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The Chemical Ecology of Tansy

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This thesis is dedicated to my beloved parents,

Lidia and Eamon Clancy,

Without whom I would not be who I am today.

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Summary

The relationship between a plant and its environment is intimate and complex, with countless pieces of information flowing in all directions. Chemical ecology is the study of how chemical and biological processes shape the interactions between plants and their environment. The relationship between plants and herbivores is multi-layered and can extend over several trophic levels. Since plants are sessile, they have developed a constantly evolving reservoir of defence mechanisms. Plants are powerful chemical factories that can synthesise hundreds of thousands of compounds that are not directly related to primary metabolism. An important group of compounds are terpenoids, a structurally very diverse class ranging from small, highly volatile organic compounds (VOCs) such as mono- and sesquiterpenes to rubber, one of the largest natural macromolecules. Plant VOCs are often used for communication with insects and microbes/fungi, but they also serve as signal substances (infochemicals) within and between plants.

One of the main objectives of this work was to investigate whether the intraspecific differences in the individual chemical profiles of Tansy plants (*Tanacetum vulgare*) influence the selection of herbivores as host plants. Tansy is an aromatic herbaceous plant that is known to have a high variation in terpenoid content and a wide range of chemotypes have been observed regionally in its range of distribution. It also hosts a specialised aphid species, *Metopeurum fuscoviride*, an obligatory myrmecophile maintained by mutualistic ants.

Manuscript one describes a field study that shows the chemotypic variation of tansy on a small spatial scale (<1 km^2) and how tansy chemotypes are related to aphid colonisation. Volatile compounds stored in special leaf structures were extracted by liquid solvent extraction, while compounds that were volatile enough to be released from the leaves into the atmosphere were bound to an adsorption material. All subsequent analyses were performed by gas chromatography - mass spectroscopy (GC-MS). Compounds detected by both extraction techniques were grouped together as a group of compounds that are constitutively emitted by undamaged plants and thus form the volatile chemical profile that is "visible" to passing herbivores and herbivore enemies. Based on the patterns and frequency distribution of these compounds, the plants of the experimental area were grouped into four chemotype groups.

During the most important dispersal phase of *M. fuscoviride* (late May/early June), when winged aphids can move freely between plants to select a host, the terpenoid patterns of the "constitutively emitted" chemotypes influenced the host selection of the aphids. Even after this, unwinged aphids can still move between plants by walking, but it was found that the “volatile” chemotype of the plant no longer had any influence on the aphid colonisation rate towards the end of the growing season. The presence of ant species (*Lasius niger* and *Myrmica rubra*) correlated with aphid colonisation towards the end of the season, when some monoterpenes correlated strongly with the presence of ants.

In the second manuscript, the non-volatile chemical profiles of the same tansy plants were investigated using non-targeted ultra-high performance liquid chromatography Qq-time of flight mass spectrometry (UHPLC-MS). This study found that the tansy plants could be classified into five metabotypes (synonymous with metabolic phenotypes) based on the composition of their non-volatile metabolites, with the metabotypes not being associated with the volatile chemotypes. Each metabotype was identified within each volatile chemotype. Across all chemotypes, aphids were more likely to successfully colonise plants belonging to both metabotype classes C and E. Compounds associated with salicylic acid-mediated defence were found to be most abundant in the colonised metabotypes (C and E). Although the presence of such compounds indicates that systemically acquired resistance (SAR) was activated in plants that were at some time colonised by aphids, it was not possible to clarify whether this was due to a long-lasting induction by aphid infestation or whether these plants had constitutively higher levels of metabolites involved in defence.

Once an understanding has been gained of how the chemo- and metabotypes of tansy can influence the behaviour of a herbivore under field conditions, the third manuscript will describe in detail how the plant defence of tansy develops dynamically during an infestation with one herbivore and with a second herbivore at a later time. This manuscript describes the changes in VOC emissions and patterns of five tansy chemotypes during the infestation by a sucking (aphids; *M. fuscoviride*) and chewing herbivore (caterpillars; *Spodoptera littoralis*) using a specially designed cuvette system, which combined GC-MS and proton transfer reaction time of flight mass spectroscopy (PTR-ToF-MS) to determine VOC emissions. These measurements were completed by the RNA-seq analysis of the transcriptome of one chemotype at three points in time of the experiment, which demonstrated a priming effect of prior aphid feeding on subsequent herbivory. The investigations revealed that some tansy chemotypes with increased VOC emissions responded to feeding by *Spodoptera* caterpillars depending on whether they had been previously in contact with aphids.

In summary, this work provides an in-depth insight into the chemical ecology of tansy. It turns out that even on a small spatial scale, tansy plants show great differences in their metabolic composition. This variation in both volatile and non-volatile compounds is a factor that influences the selection of aphid hosts. It has also been shown that the chemotypes of tansy react differently to two types of herbivores, with some chemotypes being stronger VOC emitters and thus functionally "louder" than their conspecifics. The results presented in this work, as well as the transcriptome now described for this plant species for the first time, could serve as a support for future research to better understand how plant chemotypes affect and are affected by their local insect communities.

Zusammenfassung

Die Beziehung zwischen einer Pflanze und ihrer Umgebung ist intim und komplex, wobei unzählige Informationen in alle Richtungen fließen. Die Chemische Ökologie ist die Lehre davon, wie chemische und biologische Prozesse die Wechselwirkungen zwischen Pflanzen und ihrer Umgebung gestalten. Die Beziehung zwischen Pflanzen und Pflanzenfressern ist vielschichtig und kann sich über mehrere trophische Ebenen erstrecken. Da Pflanzen sessil sind, haben sie einen sich ständig weiterentwickelndes Reservoir an Abwehrmechanismen entwickelt. Pflanzen sind kraftvolle biochemische Fabriken, die Hunderttausende von Verbindungen synthetisieren können, die nicht direkt mit dem Primärstoffwechsel in Verbindung stehen. Eine wichtige Gruppe von Verbindungen sind die Terpenoide, eine strukturell sehr vielfältige Stoffklasse, die von kleinen, leicht flüchtigen organischen Verbindungen (VOC) wie Mono- und Sesquiterpenen bis hin zu Kautschuk, einer der größten natürlichen Verbindungen, reicht. Pflanzliche VOCs werden oft in der Kommunikation mit Insekten und Mikroben/Pilzen eingesetzt, sie dienen aber auch als Signalstoffe innerhalb und zwischen Pflanzen.

