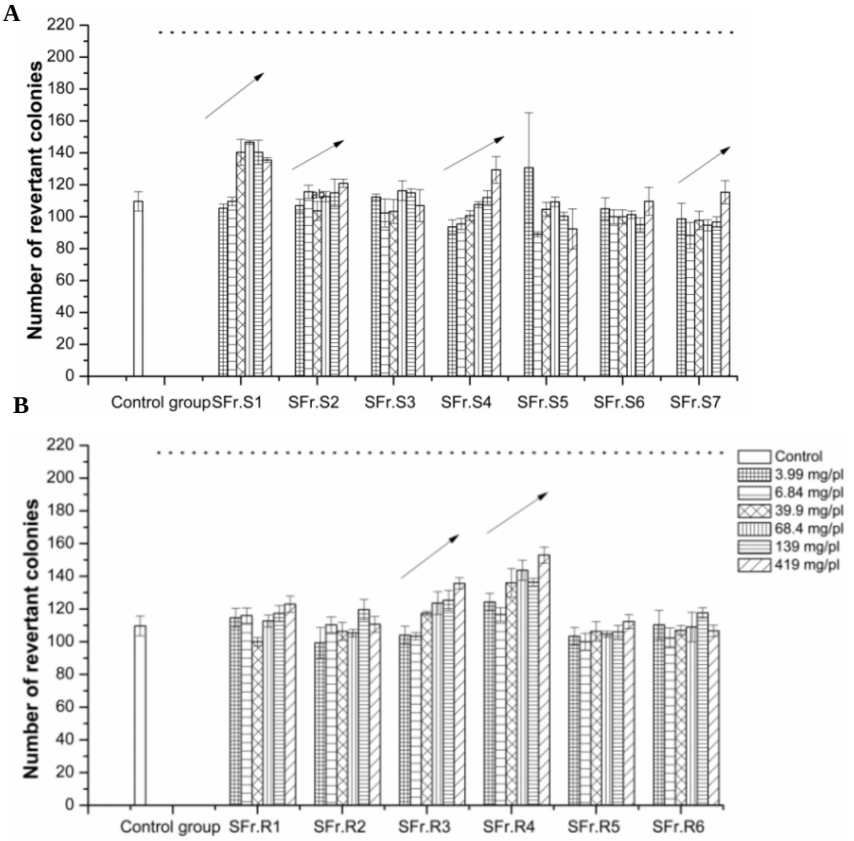


Figure 3. Number of revertant colonies (mean ± S.E., n=3) of *Salmonella typhimurium* TA100 for seven sub-fractions from *Jacobaea* $CHCl_3$ shoot extract (SFr.S1-SFr.S7) at 6 different concentrations and a control (A) and six sub-fractions from *Jacobaea n*-BuOH root extract (SFr.R1-SFr.R7) (B) (without metabolic activation). The dashed lines shows MI = 2. Arrows indicate a significant positive correlation between plant shoot/root concentration and colony numbers. Levels indicated correspond to the original amount of plant material extracted.

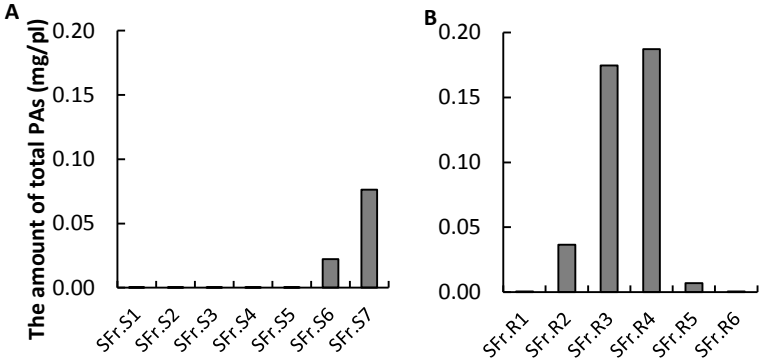


Figure 4. The total amount of naturally occurring PAs per Ames test plate for the 7 sub-fractions of the $CHCl_3$ shoot fraction (A) and the 6 sub-fractions of the *n*-BuOH root fraction (B) for the highest tested dose of *Jacobaea* plant shoot (698 mg/plate).

The reconstitution of the $CHCl_3$ shoot fraction by combining the sub-fractions in their original mass ratios restored 85% of the MI value of the original fraction (Figure 5). Reconstitution of the *n*-BuOH root sub-fractions fully restored the MI value of the original fraction (Figure 5). The MI of the restored $CHCl_3$ fraction was significantly higher than the expected MI (2.38 ± 0.29) (Figure 5) showing a synergistic interaction between the sub-fractions. Similarly, the MI of the restored *n*-BuOH fraction was significantly higher than the expected MI of 1.76 (± 0.04) (Figure 5).

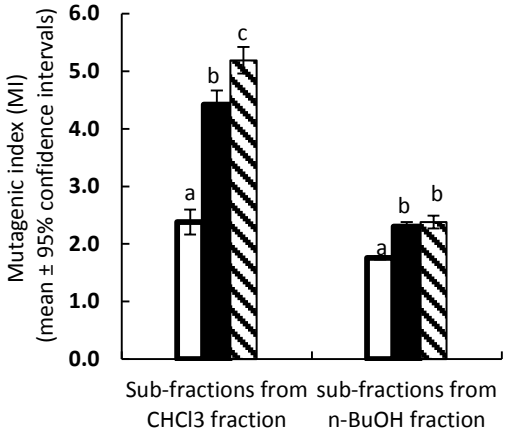


Figure 5. Observed and expected mutagenic index (MI) of the $CHCl_3$ fraction and the expected and observed MI of the recombined seven sub-fractions of the $CHCl_3$ fraction (SFr.S1-SFr.S7) and the six sub-fractions of the *n*-BuOH fraction (SFr.R1-SFr.R6) (TA100 without metabolic activation) at 418.95 mg plant material/plate. The open bar indicates the expected MI based on the MIs of the individual sub-fractions assuming no interaction (see M&M for the calculation). The solid bar gives the MI of the restored fraction obtained by recombination of the sub-fractions. The hatched bar gives the MI of the original fraction. Data are presented as means ± 95% confidence intervals (CIs) of three replicates. Different letters indicate significant differences between the three columns.

Mutagenicity of individual PAs

Overall, retrorsine and retrorsine N-oxide caused mutagenicity in a dose dependent manner (Figure 6). The effect of concentration depended on the PA tested as indicated by a significant interaction between PA and concentration, which resulted in a significant interaction in the ANOVA (Table 1).

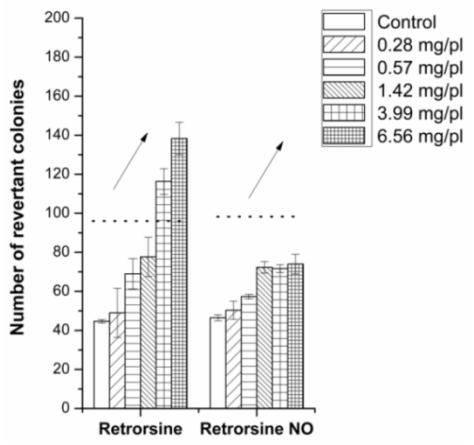


Figure 6. Number of revertant colonies (mean ± S.E.) of *Salmonella typhimurium* TA100 exposed to retrorsine and retrorsine N-oxide (without metabolic activation). Data were obtained from three replicates of the Ames test for retrorsine and retrorsine N-oxide at five concentrations and a control. Dashed lines shows MI = 2. Arrows indicate a significant positive correlation between PA concentration and colony numbers.

Table 1. Two-way ANOVA with PA (retrorsine and retrorsine N-oxide) and PA concentration as fixed factors with mutagenic index (MI) without metabolic activator S9 of the Ames test as a dependent variable.

Factors	*df*	*F*	*P*
Intercept	1,29	1194	<0.001
PA	1,29	39	<0.001
Concentration	4,29	21	<0.001
PA* Concentration	4,29	8	<0.001

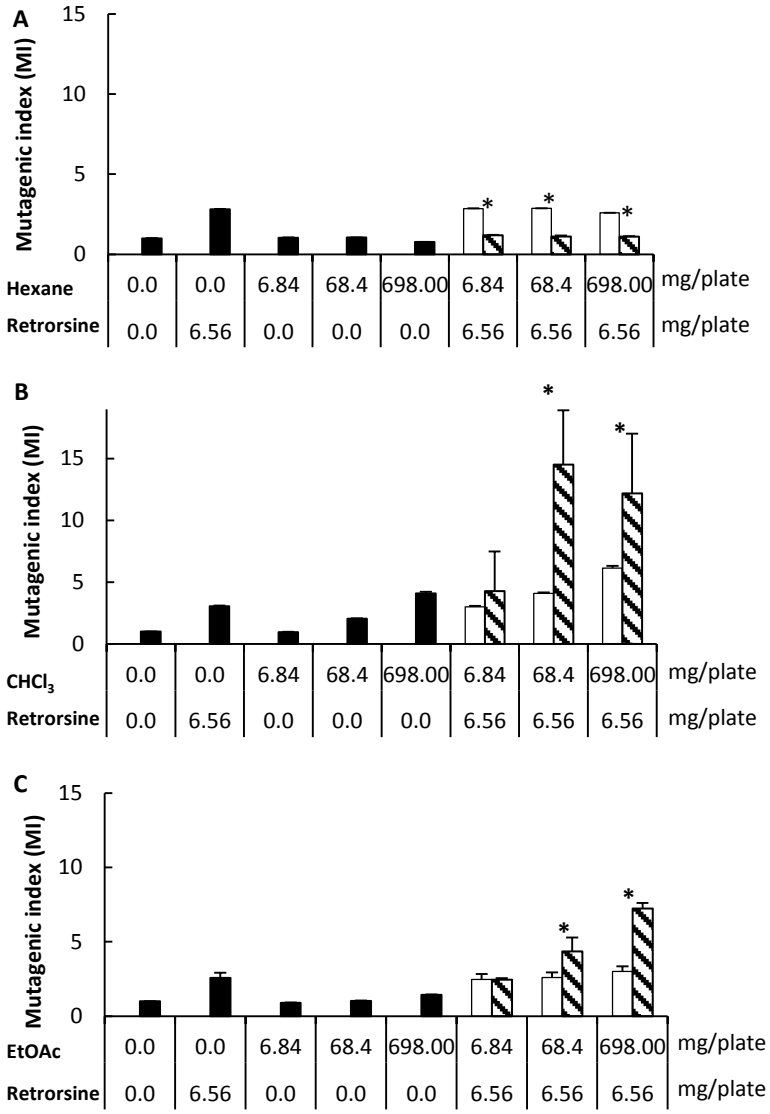
Interaction between retrorsine and *Jacobaea* shoots fractions

The model to test for interactions that we described above assumes that the relationship between the concentration of a component and its activity is linear. Inspection of (Figure S4) showed that this was only true for the two lowest concentrations of the shoot fractions. With higher concentrations the mutagenicity levels off. Consequently, if we would calculate significant interactions at the highest concentration of the fractions, such an estimate would most likely be an under estimation for synergistic interaction and an overestimation of antagonistic effects.

Retrorsine showed a MI of 2.82 (± 0.04) at 6.56 mg/plate whereas the MI for all three concentrations of the hexane shoot fractions were below 1 (Figure 7A). The combinations

of hexane fractions at 6.84 and 68.4 mg/plate with 6.56 mg/plate of retrorsine, gave an MI that was below the expected MI assuming no interaction. This significant reduction in MI compared to the expected MI indicates that an antagonistic interaction is present. The combinations of the *n*-butanol fraction and the aqueous fraction with retrorsine also showed significant antagonistic interactions (Figure 7D, 7E).

In contrast, we found synergistic effects when retrorsine was added to the $CHCl_3$ and ethyl acetate shoot fractions (Figure 7B, 7C). Therefore, the combined treatments of *Jacobaea* shoot fractions and retrorsine showed both synergistic and antagonistic interactions.



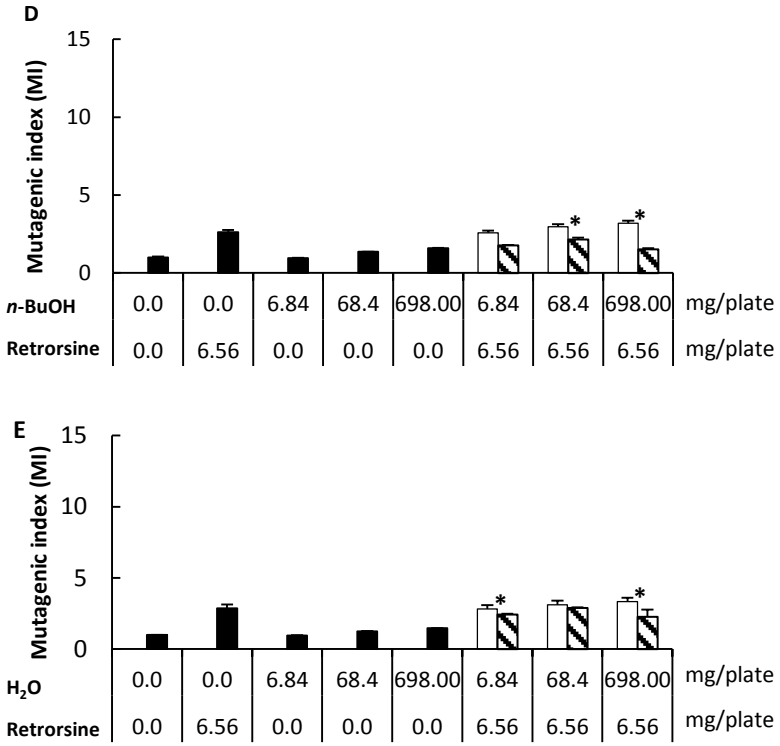


Figure 7. Mutagenic index (MI) ± 95% confidence intervals of retrorsine, 5 shoot fractions, and retrorsine added to these shoot fractions (*Salmonella typhimurium* TA100, without metabolic activation). Results represent the mean mutagenic index (MI) of three replicates. The solid bars give the observed MIs of retrorsine and fractions alone. The open bars give the expected MIs based on the MIs of retrorsine and the individual fractions assuming no interaction (see Equation 8). The hatched bars give the observed MIs of retrorsine added to plant fractions. **A**. the hexane fraction; **B**. the $CHCl_3$ fraction; **C.** the ethyl acetate fraction; **D.** the *n*-butanol fraction; **E.** the aqueous fraction. * indicates a significant difference between expected and observed MI.