Eines der Hauptziele dieser Arbeit war es zu untersuchen, ob die intraspezifischen Unterschiede der einzelnen chemischen Profile von Tansy-Pflanzen (*Tanacetum vulgare*) die Auswahl von Pflanzenfressern als Wirtspflanzen beeinflussen. Tansy ist eine aromatische krautige Pflanze, von der bekannt ist, dass sie eine hohe Variation im Terpenoid-Gehalt aufweist und regional in ihrem Verbreitungsgebiet vielfältige Chemotypen beobachtet wurden. Sie beherbergt auch eine spezialisierte Blattlausart, *Metopeurum fuscoviride*, ein obligater Myrmecophile, der von mutualistischen Ameisen gepflegt wird.

In Manuskript eins wird eine Feldstudie beschrieben, die die chemotypische Variation des Rainfarn im kleinen räumlichen Maßstab (<1 km*2*) zeigt und wie diese Chemotypen mit der Besiedlung durch Blattläuse zusammenhängen. Flüchtige Verbindungen, die in speziellen Blattstrukturen gespeichert sind, wurden mit einer Lösungsmittelextraktion extrahiert, während Verbindungen, die flüchtig genug waren, um von den Blättern in die Atmosphäre abgegeben zu werden, mittels Absorption an ein Trägermaterial gebunden wurden. Alle anschließenden Analysen wurden mit Gaschromatographie - Massenspektroskopie (GC-MS) durchgeführt. Verbindungen die mit Hilfe beider Techniken erfasst wurden, wurden als eine Gruppe von Verbindungen zusammengefasst, die konstitutiv von unbeschädigten Pflanzen emittiert werden und somit das flüchtige chemische Profil bilden, das für vorbeiziehende Pflanzenfresser und Pflanzenfresser-Feinde "sichtbar" ist. Anhand der Muster und Häufigkeitsverteilung dieser Verbindungen wurden die Pflanzen der Versuchsfläche in vier Chemotypengruppen zusammenfasst.

Während der wichtigsten Ausbreitungsphase von M. fuscoviride (Ende Mai/Anfang Juni), bei dem sich geflügelte Blattläuse zwischen den Pflanzen frei bewegen können, um einen Wirt auszuwählen, beeinflussten die Muster der "konstitutiv emittierten" Chemotypen die Wirtsselektion der Blattläuse. Auch danach können sich ungeflügelte

Blattläuse noch durch Laufen zwischen den Pflanzen bewegen, es zeigte jedoch festgestellt, dass der flüchtige Chemotyp der Pflanze gegen Ende der Saison keinen Einfluss mehr auf die Besiedlungsrate der Blattläuse hatte. Das Vorhandensein von Ameisenarten (Lasius niger und Myrmica rubra) korrelierte mit der Blattlausbesiedlung gegen Ende der Saison, als einige Monoterpene stark mit dem Vorhandensein von Ameisen korrelierten.

Im zweiten Manuskript wurden die nichtflüchtigen chemischen Profile der gleichen Rainfarn-Pflanzen mit Hilfe der nicht gezielten Ultra-Hochleistungs-Flüssigkeitschromatographie Qq-Flugzeit-Massenspektrometrie (UHPLC-MS) untersucht. Diese Studie ergab, dass die Rainfarn-Pflanzen sich anhand ihrer Zusammensetzungen an nichtflüchtigen Verbindungen in fünf Metabotypen (synonym für metabolischen Phänotyp) klassifizieren ließen, wobei die Meabotypen nicht mit den flüchtigen Chemotypen assoziiert waren. Jeder Metabotyp wurde innerhalb jedes flüchtigen Chemotyps identifiziert. Über alle Chemotypen hinweg war es wahrscheinlicher, dass Blattläuse erfolgreich Pflanzen besiedelten, die den beiden Metabotypklassen C und E angehörten. Es wurde festgestellt, dass Verbindungen, die mit der durch Salicylsäure vermittelten Abwehr assoziiert sind, am stärksten in den kolonisierten Metabotypen (C und E) vorhanden sind. Obwohl das Vorhandensein solcher Verbindungen darauf hinweist, dass eine systemisch erworbene Resistenz in Pflanzen aktiviert wurde, die zu irgendeinem Zeitpunkt von Blattläusen besiedelt waren, lies sich nicht klären, ob dies auf eine lang anhaltende Induktion durch Blattlausbefall zurückzuführen war, oder ob diese Pflanzen ein konstitutiv höheres Niveau an, an der Abwehr beteiligten Metaboliten aufwiesen.