Discussion

In this paper, we showed interacting effects between metabolites co-occurring in the same plant on a given bioactivity. We used two different methods. In the first place, we showed that sub-fractionation led to loss of activity. When the sub-fractions were recombined again the activity was restored, demonstrating synergistic interactions among plant metabolites. Secondly, we showed that adding individual metabolites to plant fractions, that already contained low amounts of that metabolite, resulted in a greater or lower level of activity than expected under the assumption that there is no interaction. This implies the presence of synergistic and antagonistic interactions, respectively.

Jacobaea fractions caused base-pair substitution mutations according to the Ames test with tester strain TA100. After screening all the fractions derived from the methanol plant

extracts, we selected the chloroform shoot fraction and the *n*-butanol root fraction for further sub-fractionation because they showed the strongest mutagenicity.

As the highest mutagenicity was observed without S9 we tested the sub-fractions only on the TA100 strain without S9. After sub-fractionation the sum of the mutagenic effects of the individual sub-fractions was much lower than that of the total fractions. Further sub-fractionation could even have resulted in a stronger decline in mutagenic activity resulting in the failure to isolate the active ingredient(s) responsible for the mutagenic activity of the fractions (Stermitz et al. 2000; Liu et al. 2003). Because the mutagenicity was restored after recombining the sub-fractions, this weaker mutagenicity cannot be attributed to a loss or a decrease in the concentration of a certain metabolites as a result of the fractionation process. We compared the contents of the total PA in fractions and sub-fractions and found that the natural occurring PAs can only partly explain the observed mutagenicity.

When attempting to identify the metabolites that are responsible for a certain bioactivity in a plant, it is possible to overlook the contribution of a metabolite because it shows a low or perhaps even no activity when tested as a pure metabolite. Such a result may be misleading if the metabolite is only active in the presence of other metabolites that are present in the plant material. Here, in 11 out of 15 cases studied, significant interactions were detected, showing that interactions between metabolites are more the rule than the exception. To study the importance of these interactions, we investigated the mutagenicity of individual metabolites in absence and presence of their natural background by adding retrorsine to plant fractions that also contain this PAs and related PAs naturally. Retrorsine is known to cause base-pair substitution mutations in the Ames test (Rubiolo et al. 1992). In general, we found a significant dose-dependent relationship between revertant colony numbers and retrorsine.

When we added retrorsine to the shoot $CHCl_3$ and ethyl acetate fractions of *Jacobaea* plants, they showed a significant synergistic interaction with retrorsine. The hexane fraction was non-mutagenic at all the concentrations that were tested. However, in combination with retrorsine, the mutagenicity of the combination was lower than that of retrorsine alone at all the concentrations, demonstrating clear antagonistic effects. For the combination of *n*-BuOH fraction and retrorsine and the combination of H_2O fraction and retrorsine, we found antagonistic effect at all concentrations. The strongest antagonistic effect was observed at the highest concentration. However, we should be careful about the apparent effect at the highest concentration, which could be overestimated because the dose-response curves for the two fractions were non-linear. This demonstrates that most likely the natural occurring PAs in fractions were not causing these interactions between retrorsine and fractions because the $CHCl_3$ fraction exhibited synergistic interactions whereas the *n*-BuOH fraction exhibited antagonistic interactions, even though a higher amount of naturally occurring total PA was detected in the *n*-BuOH fraction than in the $CHCl_3$ fraction (Figure 2).

The co-existence of numerous metabolites in plants suggests an enormous potential for interactions between plant metabolites. If it is the interaction between certain metabolites rather than a particular metabolite that contributes to a certain bioactivity of a plant, the traditional fractionation approach searching for bioactive metabolites may fail. On top of this, a central issue is how to detect the influence of interactions between plant metabolites on bioactivity given the infinite number of possible combinations of plant metabolites. Consequently, it is extremely difficult to detected interactions from regression studies without prior knowledge about the metabolites involved. The same is true for experimental studies which cannot cover all combination arrays of plant metabolites. In this regard, a natural phytochemical background, e.g. plant extract or fractions of extracts, provides a natural combination treatment and therefore offers an effective strategy to measure the potential interactions of plant metabolites.

Acknowledgments

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References

Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. Mutat Res 31:347-364

Berenbaum MR, Zangerl AR. 1993. Furanocoumarin metabolism in Papilio polyxenes: biochemistry, genetic variability, and ecological significance. Oecologia 95:370-375

Bjeldanes LF, Chang GW. 1977. Mutagenic activity of quercetin and related compounds. Science 197:577-578

Bovee TF, Helsdingen RJ, Hoogenboom RL, de Nijs MW, Liu X, et al. 2015. Are effects of common ragwort in the Ames test caused by pyrrolizidine alkaloids? Mutat Res 778:1-10

Cheng DD, Kirk H, Mulder PP, Vrieling K, Klinkhamer PGL. 2011. Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. New Phytol 192:1010-1023

Czeczot H, Tudek B, Kusztelak J, Szymczyk T, Dobrowolska B, et al. 1990. Isolation and studies of the mutagenic activity in the Ames test of flavonoids naturally occurring in medical herbs. Mutat Res 240:209-216

European Food Safety Authority. 2011. Scientific opinion on pyrrolizidine alkaloids in food and feed. EFSA Journal. Parma, Italy. 9:2406

Frei H, Luthy J, Brauchli J, Zweifel U, Wurgler FE, et al. 1992. Structure/activity relationships of the genotoxic potencies of sixteen pyrrolizidine alkaloids assayed for the induction of somatic mutation and recombination in wing cells of *Drosophila melanogaster*. Chem Biol Interact 83:1–22

Fu PP, Chou MW, Xia Q, Yang YC, Yan J, et al. 2001. Genotoxic pyrrolizidine alkaloids and pyrrolizidine alkaloid N-oxides-mechanisms leading to DNA adduct formation and tumorigenicity. Environ Carcinogen Ecotoxicol Rev C19:353

Fu PP, Xia QS, Lin G, Chou MW. 2002. Genotoxic pyrrolizidine alkaloids - mechanisms leading to DNA adduct formation and tumorigenicity. Int J Mol Sci 3:948-964

Gee JM, Wortley GM, Johnson IT, Price KR, Rutten AA, et al. 1996. Effects of saponins and glycoalkaloids on the permeability and viability of mammalian intestinal cells and on the integrity of tissue preparations in vitro. Toxicol In Vitro 10: 117-128

Hadacek F. 2002. Secondary metabolites as plant traits: current assessment and future perspectives. Crit Rev Plant Sci 21:273-322

Herrmann F, Wink M. 2011. Synergistic interactions of saponins and monoterpenes in HeLa cells, Cos7 cells and in erythrocytes. Phytomedicine 18:1191-1196

Houghton PJ. 2000. Use of small scale bioassays in the discovery of novel drugs from natural sources. Phytother Res 10:419-423

Keukens EAJ, de Vrihe T, van den Boom C. 1995. Molecular basis of glycoalkaloid induced membrane disruption. Biochem Biophys Acta 1240:216-228

Liu LJ, Alam MS, Hirata K, Matsuda K, Ozoe Y. 2008. Actions of quinolizidine alkaloids on *Periplaneta americana* nicotinic acetylcholine receptors. Pest Manage Sci 64:1222-1228

Liu RH. 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am J Clin Nutr 78:517s-520s

Maron DH, Ames BN. 1983. Revised methods for the *Samonella typhimurium* mutagenicity test. Mutat Res 113:173-215

McCann J, Spingarn NE, Kobori J, Ames BN. 1975. Detection of carcinogens as mutagens, bacterial tester strains with R factor plasmids. Proc Natl Acad Sci USA 72:979-983

Mösli Waldhauer SS, Baumann TW. 1996. Compartmentation of caffeine and related purine alkaloids depends exclusively on the physical chemistry of their vacuolar complex formation with chlorogenic acids. Phytochemistry 42:985-996

Nelson AC, Kursar TA. 1999. Interactions among plant defense compounds: a method for analysis. Chemoecology 9:81-92

Nuringtyas TR. 2014. Pyrrolizidine alkaloid variation in *Jacobaea* plants: from plant organ to cell level. PhD thesis, Leiden University, Leiden, The Netherlands

Resende FA, Vilegas W, dos Santos LC, Varanda EA. 2012. Mutagenicity of flavonoids assayed by bacterial reverse mutation (Ames) test. Molecules 17:5255-5268

Rubiolo P, Pieters L, Calomme M, Bicchi C, Vlietinck A, et al. 1992. Mutagenicity of pyrrolizidine alkaloids in the *Salmonella typhimurium*/mammalian microsome system. Mutat Res 281:143-147

Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, Lewis K. 2000. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 50-methoxyhydnocarpin, a multidrug pump inhibitor. Proc Natl Acad Sci USA 97:1433-1437

Tallarida RJ. 2001. Drug synergism: its detection and applications. J Pharmacol Exp Ther 298:865-872

Williamson EM. 2001. Synergy and other interactions in phytomedicines. Phytomedicine 8:401-409

Wink M, Schmeller T, Latz-Bruning B. 1998. Modes of action of allelochemical alkaloids: Interaction with neuroreceptors, DNA, and other molecular targets. J Chem Ecol 24:1881-1937

Wink M. 2008. Evolutionary advantage and molecular modes of action of multicomponent mixtures in phytomedicine. Curr Drug Metab 9:996-1009

Yamanaka H, Nagao M, Sugimura T, Furuya T, Shirai A, et al. 1979. Mutagenicity of pyrrolizidine alkaloids in the *Salmonella*/mammalian-microsome test. Mutat Res 68:211-216

Supplementary Materials:

Preparation of metabolic activator S9

Within organisms such as herbivores, interactions may occur not only between the original metabolites that are eaten but also between metabolites that are formed after the original compounds are metabolized. Many substances are broken down in the liver. However, in some cases the breakdown of metabolites in the liver leads to (unwanted) metabolic activation. For instance, some carcinogenic chemicals, such as aromatic amines, are biologically inactive unless they are metabolized to active forms. In humans and animals, the cytochrome-P450 metabolic system, which is present mainly in the liver, is capable of metabolizing a large number of chemicals to DNA-reactive, electrophilic forms. Since bacteria do not have this metabolic capability, an exogenous mammalian organ activation system needs to be added together with the test chemical and the bacteria in order to assess the activity after metabolization. Therefore we used a commonly used metabolic activator, lyophilized S9 from the liver of Aroclor 1254-induced male Sprague-Dawley rats (Molecular Toxicology, Lot. no. 3015, Boone, NC, USA). The S9 fraction was reconstituted in water and added to the culture medium that contained the cofactors for the generation of NADPH. The final composition of the S9 mixture was as follows: $MgCl_2$–KCl salt solution (1.65 M KCl + 0.4 M $MgCl_2$) (0.6 mL), 0.2 M sodium phosphate buffer pH 7.4 (15 mL), 0.1 M NADP (1.2 mL), 1 M glucose-6-phosphate (0.15 mL), S9 (2.1 mL), sterile distilled water (10 mL). The S9 mixture was freshly prepared and stored on ice for each experiment.