Nachdem ein Verständnis darüber gewonnen wurde, wie die Chemo- und Metabotypen des Rainfarns das Verhalten eines Pflanzenfressers unter Feldbedingungen beeinflussen können, soll im dritten Manuskript im Detail beschrieben werden, wie sich die pflanzliche Abwehr von Rainfarn dynamisch während eines Befalls mit einem Pflanzenfresser und zeitlich versetzt mit einem zweiten Herbivor entwickelt. Dieses Manuskript beschreibt die Änderungen der VOC Emissionen und -muster von fünf Rainfarn-Chemotypen während des Befalls durch einen saugenden (Blattläuse; *M. fuscoviride*) und kauenden Pflanzenfresser (Raupen; *Spodoptera littoralis*) unter Zuhilfenahme eines speziell entwickelten Küvettensystems, mit dem die VOC Emissionen über GC-MS und Protonentransfer-Reaktionszeit-Massenspektroskopie (PTR-ToF-MS) kombiniert bestimmt wurden. Vervollständigt wurden diese Messungen durch die Analyse des Transkriptoms eines Chemotyps an drei Zeitpunkten des Versuchs. Die Untersuchungen ergaben, dass einige Rainfarn Chemotypen mit erhöhten VOC-Emissionen auf den Befraß durch *Spodoptera* Raupen reagierten je nachdem, ob sie zuvor mit Blattläusen in Kontakt gekommen waren.

Zusammenfassend liefert diese Arbeit einen vertieften Blick in die chemische Ökologie des Rainfarns. Es zeigt sich, das Pflanzen des Rainfarns selbst auf einer kleinen räumlichen Skala sehr große Unterschiede in ihrer metabolischen Zusammensetzung

aufweisen. Diese Variation sowohl flüchtiger als auch nicht flüchtiger Verbindungen ist ein Faktor, der die Auswahl der Wirtspflanzen der Blattläuse beeinflusst. Es konnte auch gezeigt werden, dass die Chemotypen der Rainfarn unterschiedlich auf zwei Arten von Pflanzenfressern reagieren, wobei einige Chemotypen stärkere VOC-Emittenten und damit funktionell "lauter" als ihre Artgenossen sind. Die in dieser Arbeit vorgestellten Ergebnisse, sowie das nun erstmals für diese Pflanzenart beschriebene Transkriptom, könnten als Unterstützung für zukünftige Forschungen dienen, um besser zu verstehen, wie Pflanzen-Chemotypen ihre lokalen Insektengemeinschaften beeinflussen und von ihnen beeinflusst werden.

1 Introduction

This section gives a general introduction to chemical ecology. It describes how the chemical phenotypes of plants can affect plant-insect interactions, and gives an introductory overview of the study system used. The aims of this thesis are given at the close of this chapter.

1.1 Plant defence strategies and volatile organic compounds

Plants are sessile organisms, and being unable to run away from predators, have had to develop unique and innovative methods of self-defence. They are highly efficient metabolic factories, producing up to 200,000 separate chemical compounds (Fiehn 2001), and have utilised this ability in various ways in order to protect themselves from several forms of biotic and abiotic stress (Mithöfer and Boland 2012). Plants can defend themselves both directly and/or indirectly, using their arsenal of chemical weapons. Direct defence involves the production of compounds that make the plant an undesirable host for predators, for example in terms of diet (i.e. by producing digestibility reducers and toxins (Duffey and Stout 1996)) and reproduction (i.e. oviposition (Reymond 2013)). In contrast to direct defence, indirect defence relies on the recruitment of herbivore enemies. When plants are attacked by herbivores, they can emit a specific bouquet of volatile compounds that act as a "cry for help" (Degenhardt et al. 2003) and attract parasitic wasps or carnivorous insects in search of their host (Aartsma et al. 2019, Ye et al. 2018). These compounds are known as biogenic volatile organic compounds (VOCs), and are highly variable both across and within plant species (Maffei 2010). One very important and very diverse class of compounds are the terpenoids (Langenheim 1994). They are all based on a C5 basic building block of an isoprene skeleton, which conjugate to form larger and larger hyrdrocarbons, which are then modified in subsequent steps (Chen et al. 2011). With increasing molecule size the volatility of terpenes decreases from isoprene, a C5 hemiterpene, via monoterpenes (C10) to sesquiterpenes (C15) and to diterpenes (C20) (Zhou and Pichersky 2020b).While plants use volatile terpenoids in a variety of ways to improve their own survival, they are also highly valued in human societies for their various applications (Silva-Santos et al. 2005). Many of our most powerful and important pharmaceutical drugs are derived from terpenoids, including artemisinin (a sesquiterpene lactone naturally occurring in *Artemisia annua*, used to treat *Plasmodium falciparum* infection (Weathers et al. 2006)) and the taxanes (diterpenes, originally discovered in the genus *Taxus*, used as a chemotherapy agent (Baloglu and Kingston 1999)).

1.2 Terpenoid production in plants

Terpenoids are popularly considered to be derivatised from isoprene, a basic C_5H_8 compound; however in actuality terpenoids do not arise from isoprene, but rather from

"isoprene skeletons" (Oldfield and Lin 2012). Monoterpenes ($C_{10}H_{16}$) consist of two units of isoprene, and can be linear or cyclic (for example, β-myrcene is a linear monoterpene, whereas limonene is a cyclic monoterpene (for examples see Fig. 1). Sesquiterpenes ($C_{15}H_{24}$) are composed of three units of isoprene, and can also be acyclic or cyclic.

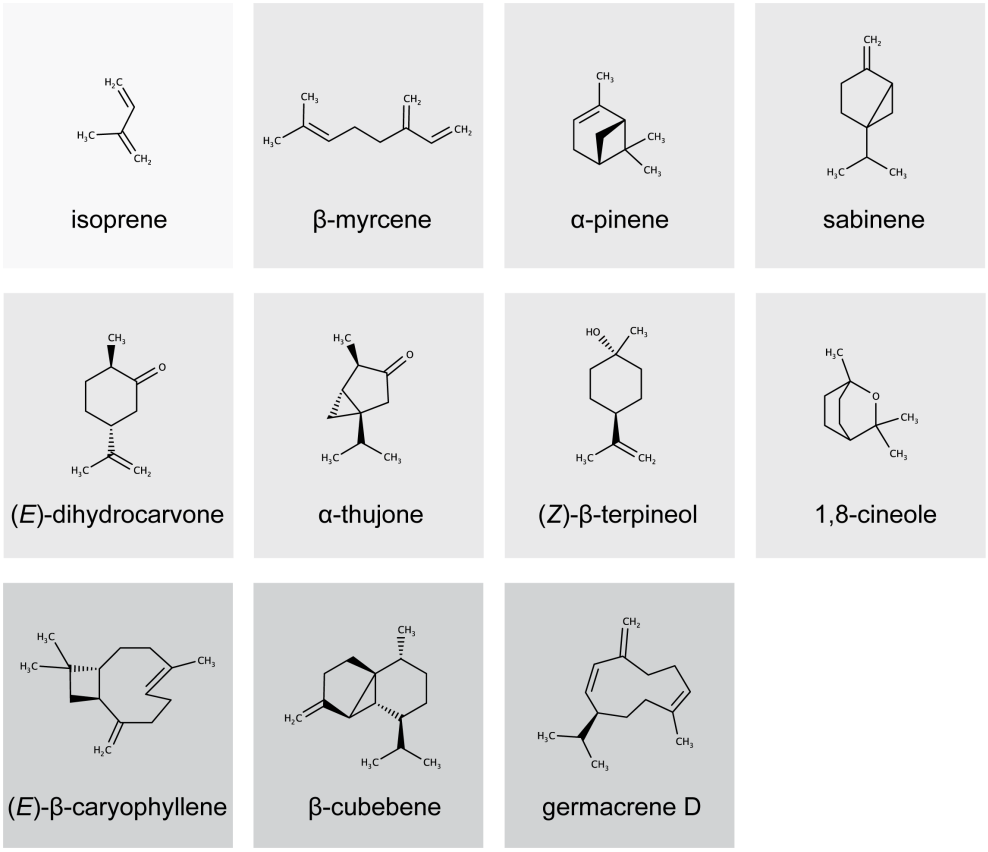

Fig. 1. Examples of common terpenoids. Light grey: isoprene; grey: monoterpenes; dark grey: sesquiterpenes.

Terpenes are formed from basic units of isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), both of which can be formed through either the cytosolic MVA (mevalonate) pathway or the plastidic MEP (methylerythritol 4-pyruvate) pathway (Fig. 2) (Rohmer 1999, Rohmer et al. 1993). In the cytosol a head-to-tail condensation reaction between two IPP units and one DMAPP unit forms farnesyl pyrophosphate (FPP), which is a precursor of sesquiterpenes. Monoterpenes however are formed from the head-to-tail condensation of a single DMAPP unit with one IPP unit, forming geranyl pyrophosphate (GPP); diterpenes are formed via one unit of DMAPP with three IPP units which gives rise

to its precursor geranyl geranyl pyrophosphate (GGPP) (Dudareva et al. 2004). While these biochemical pathways are physically separated and appear to operate independently of one another, it is now thought that limited cross-talk occurs between the MEP and MVA pathways via the trafficking of IPP and DMAPP across the inner envelope membrane of plastids (Dudareva et al. 2013, Mendoza-Poudereux et al. 2015, Rodríguez-Concepción and Boronat 2015). Interestingly, the transcriptional activation of MVA genes is correlated with the repression of MEP genes, and *vice versa* (Rodríguez-Concepción and Boronat 2015).

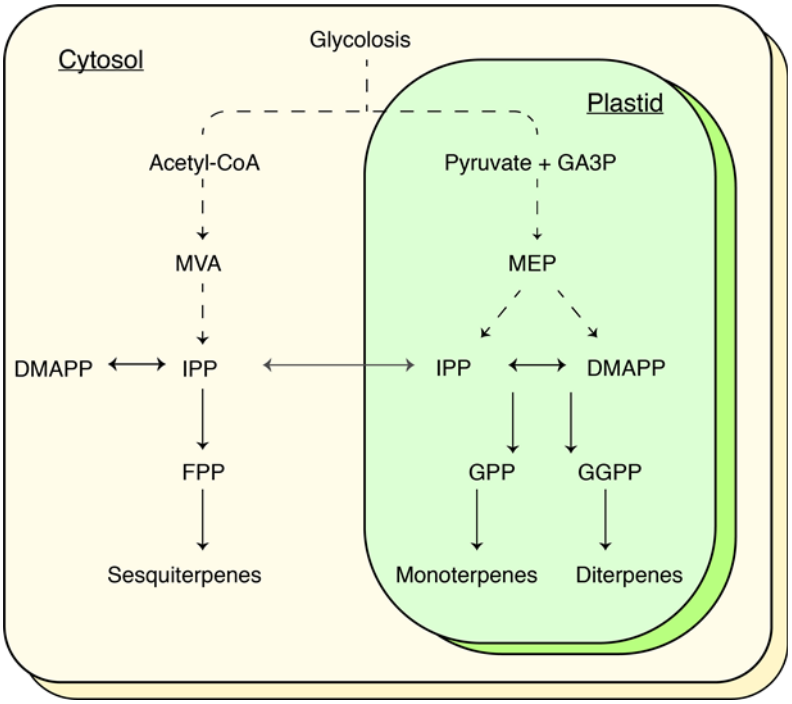

Fig. 2. Schematic of terpene biosynthesis pathways in the cell.

More than 80,000 terpenoid compounds (Christianson 2017) have been identified in all living organisms (Gershenzon and Dudareva 2007). Both mono- and sesquiterpenes can be biochemically modified in various ways which further increases the structural diversity of the terpenoid compound class. Terpene synthases (TPS) are responsible for the vast numbers of terpenes found in nature, in large part due to their ability to synthesise multiple products from a single substrate (Chen, Tholl, Bohlmann and Pichersky 2011). While most terpene synthases form one main product, a single TPS can also form multiple products. TPS genes have been divided into seven subfamilies following phylogenetic analysis; TPS-a, TPS-b, and TPS-d (specific to gymnosperms), TPS-e/f, and TPS-h (specific to

Selaginella spp.) together form type I TPSs, while TPS-c are type II TPSs (Chen, Tholl, Bohlmann and Pichersky 2011).