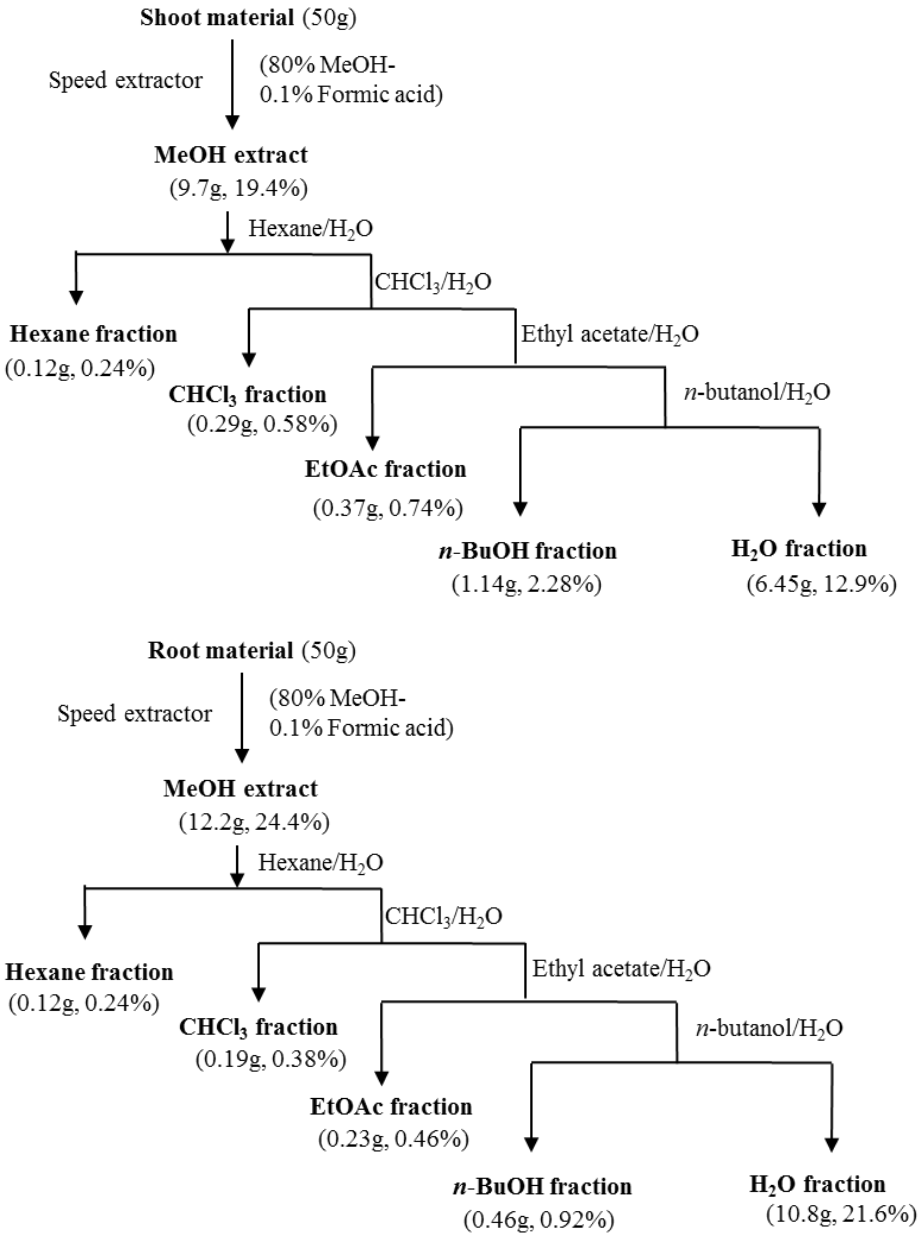


Figure S1. Extraction flowchart of the 1st batch of *Jacobaea* shoots and roots and absolute yield in g and the yield (%) in relation to the amount of starting material.

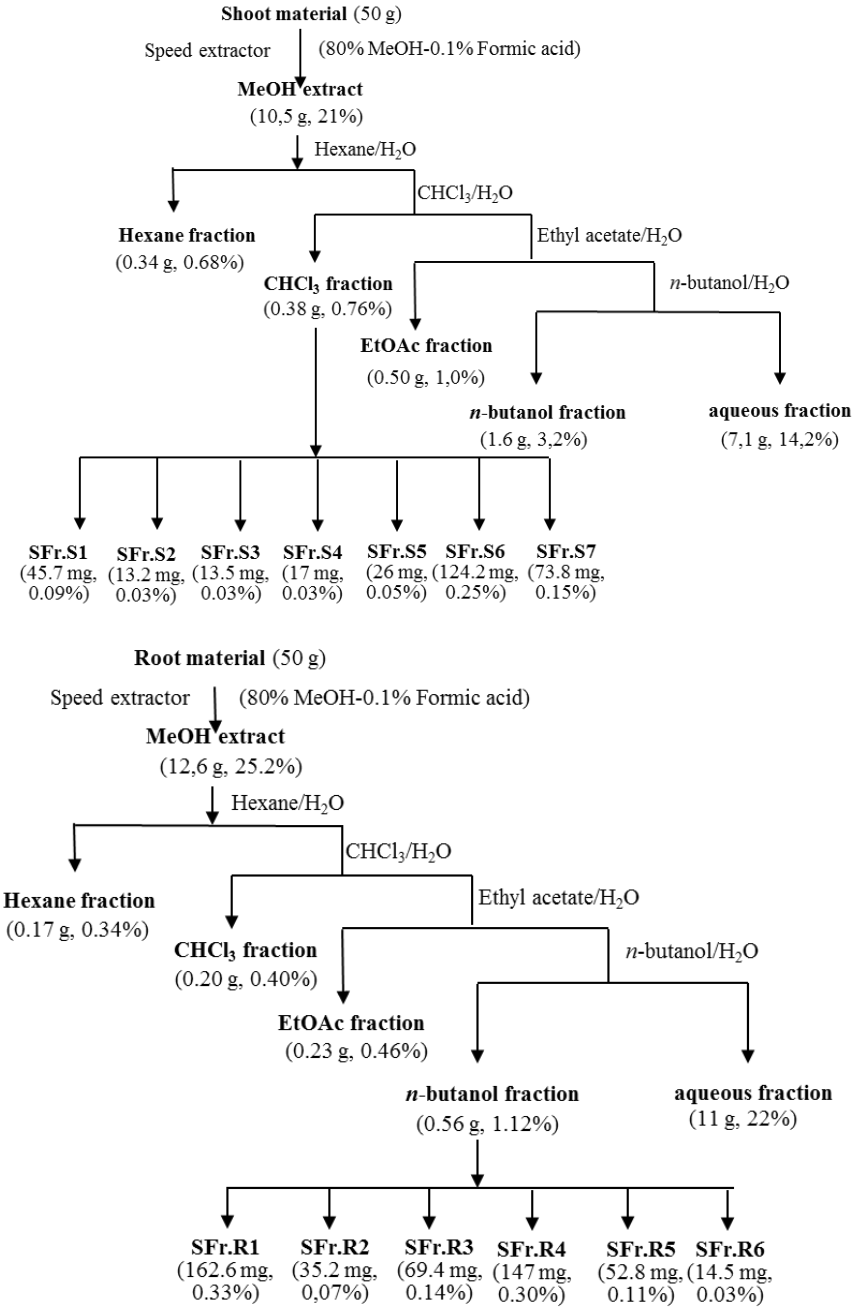
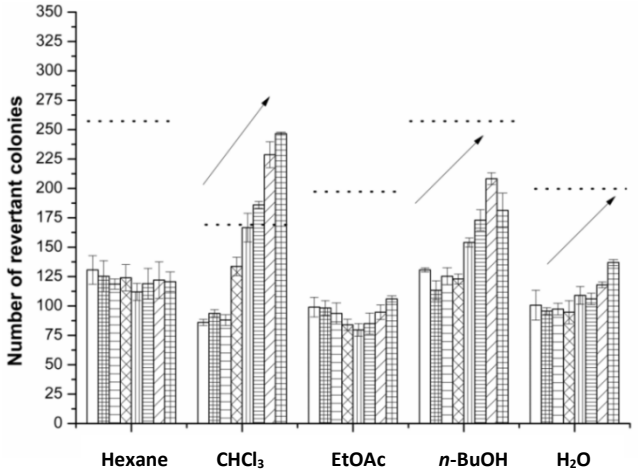


Figure S2. Extraction and fractionation flowchart of the 2nd batch of *Jacobaea* shoots and roots and absolute yield in g and the yield (%) in relation to the amount of starting material.

A



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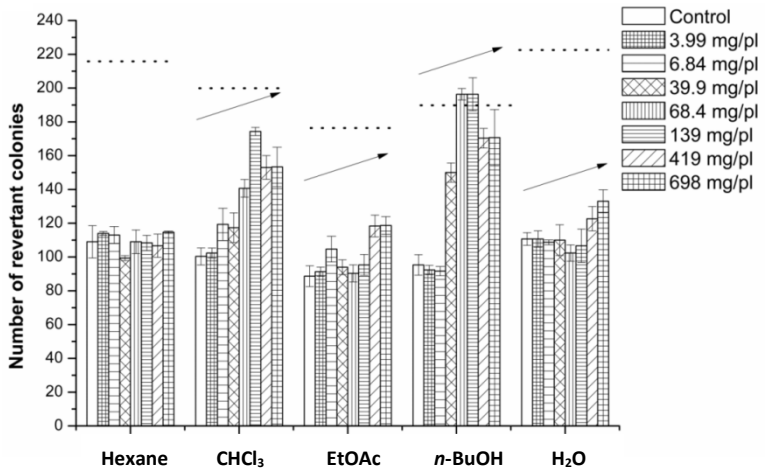

Figure S3. Number of revertant colonies (mean ± S.E.) of *Salmonella typhimurium* TA100 exposed to a control and five fractions at 7 different concentrations of *jacobaea* shoots (A) and roots (B) (with metabolic activation). Dashed lines show MI = 2. Arrows indicate a significant positive correlation between plant shoot/root concentration and colony numbers. Levels indicate the amount of original plant material per plate.

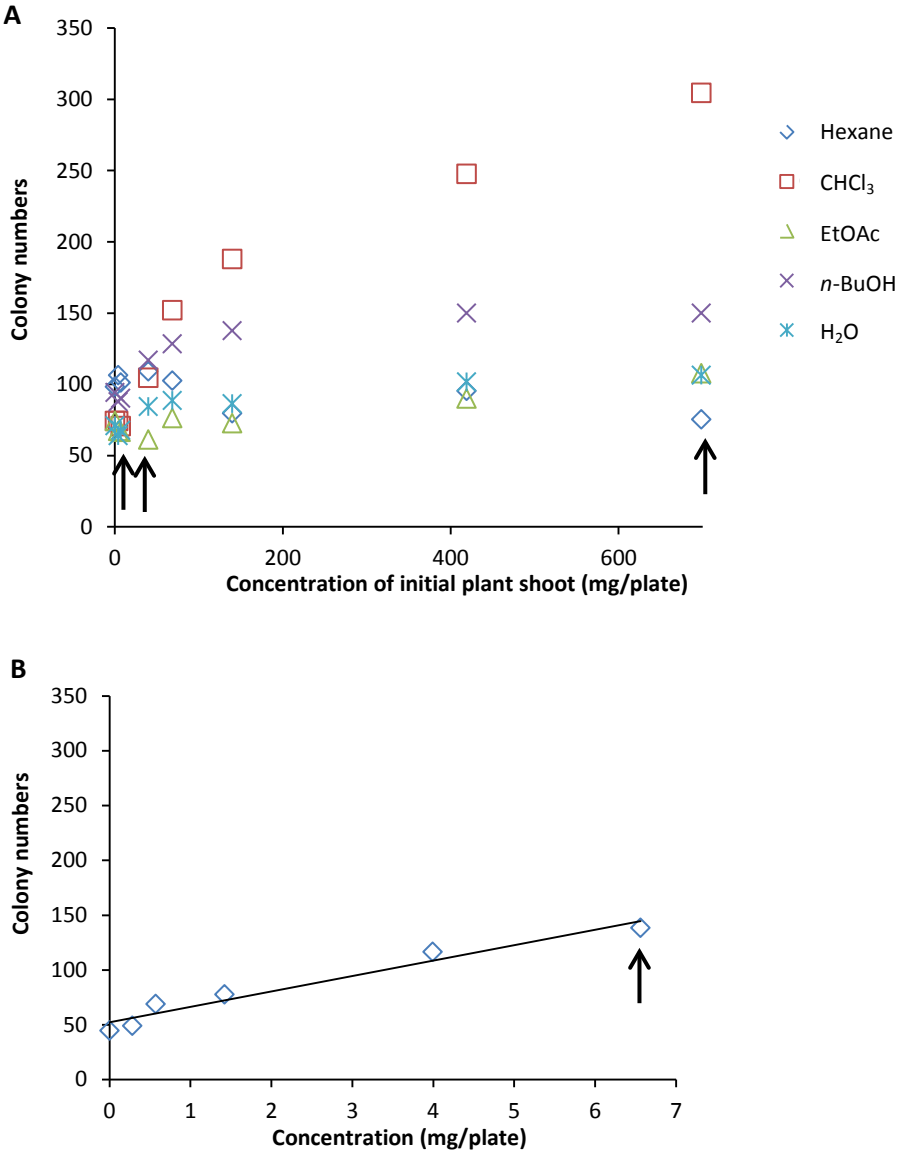

Figure S4. Colony numbers of five fractions of *jacobaea* shoots (A) and retrorsine (B) against the concentrations (mg/plate). Concentrations that were tested for interaction effects are indicated by arrows. Note that the lowest concentration tested is very close to the origin.

Chapter 7

Summary and discussion

To respond to and manipulate their natural environment, plants have developed various defense strategies, among which the chemical defenses are very powerful (Mithofer and Boland 2012). The plant kingdom has evolved an enormous number of chemically diverse metabolites (Fraenkel 1959; Hartmann 1996; Kliebenstein 2004; Mithofer and Boland 2012). The consequence of a large number of metabolites within a species is that there is a high probability of interactions. Such a co-occurrence of plant metabolites comprise a natural background where these metabolites have to function and this is often overlooked or ignored in ecology studies.

The main goal of this thesis is to understand the importance of the interactions between plant metabolites in a context of plant-insect herbivore interactions. To achieve this, I used *Jacobaea*, CGA and the pyrrolizidine alkaloids (PAs) as study objects. Here, I will first discuss the bioactivity of individual PAs. Secondly, I will discuss the interactions between plant metabolites on herbivore resistance, the effects of such interactions on herbivore resistance, and their significance in an ecological context. Third, I will briefly discuss the approach and the frame work I used in this thesis. Finally, I will draw the main conclusions of this thesis, along with perspectives for future research.

7.1 Bioactivity of individual metabolites

In Chapter 2, I found clear negative effects of PAs from *J. vulgaris* on thrips survival. This demonstrated the protective function of PAs against insect herbivores. The negative effects of individual PAs on thrips corroborate the findings of a previous correlative study with whole plants that showed that the total PA concentration was negatively correlated with herbivore performance (Cheng et al. 2011), and in particular that the jacobine-like PAs are negatively correlated with thrips damage (Leiss et al. 2009; Cheng et al. 2011).

Chapter 2 also shows that structurally related metabolites vary in their effects on thrips. Firstly, the free base PAs were found to be more toxic to thrips than their corresponding N-oxides. This finding is consistent with previous studies on the pea aphid (*Acyrthosiphon pisum*) (Dreyer et al. 1985), the snail *Arianta arbustorum* (Helicidae) (Speiser et al. 1992), the caterpillar *Spodoptera exigua* (van Dam et al. 1995), the locust *Locusta migratoria* (Macel et al. 2005), and *S. exigua* cell lines and larvae (Nuringtyas et al. 2014). The difference between the two forms of PAs in their effects on insect herbivores may be related to cell permeability. In general, a highly hydrophobic or lipophilic compound passes cell membranes easier because the membrane is composed of phospholipids. Most free base PAs are rather lipophilic and able to easily permeate bio-membranes, while N-oxides are polar hydrophilic compounds (Lindigkeit et al. 1997). Next, among the tested free base PAs, we found jacobine and erucifoline to be the most active against thrips while seneciphylline and senecionine were the least active ones. Different effects of individual PAs on herbivores have been reported for *S. littoralis* (Gonzalez-Coloma et al. 2002), thrips (Macel

et al. 2005), and *S. exigua* cell lines (Nuringtyas et al. 2014). In contrast, Dominguez et al. (2008) did not find any significant differences among the tested PAs on *Myzus persicae* or *Leptinotarsa decemlineata*. The various effects of PAs on insect herbivores could be attributed to the physiological processes in insects. For instance, the larvae of *S. littoralis* avoided the toxicity of senecionine by rapid and efficient excretion of this metabolite (Lindigkeit et al. 1997). However, knowledge about PA toxicity in herbivores is still rudimentary. We considered retrorsine, jacobine and erucifoline as more downstream in the biosynthetic pathway of PAs and senecionine and seneciphylline as PAs at the basis of the biosynthetic pathway. Senecionine N-oxide is the first PA in the biosynthesis from which the other PAs are derived (Hartmann and Dierich 1998; Pelser et al. 2005). Senecionine N-oxide and seneciphylline N-oxide co-occur in all *Senecio* species. From these two PAs, in one or two enzymatic steps, the other PAs including retrorsine N-oxide, jacobine N-oxide and erucifoline N-oxide are produced (Figure 1 in Chapters 2 and 3). In Chapter 2, I compared the effects of the more downstream free base PAs on thrips with that of the basic free base PAs. Although there was a trend that the more derived ones were more active against thrips than the senecionine and seneciphylline this was not significant. My results are partly in line with the Arms Race Hypothesis. However, to really test this hypothesis more herbivores should be tested, especially ones that co-evolved with *J. vulgaris*.

Results of bioassays on pure PAs were not always consistent with correlative studies using whole plant bioassays. Especially, for metabolites which may be effective at low amounts in plants, correlative studies might not be sensitive enough to show their bioactivity. For instance, we found that retrorsine affected thrips survival in the bioassay. However, thrips performance was not significantly correlated with retrorsine using whole *Jacobaea* plants (Cheng et al. 2011), which may be due to the low concentrations present in plants. Jacobine N-oxide was found to be significantly correlated with thrips damage in the whole plant bioassay (Cheng et al. 2011) while jacobine N-oxide did not result in strong effects on thrips when tested as a pure compound (Chapter 2). The disadvantage of correlative studies is that correlation does not imply causation. The correlation between thrips damage and jacobine N-oxide found by Cheng et al. (2011) may have resulted e.g. from the correlation between jacobine N-oxide and free base. Another possible explanation is that jacobine N-oxide becomes active in the presence of other metabolites, emphasizing the importance of studying the interactions between plant metabolites.

7.2 The interactions between plant metabolites

The co-occurrence of plant metabolites provides a natural background for individual metabolites, where there is a high possibility for them to interact with each other. Plants may manage the interactions between metabolites by accumulating metabolites in organs, tissues, cells and even cell compartments. Such a compartmentalization can be used to keep antagonistically interacting metabolites apart but may also promote synergistic interactions

between plant metabolites by storing them in the same compartment. The interactions between plant metabolites are of vital ecological significance for plants. For instance, plants can benefit from synergistic interactions by increasing their bioactivity at a lower cost (Berenbaum and Zangerl 1998; Nelson and Kursar 1999). With respect to single metabolites, the modes of action in concert with other metabolites may differ from that as a single compound. As a consequence, potential interactions may provide new insights for the bioactivity of single metabolites that may have been overlooked when tested as pure compounds. If plant metabolites interact antagonistically, compartmentalisation is a solution to avoid antagonistic interactions. Indeed, metabolite content may differ largely between plant organs (Kuhlisch and Pohnert 2015) and even between different cell layers (Nuringtyas et al. 2014; Moussaieff et al. 2013). The importance of the interactions between plant metabolites in the context of plant-insect herbivore associations and the influence of the interactions on the bioactivity of individual metabolites will be presented below in detail. In spite of the great importance, interactions between plant metabolites and their effects have not been largely investigated in an ecological context (but see references below). The rare demonstrations could be due in part to the complexity of the interactions between plant metabolites, and to the difficulty of detecting and analyzing interactions in a proper manner (Nelson and Kursar 1999).

The complexity of interactions between metabolites presents a challenge both in terms of the enormous number of metabolites in a single plant and in terms of the infinite number of potential combinations. Plants have may have reduced the interaction between metabolites by dividing them over different plant parts, tissues, cell layers, cells and even within cells. Still, it is impossible to evaluate all combination of metabolites. Investigating interactions becomes even more complex if unidentified or even unknown metabolites are involved. The unknowns account for a large part of the total amount of metabolites in a plant (Trethewey 2004). In the light of these facts, it is a great challenge to measure the interactions between plant metabolites and the influence of interactions in plant-insect herbivore associations. In this thesis, in line with the level of complexity of chemical diversity, I studied the effects of the interactions between plant metabolites on insect herbivores on three levels, i.e. interactions within a structurally related class, interactions between metabolites of different classes, and interactions occurring in the natural phytochemical backgrounds of primary metabolites (PMs) and diverse classes of secondary metabolites (SMs). I used both a bottom-up approach and a top-down approach.

7.2.1 Approaches to interaction research and a statistical framework

A way forward would be to start with combinations of classes of metabolites of which we know that the potential of interactions is high such as saponins together with metabolites that not easily pass membranes (Gee et al. 1996; Herrmann and Wink 2011). Prior information of individual metabolites provided a starting point for Chapters 2, 3 and 4. In

Chapter 2, I studied the effects of the interactions between the most abundant PA N-oxides on thrips. I wanted to investigate if the predominance of PA N-oxides in plants despite their relatively weak activity against herbivores could be explained by interactions between them. In Chapters 3 and 4, I investigated the interactions between PAs and CGA on western flower thrips *Frankliniella occidentalis*, a generalist herbivore. We choose this combination of metabolites on the basis of existing knowledge of the two metabolites: PAs and CGA are differently distributed over plant cell layers and CGA is able *in situ* to form a complex with other alkaloids. Specifically, Nuringtyas et al. (2014) found that the mesophyll of *J. vulgaris* contained high amounts of PAs while CGA was accumulated in the epidermis. CGA forms a π-molecular complex with caffeine (a purine alkaloid) (Mösli Waldhauser and Baumann 1995). An earlier study with *S. exigua* cell lines also showed an antagonistic interaction between jacobine and CGA (Nuringtyas, PhD thesis, 2014). This finding may explain why PAs and CGA are distributed differently over cell layers. This would be most relevant for single cell feeders. For chewing insects such as *Spodoptera*, distribution of PAs and CGA over different cell layers will not prevent an antagonistic effect. I, therefore, studied the interaction between CGA and PAs in their effect on thrips which is a single cell feeder. Overall, for instance, in the case of PAs and CGA, prior information is useful in terms of forming a starting point for interaction studies.

In the case of the absence of prior knowledge about the metabolites that are involved, study on interactions between metabolites can be approached in a top-down direction, from a metabolome analysis down to component-interaction analysis. In Chapters 5 and 6, I did set the first step of a top-down approach to study the interactions between metabolites with two types of bioactivity, i.e. the effects on thrips as measured by *in vitro* bioassays (Chapter 5) and the mutagenicity as determined by the Ames test (Chapter 6). I studied the bioactivity of individual PAs when they were added to their natural phytochemical background (fractions from plant extracts). This starting step provided a general impression of the importance of interactions between metabolites from a metabolome perspective. When the bioactivity of a metabolite is increased when it is present in plant fractions, this points to the importance of interactions among plant metabolites for plant defence. Further sub-fractionation and recombining sub-fractions may narrow down the candidate compounds that are involved in these interactions.

The reason that only few studies on interactions between metabolites were carried out is probably because a theoretical framework is lacking (Nelson and Kursar 1999). Here, I describe a method to calculate the expected interaction effects of two or more components, which then allows for a proper statistical testing both of the pattern and the magnitude of interaction effects. With regard to thrips survival, I formulated a multiplicative null model. This model is applicable to calculate survival, given that an individual cannot die twice (Chapter 5). Regarding to the mutagenicity as measured in the Ames test, an additive model was applied because the probability of a revertant colony is very small compared to the

initial number of cells (Chapter 6). The interaction effects then can be calculated by comparing the observed results and the expected results. Such a framework not only allows testing for the existence of interaction effects but also for differences in their magnitude. The applications of the theoretical frame work that is put forward in this thesis can be extended to other aspects of plant-environment interactions, other multi-component materials, and to the design of bioactivity studies in fields as toxicity, mutagenicity, health and plant protection.

7.2.2 **The effects of the interactions between plant metabolites and their ecological significance**

Interaction effects of metabolites within a structural related class

In plant-insect herbivore associations, examples of metabolite interactions within a structural related class include 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), the antagonistic effects of two furanocoumarins on *S. littoralis* (Diawara et al. 1993; Calcagno et al. 2002), the synergistic effects of piper amides on the mosquito *Aedes atropalpus* (Scott et al. 2002) and on *S. frugiperda* caterpillars (Dyer et al. 2003; Richards et al. 2010), the synergistic effects of two iridoid glycosides on a specialist caterpillar *Junonia coenia* (Richards et al. 2012). Only few studies have been carried out on interactions among PAs. Macel (2005) found a weak synergistic effect of a mixture of the free bases of senecionine, seneciphylline and senkirkine on *S. exigua* and of a mixture of the free bases of senecionine and seneciphylline on *L. migratoria*. Yet, Siciliano et al. (2005) did not find any synergistic effects of two PAs from *Anchusa strigosa* on *S. exigua*. These two studies have only tested the interaction effects of free bases. As PAs in plants occur mainly as PA N-oxides, it is therefore ecologically relevant to study the interaction between PA N-oxides and their effects on insect herbivores or pathogens, which however have not been studied yet. In Chapter 2, the most abundant PA N-oxides of *J. vulgaris*, senecionine N-oxide, jacobine N-oxide and erucifoline N-oxide (Joosten et al. 2011) were combined and showed no synergistic (or antagonistic) effects on thrips survival. As such, our results about the effects of interactions between PA N-oxides on thrips do not provide an explanation for why PA N-oxides are the dominant form in which PAs occur. Neither does our study explain why there are so many different PA N-oxides present in a single plant.

Interactions between metabolites of different chemical classes

Antagonistic interactions between free base PAs and CGA on thrips

In Chapter 3, I found antagonistic interactions between free base PAs and CGA on their effects on thrips for all five PAs tested. Although the strength of the interaction differed among these PAs, it suggests that this is a general pattern. From a plant's point of view, antagonistic interactions in most cases would not be an advantage to plant fitness. Therefore, from an ecological perspective, such an interaction is not easily explained. Together with a previous study, in which PAs and CGA were found to be differentially distributed over different cell layers of *Jacobaea* leaves (Nuringtyas et al. 2014), the antagonistic effects of free base PAs and CGA on thrips suggest that antagonistic interactions may represent a constraint caused by the accumulation of metabolites in plants (Nelson and Kursar 1999). It has been reported that caffeine and CGA *in situ* formed a complex at a ratio of 1:1 (Sondheimer et al. 1961; Horman and Viani, 1972; Chapman and Miller 1974). If similar complexes would be formed between free base PAs and CGA, we expect that antagonistic interactions will depend on the ratio of two components. We indeed found that the effects of the antagonistic interactions between free base PAs and CGA depended on their ratio. However, there was too much variation in the measured effects to reliably determine the optimal ratio.