Cyclic monoterpenes are formed by the initial isomerisation of the geranyl cation to a linalyl cation, which is able to be cyclised. Terpenes can be modified in various ways, including cyclisation, hydrogenation, oxidation, condensation, isomerisation, and hydration (Corma, Iborra and Velty 2007). The addition of water to the geranyl cation results in the formation of linalool and geraniol; potentially (*E*)-β-ocimene, linalool, and β-myrcene are also derivatised from the linalyl cation (see Figure 3, modified from Degenhardt, Köllner and Gershenzon 2009). The first cyclic species formed is the α-terpinyl cation, which can then undergo various changes. Proton loss catalysed by a terpene synthase leads to the formation of limonene or terpinolene from the α-terpinyl cation.

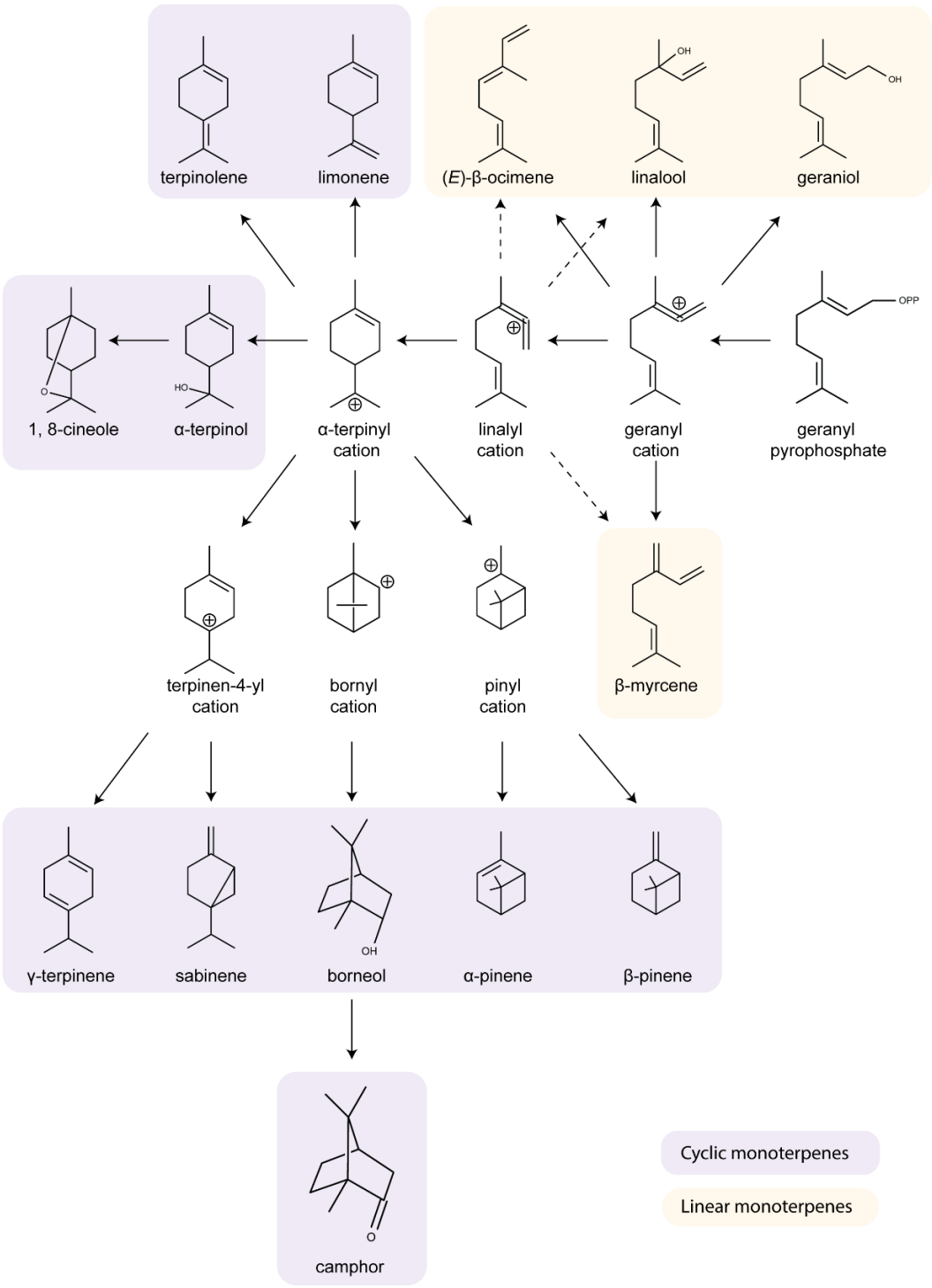

Fig. 2. Monoterpenoid biosynthesis (adapted from Degenhardt, Köllner and Gershenzon 2009). Cyclic monoterpenes are highlighted in purple, linear monoterpenes are highlighted in orange.