The fact that all the tested free base PAs showed interactions with CGA suggest that the necine part of the PA molecule involved in the interaction. It is the retronecine base which all tested free base PAs have in common, rather than the macrocyclic ring that varies in structure. This fits very well with the observation that the quinic acid part of the CGA molecule interacted antagonistically with retrorsine while the caffeoyl part did not. In Chapter 3 I only set the first steps but the results do show that that modification of functional groups, addition/elimination of specific groups, or changing the substitution pattern can be used to further reveal the mechanism from a chemical perspective.

Synergistic interactions between PA N-oxides and CGA on thrips

In Chapter 3 I studied the interaction between free base PAs and CGA on thrips survival. However, as mentioned before, in plants PAs occur mostly as N-oxides (Hartmann et al. 1989). PA N-oxides were less active than the corresponding free bases when tested alone on thrips survival (Chapter 2). Therefore, it is of ecological significance to study the interactions between PA N-oxides and other SMs such as CGA on thrips survival. In Chapter 4 I found synergistic interactions between PA N-oxides and CGA on thrips survival. Although the strength of the interaction varied, the pattern was similar for all PA N-oxides tested which again suggests a general pattern, I then compared the interaction effects of the combinations between free base PAs and CGA with that of the combinations between the corresponding PA N-oxides and CGA on thrips survival. Surprisingly, I found that the thrips survival of the two forms of PAs was reversed when they were in combination with CGA. With respect to the CGA molecule, it suggests a dual role of alleviating the bioactivity of free base PAs and of enhancing the bioactivity of PA N-oxides

on thrips survival. These findings may give an alternative explanation for the fact that PA N-oxides are more abundant than free base PAs. However this was not completely backed up by the results of Chapter 5 in which I found synergistic interactions on thrips survival between both retrorsine and retrorsine N-oxide with two fractions of a methanol extract of *Jacobaea* plants. In the case of PA N-oxides, the findings of synergistic effects on thrips survival provide a new insight into the defensive functions of PA N-oxides. In the same way, these results supply an ecological driven alternative of the predominate storage of PA N-oxides in plants, in addition to a physicochemical explanation that N-oxides are better soluble in water and are therefore more easily stored in the vacuoles and transported through the plant (von Borstel et al. 1986).

Synergistic interactions are assumed to be of ecological and evolutionary significance in the plant-environment associations. Results of Chapter 4 and other studies suggest that the bioactivity of single SMs can be enhanced in concert with others. This is meaningful for metabolites which may not be active by themselves. For instance, rutin by itself did not have a negative effect on the growth rate of the caterpillar *S. exigua*, while CGA had a slight negative effect, but together they had a strong negative effect (Stamp and Yang 1996). Synergistic interactions can also provide an advantage to plant defense in terms of producing a greater toxicity at a lower cost (Nelson and Kursar 1999; Ryabushkina 2005). In this regard, it is expected that natural selection selects for synergistic interactions.

Interactions between plant metabolites occurring in natural backgrounds

In the first chapters I studied interactions between known plant metabolites by combining them, which can be seen as a bottom-up approach. In the remaining chapters I used a top-down approach and investigated interactions between plant metabolites within the natural phytochemical background in which both PMs and SMs occur. In Chapter 5 I studied the effects on thrips survival. In Chapter 6 I used the mutagenicity as determined by the Ames test as a measure of bioactivity. Fractions of a methanol extract of *J. vulgaris* leaves differed in their bioactivity in the Ames test. Especially the chloroform fraction showed a strong mutagenicity. Upon (sub-)fractionation, this bioactivity was largely gone. Recombining the sub-fractions in their original proportion restored the bioactivity. This suggests that (sub-) fractionation led to the loss of synergistic interactions among metabolites. For thrips too, fractionation led to the loss of bioactivity. Such effects are often observed in phytochemical studies (Williamson 2001; Herrera and Amor 2011; Labuschagne et al. 2012; Inui et al. 2012).

The fractions of *J. vulgaris* plants differed in their anti-herbivore and mutagenic effects. Nevertheless, the order of bioactivity was not consistent with the order of the natural PA content of these fractions, suggesting that besides PAs, other metabolites also contributed directly or indirectly, through interacting effects, to the overall activity of plant fractions.

With the generally increasing polarity of solvents used in the fractionation process, the fractions contain different types of metabolites (Sasidharan et al. 2011). Although beyond the scope of this thesis, further sub-fractionation and identification may narrow down the potential metabolites that are responsible for the bioactivity.

In Chapters 5 and 6, I set the first step of a top-down approach by adding individual PAs to various fractions to study the interactions between plant metabolites at a whole-metabolome level, as well as to determine how natural phytochemical backgrounds shape the bioactivity of individual metabolites. In Chapter 5, both retrorsine and retrorsine N-oxide interacted synergistically with the chloroform and the *n*-butanol fractions in their effects on thrips survival. In Chapter 6, the chloroform and ethyl acetate fractions of *J. vulgaris* leaves significantly increased the mutagenicity of retrorsine while the hexane, *n*-butanol and aqueous fractions decreased the mutagenicity of retrorsine. Not surprisingly, this also suggests that the type of interaction in this case was dependent on the type of bioactivity. Considering the PA amount in these fractions, the interactions between PAs and other SMs may dominate the ultimate effects of the interactions between PAs and fractions. Altogether, the results of Chapters 5 and 6 suggest that natural backgrounds influence the bioactivity of individual SMs in both positive and negative patterns. Moreover, the strength of the interaction depended on the fractions, suggesting that the effects of plant metabolites may vary depending on the phytochemical background.

The importance of natural backgrounds

Plant extacts and fractions offer an effective approach to measure the interactions between plant metabolites as a natural combination treatment and as a natural background, without requiring prior knowledge about the metabolites involved. To study the overall efficacy of a mixture of plant metabolites is of ecological significance because insect herbivores always encounter mixtures of metabolites in nature. To study the bioactivity of individual plant metabolites against a natural background of other metabolites can assist in understanding plant-insect herbivore interactions, because a given plant metabolite is likely not to be the sole agent, but rather is likely to be imbedded as a participant in multitude of interactions that naturally occur in plants. Still, we lack sufficient evidence for the importance of natural phytochemical backgrounds in an ecological context. This thesis sets an important first step.

This study supports a commonly held notion that plant chemical defence is dependent on a variety of metabolites, which together shape the outcome of the defensive efficacy. Interactions between plant metabolites may provide additional or novel information for the bioactivity of individual SMs. Interaction effects of plant metabolites could also explain why some SMs show a certain activity in particular species while they do not show the same activity in others. For instance, CGA in *chrysanthemum* was negatively correlated with the feeding damage of thrips *Frankliniella occidentalis* (Leiss et al. 2009), while no

effect of CGA on thrips was detected in tomato *Solanum lycopersicum* (Mirnezhad 2011). Knowledge about interactions of metabolites can also be applied in other multi-component materials. For instance, the anti-proliferative activity of quercetin 3-β-D-glucoside against human breast cancer cell was increased 4 fold by combination with apple extracts (Yang and Liu 2009).

7.3 Final conclusions and future perspectives

From this thesis, the importance of interactions between plant metabolites in plant-insect herbivore associations is evident. Several conclusions emerge. First, PAs significantly decreased thrips survival, indicating the role of PAs as a plant defence against insects. Different PAs affect thrips differently with some PAs being more active than others. Secondly, combinations of plant metabolites affected thrips survival both in synergistic and antagonistic patterns. It suggests that interactions between plant metabolites should be taken into consideration in their effect on herbivore performance. Thirdly, the interactions on thrips survival and mutagenicity between plant metabolites are also observed in complex mixtures of metabolites (i.e. plant extracts and fractions). I showed that the bioactivity of individual metabolites is strongly influenced by their natural biochemical backgrounds that may potentiate or mitigate their efficacy. Therefore, the phytochemical backgrounds should be taken into account when designing bioassays. Taken as a whole, it can be argued that the bioactivity of a given metabolite is not merely dependent upon the amount and chemical structure of that metabolite, but also on the co-occurring metabolites of the natural phytochemical backgrounds.

A number of points should be considered in future studies with regard to the interactions between plant metabolites. First, there is shortfall in our current knowledge of the interaction mechanisms. Further study on physiochemical and physiological processes will likely be fruitful. From a physiochemical perspective, metabolites can alter solubility and/or the resorption rate and thereby their bioavailability (Amin et al. 2015). Physiological processes can also be critical in determining the interaction effects e.g. digestive metabolism or detoxification enzyme activity (Stermitz et al. 2000; Scott et al. 2002). Secondly, the importance of paying attention to antagonistic effects and of understanding its ecological significance should be taken into account. As evolution of plant defences occurs under selection pressures from other factors such as abiotic stresses, pathogens, other herbivores, testing the interaction effects on varied plant enemies would also be an avenue of future work. Thirdly, compartmentalization of plant metabolites between different cell organelles, cells, cell layers and organs may contribute to a plant's defence through promoting synergistic and avoiding antagonistic interactions. Therefore, as a first step it is important that chemical analysis of SMs of such compartments are carried out to identify which metabolites co-occur and which not. Finally, demonstrations of interaction effects of plant metabolites in the context of plant-herbivore associations are rare. This

likely holds true for other ecological relationships as well. To better have a better understanding of mechanistic and ecological interaction between plants and their environment a concerted and combined effort of various research fields is required. To follow up the first steps that were set in this thesis, both the bottom-up and the top-down approach are important. Considering the enormous number of plant metabolites, one of the challenges would be to measure a large number of possible combinations that are of interest. This is an impossible task with the bioassays that were used in this thesis. Development of high-throughput screening, as often applied in pharmacological studies especially in drug discovery, is essential in this respect. For example, receptor-based approaches have been widely applied in drug discovery. From a mechanistic perspective, the effects of interactions between compounds can be caused by blocking or disturbing the membrane-bound receptor function. For instance, ramipril inhibits the angiotensin receptor, thereby facilitating the antihypertensive effect of candesartan-cilexetil (Raasch et al. 2004). As such, approaches based on receptor activity would be a screening strategy. Another approach is the use of cell lines for bioactivity screening. While cell lines of thrips are presently not available, we can start using cell lines of other herbivores, e.g. the beet armyworm, *S. exigua*. Such cell lines have been used in our group (Nuringtyas et al. 2014). The effects of the interactions between free base PAs and CGA on *S. exigua* cell lines (Nuringtyas et al. 2014) were consistent with that on thrips larvae (Chapter 3). Bioassays on cell lines, however, cannot account for metabolomic changes that may occur during digestion and absorption (see a review by Yoon et al. 2012). Despite this disadvantage, cell lines can still be employed for initial screening and selecting candidate metabolites or combinations of interest to be further used in bioassays with living organisms.

References

Amin MU, Khurram M, Khattak B, Khan J. 2015. Antibiotic additive and synergistic action of rutin, morin and quercetin against methicillin resistant Staphylococcus aureus. BMC Complementary and Alternative Medicine 15:59

Berenbaum MR, Zangerl AR 1998. Chemical phenotype matching between a plant and its insect herbivore. Proc Natl Acad Sci USA 95:13743-13748

Calcagno MP, Coll J, Lloria J, Faini F, Alonso-Amelot ME. 2002. Evaluation of synergism in the feeding deterrence of some furanocoumarins on *Spodoptera littoralis*. J Chem Ecol 28:175-191

Chapman RA, Miller DJ. 1974. The effects of caffeine on the contraction of the frog heart. J Physiol 242:589-613

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011. The relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance in F(2) hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. J Chem Ecol 37:1071-1080

Diawara M, Trumble J, White K, Carson W, Martinez L. 1993. Toxicity of linear furanocoumarins to *Spodoptera exigua*: evidence for antagonistic interactions. J Chem Ecol 19:2473-2484

Domínguez DM, Reina M, Santos-Guerra A, Santana O, Agulló T, et al. 2008. Pyrrolizidine alkaloids from Canarian endemic plants and their biological effects. Biochem Syst Ecol 36:153-166

Dreyer DL, Jones KC, Molyneux RJ. 1985. Feeding deterrency of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrthosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. J Chem Ecol 11:1045-1051

Dyer LA, Dodson CD, Stireman JO, Tobler MA, Smilanich AM, et al. 2003. Synergistic effects of three Piper amides on generalist and specialist herbivores. J Chem Ecol 29:2499-2514

Fraenkel GS. 1959. The raison d'être of secondary plant substances. Science 129:1466-1470

Gee JM, Wortley GM, Johnson IT, Price KR, Rutten AA, et al. 1996. Effects of saponins and glycoalkaloids on the permeability and viability of mammalian intestinal cells and on the integrity of tissue preparations in vitro. Toxicol In Vitro 10:117-128

Gonzalez-Coloma A, Valencia F, Martín N, Hoffmann JJ, Hutter L, et al. 2002. Silphinene sesquiterpenes as model insect antifeedants. J Chem Ecol 28:117-129

Hartmann T, Dierich B. 1998. Chemical diversity, variation of pyrrolizidine alkaloids of the senecionine type, biological need or coincidence? Planta 206:443-451

Hartmann T, Ehmke A, Eilert U, von Borstel K, Theuring C. 1989. Sites of synthesis, translocation and accumulation of pyrrolizidine alkaloid N-oxides in *Senecio vulgaris*. Planta 177:98-107