1.3 Classifying plants by chemical phenotype

Terpenoids can be released from specialised storage structures, or else actively synthesised following biotic (e.g. herbivory, pathogen infection, etc.) or abiotic (e.g. drought, flooding, etc.) stress (Kleine and Müller 2014). Many plants possess reservoir glands (Kleine and Müller 2013, Takabayashi et al. 1994, Tholl 2015) that contain mixtures of various terpenoids that are highly inter- and intraspecific. Blends of these volatile terpenoids may allow plants to send specific signals, and thus might be important for communication. Analysing the profiles of mono- and sesquiterpenoids in aromatic plants has given rise to a method known in chemical ecology as "chemotyping" (Trindade et al. 2018). Chemical variation in plant VOCs is apparent across a variety of plant species and types. Intraspecific variation in terpenoids has been recorded in tree species; e.g. two chemotypes were described in the treelet *Myrceugenia cucullata* (Limberger et al. 2005), while four distinct chemotype groupings were found in *Eucalyptus camaldulensis* trees (Bustos-Segura, Dillon, et al. 2017). Numerous plant species have specialised reservoir glands where terpenoids are stored, such as secreting glandular trichomes (Wagner 1991). Many herbaceous plant genera belonging to the Lamiaceae family have been profiled into chemotypes according to their intraspecific terpenoid diversity, for example *Thymus* (reviewed by Trindade, Pedro, Figueiredo and Barroso (2018)) and *Lavandula* (Aprotosoaie et al. 2017). As it is known that plant volatiles vary considerably both across and within species, an important question to consider is *why* plants exhibit such extraordinary differences in their VOC phenotypes.

In their natural environment, plants are subjected to abiotic and biotic stresses, and it has been shown that intrinsic chemodiversity among populations results in differing levels of defence responses. For example, Kigathi et al. found that the number of VOCs emitted to the headspace by *Trifolium pratense* increased with increasing plant species richness; plants growing in monocultures emitted lower amounts of VOCs than those growing in polycultures (Kigathi et al. 2019). Bustos-Segura and colleagues showed that with increased plant diversity, plants were bigger and housed more herbivores, but exhibited less damage than plants grown in monocultures (Bustos-Segura, Poelman, et al. 2017).

When attacked by antagonists such as herbivores and pathogens, plants exhibit local and

systemic defence responses resulting in changes to their metabolic profiles. Plant-plant communication is also facilitated by the VOCs released following damage caused by an antagonist (e.g. loss of tissue caused by a chewing herbivore); defences in neighbouring undamaged plants can be increased following exposure these VOC blends (Guerrieri 2016, Karban et al. 2000, Paré and Tumlinson 1999).

1.4 Chemical ecology of tansy

Tansy (*Tanacetum vulgare* L.) is a highly aromatic plant belonging to the Asteraceae family that is well known to exhibit high levels of variation in terpenoid content (Fig. 4). It has long been recognised for its medicinal properties. First records indicate that tansy was used by the ancient Greeks; it was also present in the gardens of Swiss Benedictine monks and Charlemagne the Great (Barceloux 2008). Common historical uses for tansy include treating intestinal worms and fevers, as well as repelling insects from houses and foods (Roecklein and Sun Leung 1987). Tansy was also an extremely important medicinal plant for women around the globe, as in large doses it acts as an abortifacient. It is sometimes used in rituals celebrating womanhood, and is associated with the Virgin Mary (Müller-Ebeling et al. 2003).

Tansy is known to exhibit high levels of variation in terpenoid content. This rich production of volatile compounds has led to tansy gaining a reputation for being an effective insect repellent; it is frequently used as a companion plant from hobby gardening to large-scale agriculture (Cline et al. 2008, Gabel et al. 1992, Latheef and Ortiz 1983). While tansy may act as a repellent in some cases, it is also host to several species of aphids. One of these species, *Metopeurum fuscoviride* Stroyan, feeds on tansy plants almost exclusively. *M. fuscoviride* are obligate myrmecophiles, meaning that they require ants to tend to them (Stadler 2004). Ant colonies are therefore frequently found adjacent to colonised tansy plants, and provide protection to the aphids from predators in exchange for honeydew (Senft et al. 2017). Although this species of aphid essentially lives only on tansy plants, they often do not colonise every single plant within a field site (Weisser 2000).

Fig. 4. Botanical drawing of tansy from Svensk Botanik by J.W. Palmstruch and O. Swartz, 1803.

The most common types of terpenoids found in tansy are mono- and sesquiterpenoids. Like other aromatic plants, tansy uses its store of VOCs for communication and defence. Not all tansy plants "smell" the same however; huge variation in terpenoid content gives rise to the ability to classify plants into chemical phenotype (chemotype) classes, based on their chemical profiles (Benedek et al. 2015, Holopainen et al. 1987, Keskitalo et al. 2001, Kleine and Müller 2011). Traditional methods for identifying chemotypes have relied on quantifying the most dominant compounds found in the VOC profile of an individual plant. The body of work presented in my PhD thesis aims to develop a more holistic approach to chemotyping by describing a novel method for assigning chemotype. It has been demonstrated that tansy chemotypes can be wildly different from one another; one aim of

this thesis is to show that even within one field site (< 1 km^2) tansy chemotypes are significantly different to one another, to the point where they influence aphid host plant selection.

1.5 Volatile and non-volatile chemical diversity

Chemical diversity in plants is not limited to only volatile compounds. Primary metabolism is responsible for the production of metabolites that are necessary for normal plant growth, development, and reproduction, however it also provides the necessary precursors for secondary metabolism, which produces compounds that are central to how a plant interacts with its environment. Secondary metabolites produced by plants span several compound classes, including terpenoids, phenolics and nitrogen/sulphur containing compounds such as alkaloids (Aharoni and Galili 2011). There have been studies published showing that both volatile and non-volatile metabolites influenced insect behaviour in *Eucalyptus* species (Matsuki et al. 2011) and soybean (Silva et al. 2013). Extraordinarily high levels of chemical diversity (volatile and non-volatile) among individuals of the same species have been recorded, for example, in tea tree (*Melaleuca alternifolia*; (Bustos-Segura and Foley 2018)) and some eucalyptus species (*Eucalyptus camaldulensis*; (Bustos-Segura, Dillon, Keszei, Foley and Külheim 2017)). It has been shown that these differences among individuals can have consequences for plant performance. Individuals grown in mixed stands have been shown to perform better than plants grown in monoclonal stands (Bustos-Segura, Poelman, Reichelt, Gershenzon and Gols 2017, Crutsinger et al. 2006). Plant metabolites and the presence or absence of certain secondary metabolites strongly influences herbivore feeding dynamics (Schoonhoven et al. 2005) and complexity of the associated invertebrate community. As metabolites are direct products of modifications to gene, transcript, and protein expression, analysis of the metabolome gives us an insight into the impact of various stresses on plant biochemical processes. Using a combination of volatile and non-volatile compounds, specialist herbivores locate their preferred host species, and also the most suitable individual plant.

Although the odour profile of tansy plants can influence aphid host selection, further analysis into the non-volatile metabolome is warranted. Aphids are phloem sucking herbivores and feed off plant sap. While this does not cause the same level of visible

damage as chewing herbivores inflict, they deplete nutrients and induce defence responses in the plant. Aphid growth is strongly affected by both the nutrient status and genotype of a host plant (Zytynska and Weisser 2016). From the same field site mentioned previously, tansy plants were analysed using non-targeted metabolomics. Although innovative advances have been made in the field of metabolomics, the proportion of unknown molecular features (reported to be between 70% and 90%) means that compound classification can be very difficult and limited. Methods have been developed that encompass this percentage of unknowns into chemical profiling and analytical interpretations. Mass difference enrichment analysis (MDEA) (Kaling et al. 2018, Moritz et al. 2017) is used to analyse mass difference networks (MDiNs) (Breitling et al. 2006), which represent accurate *m/z* features as nodes that are connected by mass-difference building blocks (MDBs) as edges. These can be used in conjunction with MDEA for future identification of mass features (Moritz, Kaling, Schnitzler and Schmitt-Kopplin 2017).

One aim of this thesis is to answer the question of whether tansy plants can be grouped into discriminant categories based on their non-volatile metabolome, and whether the metabolome correlates to the volatilome. Non-targeted metabolomics has great potential to further our understanding in the field of chemical ecology as it provides an unbiased and complete overview of the physiological responses of a plant to herbivory. This thesis provides the first report on the tansy metabolome.

1.6 Volatile and non-volatile responses to herbivory

Differences in chemotype are known to influence single species of herbivore, with more and more information becoming available on how plant volatile emissions influence or are influenced by herbivore attack by multiple species. Plants have different defence strategies depending on what type of damage is being inflicted on them, and ramp up induction of specific biochemical pathways in response. Chewing herbivores that consume leaf tissue typically induce the jasmonic acid (JA) defence pathway (in conjunction with the volatile phytohormone ethylene); herbivores that feed off plant sap (e.g. aphids) generally induce the salicylic acid (SA) defence pathway (Schweiger et al. 2014). These pathways are not completely distinguished from one another as was previously thought. Interactions between both pathways have generally been categorised as being antagonistic, however there are

several reports of synergistic or additive interactions (Davidson-Lowe et al. 2019, Devadas et al. 2002, Johnson et al. 2020, Thaler et al. 2012). The defence pathways mediated by these phytohormones give rise to far-reaching changes across the transcriptome, metabolome, and proteome. Damage caused by chewing herbivores can be severe and result in large changes to both primary and secondary metabolism (Poelman and Kessler 2016). The level of damage a plant experiences due to chewing herbivores is dependent on multiple factors, including the relationship of the plant-herbivore interaction (i.e. generalist versus specialist), species, and larval stage (Zhou et al. 2015). Although plant sap feeders such as aphids cause less obviously apparent damage to plants, comprehensive induction of various defence pathways both locally and systemically are observed (Tzin et al. 2015). Feeding by sucking and chewing herbivores results in the induction of defence pathways that can interact in antagonistic or synergistic manners, and so could possibly involve defence priming.

Plant defence priming refers to an inducible state of heightened defence that can provide protection to the plant, and can be triggered by volatile cues and pathogenic and non-pathogenic infection for example (Mauch-Mani et al. 2017). These triggers can lead to changes to the plant at several levels, including physiological, metabolic, and transcriptional. This priming can be induced by the presence of a variety of chemicals such as monoterpenes (Riedlmeier et al. 2017), JA, ethylene, SA, azelaic acid, pipecolic acid, as well as the methyl esters of JA and SA (MeJA and MeSA respectively) (Tripathi et al. 2019, Yuan et al. 2019). Plants initially respond very quickly to wounding by rapid emission of LOX pathway (lipoxygenase) products which are known as green leaf volatiles (GLVs) (Scala et al. 2013b). However, following a local pathogenic infection a long-lasting form of immunity known as systemic acquired resistance (SAR) can be triggered throughout a plant. SAR is associated with high levels of salicylic acid accumulation across the whole plant (Gao et al. 2015). Volatile monoterpenes are required for SAR related signalling within a plant (Riedlmeier, Ghirardo, Wenig, Knappe, Koch, Georgii, Dey, Parker, Schnitzler and Vlot 2017), and could even act as long-distance signalling molecules between plants (Wenig et al. 2019).

The final question this thesis aims to answer is whether the chemotype of a tansy plant has

an effect on its volatile response to herbivory by two species (aphid and caterpillar; *Metopeurum fuscoviride* Stroyan and *Spodoptera littoralis* Boisduval) of herbivores that have markedly different feeding styles (sucking and chewing respectively). Using various techniques to analyse the volatilome, this thesis aimed to determine whether tansy chemotypes respond to dual species herbivory in a similar manner to one another. RNA-seq was used to investigate transcriptional responses (i.e. expression/suppression of genes) of tansy to the combinations of herbivore treatments on a transcript level; also providing the first report to date of a tansy transcriptome.