Hartmann T. 1996. Diversity and variability of plant secondary metabolism: a mechanistic view. Entomol Exp Appl 80:177-188

Herrera AA, Amor EC. 2011. Antiangiogenic activity of extracts and fractions from an endemic plant *Ardisia pyramidalis* (Cav.) Pers. From Bataan, Philippines using duck in ovo chorioallantoic membrane assay. J Med Plants Res 13:2637-2646

Herrmann F, Wink M. 2011. Synergistic interactions of saponins and monoterpenes in HeLa cells, Cos7 cells and in erythrocytes. Phytomedicine 18:1191-1196

Horman I, Viani R. 1972. The nature and complexation of the caffeine–chlorogenate complex of coffee. J Food Sci 37:925-927

Inui T, Wang Y, Pro SM, Franzblau SG, Pauli GF. 2012. Unbiased evaluation of bioactive secondary metabolites in complex matrices. Fitoterapia 83:1218-1225

Joosten L, Cheng D, Mulder PPJ, Vrieling K, van Veen JA, et al. 2011. The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. Phytochemistry 72:214-222

Kliebenstein DJ. 2004. Secondary metabolites and plant/environment interactions: a view through Arabidopsis thaliana tinged glasses. Plant Cell Environ 27:675-684

Kuhlisch C, Pohnert G. 2015. Metabolomics in chemical ecology. Nat Prod Rep 32:937-955

Labuschagne A, Hussein AA, Rodriguez B, Lall N. 2012. Synergistic antimycobacterial actions of *Knowltonia vesicatoria* (L.f) Sims. Evid Based Complement Alternat Med 2012:1-9

Leiss KA, Choi YH, Abdel-Farid I, Verpoorte R, Klinkhamer PGL. 2009. NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. J Chem Ecol 35:219-229

Lindigkeit R, Biller A, Buch M, Schiebel HM, Boppre M, et al. 1997. The two faces of pyrrolizidine alkaloids: the role of tertiary amine and its N-oxide in chemical defense of insects with acquired plant alkaloids. Eur J Biochem 245:626-636

Macel M, Bruinsma M, Dijkstra SM, Ooijendijk T, Niemeyer HM, et al. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. J Chem Ecol 31:1493-1508

Mithofer A, Boland W. 2012. Plant defense against herbivores: chemical aspects. Annu Rev Plant Biol 63:431-450

Mösli Waldhauer SS, Baumann TW. 1996. Compartmentation of caffeine and related purine alkaloids depends exclusively on the physical chemistry of their vacuolar complex formation with chlorogenic acids. Phytochemistry 42:985-996

Moussaieff A, Rogachev I, Brodsky L, Malitsky S, Toal TW, et al. 2013. High-resolution metabolic mapping of cell types in plant roots. Proc Natl Acad Sci USA 3:E1232-E1241

Nelson AC, Kursar TA. 1999. Interactions among plant defense compounds: a method for analysis. Chemoecology 9:81-92

Nenaah GE. 2011. Toxic and antifeedant activities of potato glycoalkaloids against *Trogoderma granarium* (Coleoptera: Dermestidae). J Stored Prod Res 47:185-190

Nuringtyas TR, Verpoorte R, Klinkhamer PGL, van Oers MM, Leiss KA. 2014. Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* using insect cell lines and injection bioassays. J Chem Ecol 40:609-616

Nuringtyas TR. 2014. Pyrrolizidine alkaloid variation in *Jacobaea* plants: from plant organ to cell level. PhD thesis, Leiden University, The Netherlands

Pelser PB, de Vos H, Theuring C, Beuerle T, Vrieling K, et al. 2005. Frequent gain and loss of pyrrolizidine alkaloids in the evolution of *Senecio* section *Jacobaea* (*Asteraceae*). Phytochemistry 66:1285-1295

Raasch W, Johren O, Schwartz S, Gieselberg A, Dominiak P. 2004. Combined blockade of AT1-receptors and ACE synergistically potentiates antihypertensive effects in SHR. J Hypertens 22: 611-618

Richards LA, Dyer LA, Smilanich AM, Dodson CD. 2010. Synergistic effects of amides from two Piper species on generalist and specialist herbivores. J Chem Ecol 36:1105-1113

Richards LA, Lampert EC, Bowers MD, Dodson CD, Smilanich AM, et al. 2012. Synergistic effects of iridoid glycosides on the survival, development and immune response of a specialist caterpillar, *Junonia coenia* (Nymphalidae). J Chem Ecol 38:1276-1284

Ryabushkina NA. 2005. Synergism of metabolite action in plant responses to stresses. Russ J Plant Physiol 52:547-552

Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L. 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med 8:1-10

Scott IM, Puniani E, Durst T, Phelps D, Merali S, et al. 2002. Insecticidal activity of Piper tuberculatum *Jacq.* extracts: synergistic interaction of piperamides. Agr Forest Entomol 4:137-144

Siciliano T, Leo MD, Bader A, Tommasi ND, Vrieling K, et al. 2005. Pyrrolizidine alkaloids from *Anchusa strigosa* and their antifeedant activity. Phytochemistry 66:1593-600

Smith DB, Roddick JG, Jones LJ. 2001. Synergism between the potato glycoalkaloids α-chaconine and α-solanine in inhibition of snail feeding. Phytochemistry 57:229-234

Sondheimer E, Covitz F, Marquisee MJ. 1961. Association of naturally occurring compounds, the chlorogenic acid-caffeine complex. Arch Biochem Biophys 93:63-71

Speiser B, Harmatha J, Rowell-Rahier M. 1992. Effects of pyrrolizidine alkaloids and sesquiterpenes on snail feeding. Oecologia 92:257-265

Stamp NE, Yang Y. 1996. Response of insect herbivores to multiple allelochemicals under different thermal regimes. Ecology 77:1088-1102

Stermitz FR, Lorenz P, Tamara JN, Zenewicz LA, Lewis K. 2000. Synergy in the medicinal plant: antimicrobial action of berberine potentiated by 5'-Methoxyhydrocarpin, a multidrug pump inhibitor. Proc Natl Acad Sci USA 97:1433-1437

Trethewey R. 2004. Metabolite profiling as an aid to metabolic engineering. Curr Opin Plant Biol 7:196-201

van Dam NM, Vuister LWN, Bergshoeff C, de Vos H, van der Meijden E. 1995. The ‘‘raison d'être’’ of pyrrolizidine alkaloids in *Cynoglossum officinale*: deterrent effects against generalist herbivores. J Chem Ecol 21:507-523

Wiedenfeld H. 2011. Plants containing pyrrolizidine alkaloids: toxicity and problems. Food Addit Contam 28:282-292

Williamson EM. 2001. Synergy and other interactions in phytomedicine. Phytomedicine 8:400-409

Yang J, Liu RH. 2009. Synergistic effect of apple extracts and quercetin 3-β-d-glucoside combination on antiproliferative activity in MCF-7 human breast cancer cells in vitro. J Agric Food Chem 57:8581-8586

Yoon M, Campbell JL, Andersen ME, Clewell HJ. 2012. Quantitative in vitro to in vivo extrapolation of cell based toxicity assay results. Crit Rev Toxicol 42:633-652

Samenvatting en discussie

Planten hebben verschillende afweer mechanismen ontwikkeld om te reageren op prikkels uit hun natuurlijke omgeving. Dit heeft geresulteerd in een groot scala van chemische inhoudsstoffen (Fraenkel 1959; Hartmann 1996; Kliebenstein 2004; Mithofer and Boland 2012). De gevolgen van het voorkomen van veel verschillende metabolieten in een plant is dat er een grote kans op interacties bestaat tussen deze metabolieten. Het samen voorkomen van plant metabolieten creëert een natuurlijke chemische achtergrond waar deze metabolieten in functioneren. Dit laatste wordt vaak over het hoofd gezien in ecologische studies.

Het hoofddoel van dit proefschrift is om het belang van de interacties tussen plant metabolieten in de context van plant-insect herbivoor interacties te begrijpen. Hiervoor heb ik *Jacobaea spp.*, en het daarin voorkomende chlorogeen zuur (CGA) en pyrrolizidine alkaloiden (PA's) als studie object gebruikt. In deze samenvatting van mijn proefschrift bespreek ik als eerste de bioactiviteit van individuele PA's. Als tweede zal ik de interacties tussen plant metabolieten bespreken in relatie tot herbivoor resistentie. Als derde zal ik kort de aanpak waarmee en het kader waarin ik dit onderzocht heb bespreken. Als laatste zal ik de hoofdconclusies van dit proefschrift samenvatten, en bespreken welke vooruitzichten zij bieden voor verder onderzoek.

1 Bioactiviteit en individuele metabolieten

In hoofdstuk 2 vind ik duidelijke negatieve effecten van PA's van *J. vulgaris* op trips overleving. Dit laat de beschermende functie van PA's tegen insect herbivoren zien. Het negatieve effect op trips ondersteunt eerdere bevindingen in een correlatieve studie met hele planten die liet zien dat de totale PA concentratie negatief gecorreleerd was met resistentie tegen verschillende herbivoren (Cheng et al. 2011), en meer specifiek dat met name de jacobine-achtige PA's negatief gecorreleerd waren met trips schade (Leiss et al. 2009; Cheng et al. 2011).

In hoofdstuk 2 laat ik ook zien dat metabolieten met een vergelijkbare chemische structuur verschillen in de sterkte van hun effect op trips. Als eerste blijken de PA's met een vrije base meer toxisch in vergelijking met hun corresponderende N-oxides. Dit is consistent met eerdere studies in erwtenbladluis (*Acyrthosiphon pisum*) (Dreyer et al. 1985), de slak *Arianta arbustorum* (Helicidae) (Speiser et al. 1992), de rups *Spodoptera exigua* (van Dam et al. 1995), de sprinkhaan *Locusta migratoria* (Macel et al. 2005), en met resultaten met *S. exigua* cel lijnen en rupsen (Nuringtyas et al. 2014). Het verschil tussen de twee vormen van PA's en hun effect op insect herbivoren is mogelijk gerelateerd aan cel permeabiliteit. In het algemeen passeren hydrofobe of lipofiele componenten de cel membraan makkelijker omdat het membraan uit fosfolipiden bestaat. De meeste vrije base PA's zijn lipofiel en

kunnen daardoor makkelijk een bio-membraan passeren, terwijl N-oxides polair en hydrofiel zijn (Lindigkeit et al. 1997). Daarnaast hebben ik gevonden dat de vrije base PA's jacobine en erucifoline de meest actieve PA's tegen trips waren, terwijl seneciphylline en senecionine het minst actief waren. Eerdere studies met individuele PA's lieten zien dat deze verschilden in hun effecten op *Spodopteria littoralis* (Gonzalez-Coloma et al. 2002), trips (Macel et al. 2005) en *S. exigua* cel lijnen (Nuringtyas et al. 2014). Daarentegen vonden Dominiguez et al. (2008) geen significante verschillen tussen de effecten van de geteste PA's op *Myzus persicae* en *Leptinotarsa decemlineata*. Fysiologische processen in de herbivoor kunnen de verschillende PA's anders beïnvloeden. De larven van *S. littoralis* kunnen bijvoorbeeld de toxiciteit van senecionine omzeilen door deze stof snel en efficiënt uit te scheiden (Lindigkeit et al. 1997). Er is echter nog weinig kennis over de toxiciteit van PAs in herbivoren. De metabolieten senecionine en seneciphylline staan aan de basis van de biosynthetische route van PA's. Retrorsine, jacobine en erucifoline worden verderop in de route gesynthetiseerd. Senecionine N-oxide is het eerste PA in de biosynthese waarvan de andere PA's zijn afgeleid (Hartmann en Dierich 1998; Pelser et al. 2005). Senecionine N-oxide en seneciphylline N-oxide komen beide voor in alle *Senecio* soorten. Van deze PA's worden in een of twee enzymatische stappen de andere PA's gesynthetiseerd, waaronder retrorsine N-oxide, jacobine N-oxide en erucifoline N-oxide (Figuur 1 in hoofdstuk 2 en 3). In hoofdstuk 2 vergelijk ik de effecten op trips van de vrije base PA's verderop in de biosynthese route met die van de vrij base PA's aan het begin van de biosynthese route. Hoewel er een trend was te zien dat de afgeleide PA's meer actief waren tegen trips dan senecionine en seneciphylline, vond ik geen echt significant verschil. Resultaten van experimenten met pure PA's kwamen niet altijd overeen met de resultaten van correlatieve studies met hele planten. Voor metabolieten die effectief zijn bij lage concentraties zijn correlatieve studies niet altijd gevoelig genoeg om bio-activiteit aan te tonen. Ik heb bijvoorbeeld gevonden dat retrorsine effect heeft op trips overleving in bioassays, terwijl er geen significante correlatie gevonden werd met trips schade op *Jacobaea* planten (Cheng et al. 2011). Jacobine N-oxide is positief gecorreleerd met trips schade in bioassays met hele planten (Cheng et al. 2011), terwijl er geen sterke effecten op trips waren in experimenten met zuivere metabolieten die werden toegevoegd aan een artificieel dieet (hoofdstuk 2). Een nadeel van correlative studies is dat een correlatie niet altijd een oorzakelijk verband aangeeft. De correlatie tussen trips schade en de concentratiie van jacobine N-oxide die gevonden is door Cheng et al. (2011) kan het gevolg zijn van een correlatie tussen jacobine N-oxide en een of meer andere PA's. Een andere mogelijke verklaring is dat jacobine N-oxide geactiveerd wordt in aanwezigheid van andere metabolieten. Dit benadrukt het belang van onderzoek naar de interacties tussen plant metabolieten.

2 Interactie tussen plant metabolieten

Het metaboloom van de plant vormt de natuurlijke chemische achtergrond voor individuele metabolieten. Doordat er zeer veel verschillende metabolieten in een plant voorkomen bestaat er een grote kans op interactie tussen de metabolieten. Planten kunnen die interacties beïnvloeden door opslag van metabolieten in verschillende organen, weefsels, cellen en cel compartimenten. Compartimentering kan gebruikt worden om metabolieten die antagonistisch interacteren van elkaar te scheiden. Planten kunnen profiteren van synergistische interacties doordat deze de bioactiviteit doen toenemen tegen lagere kosten (Berenbaum en Zangerl 1998; Nelson en Kursar 1999). Individuele metabolieten kunnen een andere activiteit vertonen dan wanneer zij interacteren met andere metabolieten. Daarom geven potentiele interacties mogelijk nieuwe inzichten in de bio-activiteit van individuele metabolieten die tot nu toe over het hoofd gezien zijn tijdens het testen van de zuivere stoffen. Het is bekend dat de concentratie van metabolieten sterk kan verschillen tussen plant organen (Kuhlisch en Pohnert 2015) en zelfs tussen verschillende cel lagen (Nuringtyas et al. 2014; Moussaieff et al. 2013). Het belang van de interacties tussen plant metabolieten in de context van plant-insect herbivoor interacties en de invloed van die interacties op bio-activiteit van individuele metabolieten zal hieronder beschreven worden. Ondanks dat interacties tussen plant metabolieten belangrijk zijn, is er nog weinig onderzoek gedaan naar deze interacties en de effecten die ze hebben in een ecologische context (zie enkele referenties hieronder). Dit kan mogelijk verklaard worden door de complexiteit van de interacties, en de moeilijkheid deze te detecteren en analyseren op een juiste manier (Nelson en Kursar 1999).

De studie van interacties tussen plant metabolieten vormt een grote uitdaging door het schier oneindige aantal mogelijke combinaties. Het onderzoek naar de interacties wordt nog complexer doordat een groot aantal metabolieten nog niet bekend of geïdentificeerd is (Trethewey 2004). In dit proefschrift, met inachtneming van de mate van complexiteit van de chemische diversiteit, heb ik de effecten van de interacties tussen plant metabolieten op insect herbivoren bestudeerd op drie niveaus, namelijk 1) interacties tussen metabolieten uit een zelfde chemische klasse, 2) interacties tussen metabolieten uit verschillende chemische klassen en 3) interacties die plaatsvinden in combinatie met de natuurlijke chemische achtergrond van primaire metabolieten (PM's) en verschillende klassen van secundaire metabolieten (SM's).

2.1 Onderbouwing voor onderzoek aan interactie tussen metabolieten

Een mogelijke aanpak is om te starten met combinaties van metabolieten waarvan bekend is dat ze potentieel interacteren. Een voorbeeld hiervan wordt gevormd door saponinen in combinatie met metabolieten die moeilijk membranen passeren (Gee et al. 1996; Herrmann en Wink 2011). Ik heb de interacties in bioactiviteit onderzocht van PA's en CGA. We hebben deze combinatie gekozen op basis van kennis over deze twee metabolieten. Nuringtyas et al. (2014) beschrijven dat het mesofyl van *J. vulgaris* grote hoeveelheden

PA's bevat, terwijl CGA ophoopt in de epidermis. CGA vormt een π-moleculair complex met cafeïne (een purine alkaloid) (Mösli, Waldhauser en Baumann 1995). Een eerdere studie in *S. exigua* cel lijnen liet een antagonistische interactie zien tussen jacobine en CGA (Nuringtyas, PhD thesis, 2014). Dit verklaart mogelijk waarom PA's en CGA verdeeld zijn over verschillende cel lagen. Dit is van belang voor insecten die specifieke cellen eten. Voor knagende insecten zoals *S. exigua* zal verspreiding van PA's en CGA over verschillende cel lagen een antagonistisch effect niet voorkomen. Daarom heb ik de interactie tussen PA's en CGA bestudeerd in relatie met trips, een herbivoor die individuele cellen leeg zuigt. Ook bestuurde ik de effecten van de meest voorkomende PA N-oxides in *J. vulgaris* op Californische trips (*Frankliniella occidentalis*), een generalistische herbivoor. Ik wilde onderzoeken of het veel voorkomen van PA N-oxides in planten, ondanks de relatief lage activiteit tegen herbivoren, verklaard kan worden door interacties tussen deze PA's.

In de gevallen waar geen voorkennis over metabolieten beschikbaar is, kan een 'top-down' benadering worden gebruikt: van metaboloom analyse naar component-interactie analyse. In hoofdstukken 5 en 6 heb ik de eerste stappen gezet voor een 'top-down' benadering om de interacties tussen metabolieten te bestuderen met twee soorten bioactiviteit, namelijk de effecten op trips zoals gemeten met een *in vitro* bio-assay (hoofdstuk 5) en mutageniteit zoals bepaald met de Ames test (hoofdstuk 6). Ik heb de bio-activiteit van individuele PA's bestudeerd wanneer deze waren toegevoegd aan hun natuurlijke chemische achtergrond (fracties van plant extracten). Deze eerste stap heeft een algemene indruk gegeven van het belang van interacties tussen metabolieten vanuit het perspectief van het metaboloom. Wanneer de bio-activiteit van een metaboliet vergroot wordt als het wordt toegevoegd aan een planten extract of een fractie daarvan, geeft dit een indicatie van het belang van interacties tussen de verschillende plant metabolieten voor die activiteit. Verdere sub-fractionering en recombineren van sub-fracties kan het aantal mogelijke kandidaten betrokken bij deze interacties reduceren.

Ik beschrijf in mijn proefschrift een methode om de sterkte van interacties tussen twee of meer componenten te berekenen. De ontwikkelde methode maakt het mogelijk om het patroon en de grootte van de interacties statistisch te toetsen. Voor de trips overleving heb ik een multiplicatief nulmodel geformuleerd. Dit kan gebruikt worden om de verwachte overleving te berekenen onder de aanname dat er geen interacties plaatsvinden. De gevonden overleving kan dan getoetst worden tegen de verwachting waarbij een hogere overleving dan verwacht duidt op antagonistische interacties en een lagere overleving op synergistische interacties (hoofdstuk 5). Voor de mutageniteit zoals gemeten met de Ames test is een additioneel model toegepast, omdat de kans op een terug mutatie erg klein is in vergelijking met het initiële aantal cellen (hoofdstuk 6). Een dergelijk kader staat niet alleen het testen van het bestaan van interactie effecten toe, maar ook van de verschillen in grootte.

2.2 Het effect van interacties tussen plant metabolieten en hun ecologisch belang

Interactie effecten van metabolieten binnen klassen van planten stoffen

Voorbeelden van interacties tussen metabolieten binnen een chemische klasse in plant-insect herbivoor associaties zijn de synergistische effecten van twee aardappel glycoalkaloïden op de slak *Helix aspersa* (Smith et al. 2001) en op de Khapra kever *Trogoderma granrium* (Nenaah 2011), het antagonistische effect van twee furanocoumarinen op *S. littoralis* (Diawara et al. 1993; Calcagno et al. 2002), het synergistische effect van piper amides op de mug *Aedes atropalpus* (Scott et al. 2002) en op *S. frugiperda* rupsen (Dyer et al. 2003; Richards et al. 2010) en het synergistische effect van twee iridoïde glycosiden op een specialistische rups *Junonia coenia* (Richards et al. 2012). Er zijn slechts enkele studies uitgevoerd naar interacties tussen PA's. Macel (2005) heeft een zwak synergistisch effect gevonden van een combinatie van vrije basen van senecionine, seneciphylline en senkirkine op *S. exigua* en van een mix van vrije basen van senecionine en seneciphylline op *L. migratoria*. Daarentegen heeft Siciliano et al. (2005) geen synergistisch effect gevonden van twee PA's van *Anchusa strigosa* op *S. exigua*. Beide studies hebben alleen interacties tussen vrije basen bestudeerd. Echter PA's komen in de plant voornamelijk voor als PA N-oxides en daarom is het ecologische relevanter om deze interacties te bestuderen. In hoofdstuk 2 zijn de meest voorkomende PA N-oxides van *J. vulgaris* gecombineerd, namelijk senecionine N-oxide, jacobine N-oxide en erucifoline N-oxide (Joosten et al. 2011). Tussen deze N-oxiden vonden we geen synergistisch (of antagonistisch) effect op trips overleving. Deze resultaten geven daarom geen verklaring waarom PA N-oxides de meest voorkomende vorm van PA's zijn in de plant. Ook geeft deze studie geen verklaring voor waarom er zo veel verschillende PA N-oxides aanwezig zijn in een enkele plant.

Interacties tussen metabolieten van verschillende chemische klassen

Antagonistische interacties tussen vrije base PA's en CGA.

In hoofdstuk 3 beschrijf ik antagonistische interacties tussen vrije base PA's en CGA van alle vijf geteste PA's in de effecten op trips. Vanuit de plant gezien vormen antagonistische interacties geen fitness voordeel. Daarom zijn deze interacties vanuit een ecologisch perspectief moeilijk te verklaren. Dit, en de eerdere bevinding dat PA's en CGA verdeeld zijn over verschillende cel lagen van *Jacobaea* bladeren (Nuringtyas et al. 2014) zou kunnen betekenen dat antagonistische effecten van vrije base PA's en CGA op trips een negatief gevolg zijn van de accumulatie van verschillende metabolieten in de plant (Nelson en Kursar 1999). Voor cafeïne en CGA is *in situ* beschreven dat ze een complex vormen met een ratio van 1:1 (Sondheimer et al. 1961; Horman en Viani, 1972; Chapman en Miller 1974). Als soortgelijke complexen gevormd worden tussen vrije base PA's en CGA dan is

te verwachten dat antagonistische interacties afhangen van de ratio van twee componenten. Ik heb inderdaad gevonden dat het effect van antagonistische interacties tussen vrije base PA's en CGA afhangt van de ratio. Er was echter te veel variatie in de metingen om de ratio die het sterkste antagonistische effect liet zien nauwkeurig genoeg te kunnen bepalen.

Het feit dat alle geteste vrije base PA's interacteerden met CGA duidt erop dat het necine deel van het PA molecuul betrokken is bij de interactie. Alle geteste vrije base PA's hebben een overeenkomende retronecine base, in tegenstelling tot de macrocyclische ring die verschillend van structuur is. Het kinazuur (quinic acid) deel van het CGA molecuul interacteerde antagonistisch met retrorsine, terwijl het caffeoyl deel dit niet doet. In hoofdstuk 3 heb ik de eerste stappen gezet tot modificatie van de functionele groepen van het PA molecuul, door additie of eliminatie van specifieke groepen of door wijzigingen van het substitutiepatroon, die gebruikt kunnen worden om de bindingsmechanismen verder te onderzoeken vanuit een chemisch perspectief.

Synergistische interacties tussen PA N-oxides en CGA op trips

Zoals eerder vermeld komen PA's in planten voornamelijk voor als N-oxides (Hartmann et al. 1989). PA N-oxides waren minder actief dan de corresponderende vrije basen wanneer deze individueel werden getest op trips overleving (hoofdstuk 2). Daarom is het van ecologisch belang om interacties tussen PA N-oxides en andere SM's zoals CGA op trips overleving te bestuderen. In hoofdstuk 4 vond ik synergistische interacties tussen PA N-oxides en CGA op trips overleving. Hoewel de sterkte van de interacties varieerde, was het patroon voor alle geteste PA N-oxides hetzelfde. Vervolgens heb ik de interactie effecten van de combinaties van vrije base PA's en CGA vergeleken met de combinaties van overeenkomende PA N-oxides en CGA op trips overleving. Verrassend genoeg vond ik dat welke vorm van de PAs het meest effectief was tegen trips afhing van de concentratie van CGA. Het CGA molecuul verminderde de bio-activiteit van de vrije base PA's en vergrootte de bio-activiteit van PA N-oxides tegen trips. Deze bevindingen geven een alternatieve verklaring voor waarom PA N-oxides in grotere hoeveelheden voorkomen in planten dan vrije base PA's. Deze verklaring wordt echter niet ondersteund door de resultaten van hoofdstuk 5 waarin ik synergistische interacties op trips overleving heb gevonden tussen zowel retrorsine als retrorsine N-oxide en twee fracties van een methanol extract van *Jacobaea* planten. In het geval van PA N-oxides geeft het vinden van synergistische effecten een nieuw inzicht in de afweer functies van PA N-oxides. Ook hier geven de resultaten een ecologisch gedreven alternatief voor het in grote hoeveelheden opslaan van PA N-oxides in planten, als aanvulling op de fysisch-chemische verklaring dat N-oxides beter oplosbaar zijn in water en daarom makkelijker opgeslagen kunnen worden in de vacuole of getransporteerd kunnen worden door de plant (von Borstel et al. 1986).

Er wordt verondersteld dat synergistische interacties van ecologisch en evolutionair belang zijn in de relatie tussen planten en hun omgeving. Resultaten van hoofdstuk 4 en andere studies suggereren dat de bioactiviteit van individuele SM's versterkt kan worden in samenwerking met andere SM's. Rutine bijvoorbeeld heeft op zichzelf geen negatief effect op de groei van de rups *S. exigua*, terwijl CGA een licht negatief effect heeft, maar samen hebben ze een sterk negatief effect (Stamp en Yang 1996). Synergistische interacties kunnen voordelig zijn voor de verdediging van de plant doordat ze tot een hogere toxiciteit leiden tegen lagere kosten (Nelson en Kursar 1999; Ryabushkina 2005). Op basis daarvan is het te verwachten dat natuurlijke selectie er toe leidt dat synergistische interacties tussen metabolieten in plant-herbivoor interacties vaker voorkomen dan antagonistische.

Interacties tussen plant metabolieten met hun natuurlijke chemische achtergrond

In de eerste hoofdstukken bestudeerde ik de interacties tussen bekende plant metabolieten door ze te combineren, volgens een 'bottom-up' benadering. In de overige hoofdstukken heb ik een 'top-down' benadering gebruikt om interacties tussen plant metabolieten in hun natuurlijke chemische achtergrond te onderzoeken. In hoofdstuk 5 heb ik de effecten op trips overleving bestudeerd. In hoofdstuk 6 heb ik de mate van mutageniteit gebruikt. De laatste heb ik bepaald in de Ames test. Fracties van een methanol extract van *J. vulgaris* blad verschilden in bio-activiteit in de Ames test. Vooral de chloroform fractie vertoonde een hogere mutageniteit. Na (sub-) fractionering konden we geen bio-activiteit meer meten. Door de fracties opnieuw samen te voegen werd de activiteit weer hersteld. Dit wijst er op dat door (sub-) fractionering de synergistische interacties tussen de metabolieten werden verloren. Ook in de experimenten met trips gaf fractionering een verlies van bio-activiteit. De fracties van *J. vulgaris* planten verschilden in hun mutageniteit en hun anti-herbivoor effect. We vonden geen correlatie tussen de bio-activiteit van de fracties en hun alkaloide gehaltes. Dit duidt er op dat naast alkaloiden andere metabolieten een grote rol spelen in de bio-activiteit van de geteste fracties. Hoewel het buiten de scope van dit proefschrift valt, zou verdere sub-fractionering en identificatie het mogelijke aantal metabolieten betrokken bij deze bio-activiteit verder kunnen reduceren.

In hoofdstuk 5 en 6 heb ik de eerste stappen gezet van een 'top-down' benadering door individuele PA's toe te voegen aan verschillende fracties om zo de interacties tussen plant metabolieten op metaboloom niveau te bestuderen, en tegelijkertijd te bepalen hoe de natuurlijke chemische achtergrond de bio-activiteit van de individuele metabolieten beïnvloedt. In hoofdstuk 5 interacteren zowel retrorsine als retrorsine N-oxide synergistisch met de chloroform en *n*-butanol fracties in hun effect op trips overleving. In hoofdstuk 6 beschrijf ik hoe de chloroform en ethyl acetaat fracties van *J. vulgaris* spruiten de mutageniteit van retrorsine liet toenemen, terwijl hexaan, *n*-butanol en water fracties de mutageniteit van retrorsine lieten afnemen. Gezien de PA hoeveelheid in deze fracties lijkt het er op dat de interacties tussen PA's en andere SM's het uiteindelijke effect van de

interacties tussen PA's en de fracties domineren. Samengenomen wijzen de resultaten van hoofdstuk 5 en 6 erop dat de natuurlijke chemische achtergrond een sterke invloed heeft op de bio-activiteit van individuele SM's in zowel positieve als negatieve zin.

Het belang van de natuurlijke achtergrond

Plant extracten en fracties kunnen effectief gebruikt worden om interacties tussen plant metabolieten te meten in een natuurlijke achtergrond, zonder dat er voorkennis over de betrokken metabolieten nodig is. Het kan helpen de bio-activiteit van individuele plant metabolieten in een natuurlijke achtergrond te bestuderen om plant-insect herbivoor interacties beter te begrijpen. Tot nu toe er is nog slechts anekdotisch bewijs voor het belang van de natuurlijk achtergrond in een ecologische context. Dit proefschrift heeft hiervoor een belangrijke eerste stap gezet.

Dit proefschrift ondersteunt de algemene opvatting dat chemische afweer van een plant afhangt van de variatie in aanwezige metabolieten, die samen vorm geven aan de effectiviteit van de afweer. Interacties tussen plant metabolieten kunnen nieuwe of aanvullende informatie geven over de bioactiviteit van individuele SM's. Interactie effecten van plant metabolieten kunnen ook verklaren waarom sommige SM's activiteit vertonen in bepaalde plant soorten terwijl ze dit niet doen in andere. Bijvoorbeeld CGA in *chrysanthemum* is negatief gecorreleerd met voedingsschade door trips *Frankliniella occidentalis* (Leiss et al. 2009), terwijl geen effect van CGA op deze trips is gemeten in tomaat *Solanum lycopersicum* (Mirnezhad 2011). Kennis over interacties van metabolieten kan ook toegepast worden op multi-component materialen. Bijvoorbeeld de anti-proliferatieve activiteit van quercetin 3-β-D-glucoside tegen borstkankercellen in mensen wordt verviervoudigd in combinatie met appel extracten (Yang en Liu 2009).

3 Hoofdconclusies en toekomst perspectieven

Verder onderzoek naar de interacties tussen plant metabolieten moet met een aantal punten rekening houden. Ten eerste is er een tekort aan kennis over de mechanismen die tot interacties leiden. Verder onderzoek naar fysisch-chemische en fysiologische processen zal waardevolle informatie opleveren. Vanuit een fysisch-chemisch perspectief kunnen metabolieten de oplosbaarheid en/of de resorptiegraad van andere metabolieten en daarmee de biologische beschikbaarheid er van beïnvloeden (Amin et al. 2015). Ook fysiologische processen, zoals de afbraakstofwisseling of de activiteit van enzymen die betrokken zijn bij de detoxificatie in het insect kunnen bepalend zijn voor interactieve effecten, (Stermitz et al. 2000; Scott et al. 2002). Ten tweede moet het belang van antagonistische effecten in acht genomen worden. Als derde kan compartimentering van plant metabolieten tussen verschillende cel organellen, cellen, cel lagen en andere organen bijdragen aan plant afweer door het stimuleren van synergistische of het voorkomen van antagonistische interacties.

Daarom is het belangrijk om in eerste instantie chemische analyses van SM's in deze compartimenten uit te voeren, om te identificeren welke metabolieten tegelijk voorkomen en welke niet. Ten slotte zijn er weinig voorbeelden van interactieve effecten van plant metabolieten in de context van plant-herbivoor associaties. Dit is waarschijnlijk ook het geval voor andere ecologische relaties. Om plant-omgeving interacties mechanistisch en ecologisch te kunnen begrijpen is een gezamenlijke inspanning nodig van verschillende onderzoeksvelden. In dit proefschrift zijn de eerste stappen gezet voor zowel een 'bottom-up' als een 'top-down' benadering. Gezien het enorme aantal plant metabolieten is het een uitdaging om het grote aantal mogelijke combinaties die van belang zijn te onderzoeken. Deze taak is onmogelijk met de bio-assay's die ontwikkeld zijn voor dit proefschrift. Ontwikkeling van high-throughput screening methoden, zoals veel toegepast in farmacologische studies, is daarvoor van essentieel belang. Bijvoorbeeld receptorgerichte benaderingen zijn op grote schaal toegepast in geneesmiddel ontwikkeling. Vanuit een mechanistisch perspectief kunnen de effecten van interacties tussen metabolieten veroorzaakt worden door blokkeren of verstoren van een membraan gebonden receptor functie. Ramipril bijvoorbeeld remt de angiotensine receptor waardoor de anti hoge bloeddruk werking van candesartan-cilexetil wordt gefaciliteerd (Raasch et al. 2004). Benaderingen gebaseerd op receptor activiteit zouden kunnen worden gebruikt als screening strategie. Een andere benadering is het gebruik van cel lijnen voor bio-activiteit screening. Hoewel cel lijnen voor trips momenteel nog niet beschikbaar zijn, is het wel mogelijk cel lijnen te gebruiken van andere herbivoren, zoals die voor de floridamot *S. exigua*. Dergelijke cel lijnen zijn eerder gebruikt in de onderzoeksgroep (Nuringtyas et al. 2014). De effecten van de interacties tussen vrije base PA's en CGA in *S. exigua* cel lijnen (Nuringtyas et al. 2014) kwamen overeen met die in trips larven (hoofdstuk 3). Bioassay's in cel lijnen zijn echter niet representatief voor metaboloom veranderingen die kunnen plaatsvinden door afbraak stofwisseling of opname (zie review van Yoon et al. 2012). Ondanks dit nadeel kunnen cel lijnen wel goed toegepast worden bij initiële screening en selectie van kandidaat metabolieten of combinaties van metabolieten, die vervolgens verder onderzocht kunnen worden in levende organismen.

Dit proefschrift maakt duidelijk dat de interacties tussen plant metabolieten belangrijk zijn bij plant-insect herbivoor relaties. Verschillende conclusies komen naar voren. Ten eerste dat PA's de trips overleving verlagen, hetgeen wijst op een rol van PA's bij de afweer tegen insecten. Sommige PA's zijn actiever dan andere. Ten tweede beïnvloeden combinaties van plant metabolieten trips overleving meer of minder dan op basis van de activiteit van de afzonderlijke metabolieten verwacht kan worden. Dit geeft aan dat interacties tussen plant metabolieten in acht genomen moeten worden als we het afweer systeem van planten bestuderen. Als derde spelen interacties van plant metabolieten in plant extracten en fracties daarvan een rol bij trips overleving en mutageniteit. Ik laat zien dat de bio-activiteit van individuele metabolieten sterk beïnvloedt wordt door hun natuurlijke biochemische

achtergrond, die de effectiviteit kan versterken of verzwakken. Daarom moet er rekening gehouden worden met de chemische achtergrond bij het ontwerpen van bio-assay's.

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Xiaojie 晓节

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Curriculum Vitae

Xiaojie Liu was born on October 26th, 1987, in Shanxi, China. She got her Bachelor degree in the Department of Pharmacy, School of Chemistry at Shanxi University in 2008. Her undergraduate research project was about assessing the quality of two species used as *Radix Bupleuri* and describing their metabolic fingerprinting, supervised by Prof. Xuemei Qin. She received a recommendation to be exempted from the admission exam to her Master study which she followed in the Modern Research Center for Traditional Chinese Medicine, Shanxi University from 2008 to 2011. During the M.Sc, she studied the anti-depressive effect of a Chinese herbal prescription, Xiaoyansan, with NMR based metabolomics and the quality assessment of herbal medicine under the supervision of Prof. Dr. Xuemei Qin. In 2011, she did research on uncovering the underlying mechanism of hepatitis B and the treatment effects of Chinese medicine by using metabolomics at the 302 Militry Hospital of China.

In 2012, she was supported by the Chinese Scholarship Council to conduct a Ph.D study at the section of Plant Ecology and Phytochemistry at the Institute of Biology Leiden (Leiden University, The Netherlands) under the supervision of Prof. Dr. Peter G. L. Klinkhamer, Dr. Klaas Vrieling and Dr. Patrick P. J. Mulder. Her doctorate dissertation consisted of three main projects, which were the effects of individual metabolites, the effects of the interactions between well-characterized metabolites and the influence of natural phytochemical backgrounds on the activity of individual metabolites. During her Ph.D study from 2012 to 2016, Xiaojie Liu together with her supervisors collaborated with RIKILT, Wageningen UR and EXPLANT, Leiden, where she acquired various research skills in mutagenic and phytochemical studies.

Publications

Bovee TF, Helsdingen RJ, Hoogenboom RL, de Nijs MW, **Liu X**, et al. 2015. Are effects of common ragwort in the Ames test caused bypyrrolizidine alkaloids? *Mutat Res* 778:1–10

Liu X, Zhou Y, Li Z, Cui J, Li Z, et al. 2012. Anti-depressant effects of Xiaoyaosan on rat model of chronic unpredictable mild stress: a plasma metabonomics study based on NMR spectroscopy. *J Pharm Pharmacol* 4:578-588

Liu X, Li Z, Gao X, Zhou Y, Sun H, et al. 2012. Urinary metabonomic study using a CUMS rat model of depression. *Magn Reson Chem* 3:187-192

Liu X, Hu J, Li Z, Qin X, Zhang L, et al. 2011. Species classification and quality assessment of Chaihu (*Radix Bupleuri*) based on high-performance liquid chromatographic fingerprint and combined hemometrics methods. *Arch Pharm Res* 6:961 -969

Zhou Y, Zheng X, **Liu X**, Li Z, Gao X, et al. 2012. Metabonomic analysis of urine from chronic unpredictable mild stress rats using gas chromatography-mass spectrometry. *Chromatographia* 3-4:157-164

Qin X, Dai Y, Liu NQ, Li Z, **Liu X**, et al. 2012. Metabolic fingerprinting by (HNMR)-H-1 for discrimination of the two species used as Radix Bupleuri. *Planta Med* 9:926-933