1.7 Aims of Thesis

This thesis aims to deepen understanding of chemical diversity in *Tanacetum vulgare*. The specific aims are:

- To develop a method of chemotyping that effectively describes the differences in odour profiles in a population of tansy;
- To study the non-volatile metabolome of tansy and to determine whether the volatilome and metabolome are correlated;
- To determine whether tansy chemotypes respond differently to dual attack herbivory, and to simultaneously observe changes over time of volatile emissions;
- To identify terpenoid biosynthesis related genes in tansy and gain insight on the transcriptional changes that follow herbivory by aphid and caterpillar feeding.

This body of work is a cumulative dissertation of published articles and an article submitted for publication, which are found in this thesis.

2 Study system and methods

This section contains an overview of the study system and the methods used in experimental work. More detailed descriptions of the methods can be found in each relevant manuscript.

2.1 Description of systems

2.1.1 Plant

Tanacetum vulgare L. (commonly known as tansy) is a highly aromatic herbaceous plant that was selected for the present study due to its high terpenoid content and recorded variation in chemotypes. Its leaves are alternate and pinnately lobed; each lobe is saw-toothed, which gives it a fern-like appearance. The flowers are yellow, round, and button-like, forming terminal inflorescence clusters from mid-late summer on.

2.1.2 Insects

Metopeurum fuscoviride Stroyan is an aphid species that frequently colonises tansy plants. It is autoecious and produces unwinged morphs at the end of the season (October - November, (Mehrparvar et al. 2013)). Eggs are laid by mated females at the base of plants, from which new colonies emerge the following spring in April (Mehrparvar, Zytynska and Weisser 2013). *M. fuscoviride* is an obligate myrmecophile, meaning that it must be tended by ants in order to ensure survival (Stadler 2004). It is commonly tended by *Lasius niger* L. and *Myrmica rubra* L. (Formicidae), and predated upon by aphidophagous predators such as the ladybird (Coccinellidae) and soldier beetle (Cantharidae).

M. fuscoviride aphids were collected by M. Senft (Technical University of Munich (TUM)) from random tansy plants in the vicinity of the TUM at Weihenstephan, Freising, Germany. Second instar *Spodoptera littoralis* Boisduval larvae were obtained from Jena (Max Planck Institute for Chemical Ecology) reared on commercial lettuce leaves. *S. littoralis* is a highly polyphagous organism and as such must be kept under quarantine conditions (CABI and EPPO). Following each experimental round, I destroyed all *S. littoralis* larvae by freezing in liquid nitrogen.

2.2 Field experiment

A weekly field survey of tansy plants was conducted by M. Senft (TUM) in 2014 at a field site at Altenhausen, situated north of Freising, Germany. I harvested tansy leaf material from this field site for chemotypic and metabolomic analysis in July of 2014. In experiments where plants were grown from seed, the seeds were collected from dried flower heads at Altenhausen in late Autumn 2013 (by S. Zytynska, TUM). We tagged 176

individual plants using weatherproof labels, and recorded their location using GPS coordinates.

2.2.1 Harvesting plant material

Individual tansy plants were sampled at the field site in Altenhausen in July of 2014. I cut off fresh leaf material from each plant and placed the harvested material into individually labelled Eppendorf tubes containing two small ceramic balls. I froze the plant material immediately on dry ice, and later stored it at -80 ºC.

See manuscripts I and II for further details.

2.2.2 Chemotyping

2.2.2.1 Liquid extraction method for GC-MS analysis

I ground frozen leaf material into powder under liquid nitrogen, and added 1 mL GC-MS grade hexane to ~500 mg of the frozen leaf powder in a 1.5 mL glass vial. I then vortexed the sample stored it at 4 ºC for 24 hours. Using a glass syringe (Hamilton Company Inc, Nevada, USA) I then removed 500 µL of liquid extract and stored it at 4 ºC. 500 µL of fresh hexane was again added to the leaf material; the sample was then vortexed and then stored at 4 ºC for a further 24 hours. Again, I carefully removed 500 µL of liquid extract and combined it with the already collected extract. Liquid extracts were then stored at -80 ºC in 1.5 mL amber glass vials with teflon screw-top lids until further use. I dried remaining plant material and stored it short-term at -80 ºC until further extraction (see methods section 2.2.3.1).

See manuscript I for further details.

2.2.2.2 Stir Bar Sorptive Extraction method for GC-MS analysis

The headspace of 28 plants in the field were assessed using the stir bar sorptive extraction method (non-polar polydimethylsiloxane coated stir bars; Twister, film thickness 0.5 mm, length 10 mm, Gerstel, Mülheim an der Ruhr, Germany) in 2013 by S. Zytynska (TUM). VOCs were collected by suspending a twister in the headspace of undamaged leaves that were encased in transparent bags (40 cm length x 31 cm diameter, PET film, secured at each end; Toppits Bratschlauch, Melitta Group, Minden, Germany). Time period of VOC

collection was 3 hours during daytime, between 10:00 to 16:00 (24/07/2013; temperature range 22.7-29.9 C; humidity ~60%; no precipitation. Weather data publicly available from weather station at 48°24′32′, E 11°43′20′ provided by the Bavarian State Research Centre for Agriculture.

See manuscript I for further details.

2.2.3 Metabotyping

2.2.3.1 Liquid extraction method for UPLC-MS analysis

Plant material that had initially been extracted with hexane was fully dried. In a 1.5 mL Eppendorf tube, I added 1 mL extraction solvent A (methanol/water 7:3, [v/v]) at -20 ºC to ~200 mg of the dried leaf powder. After 30 min in a shaker on ice the mixture was centrifuged at 10,000 *g* for 10 min at 4 ºC. I dried the supernatant under vacuum, then added 1 ml solvent B (acetonitrile/water 1:1, [v/v]) at -20 ºC and vortexed. I then centrifuged the solution at 10,000 *g* for 10 min at 4 ºC. I collected the supernatant and diluted it with solvent B at a ratio of 1:50 [v/v].

See manuscript II for further details.

2.3 Cuvette experiment

Tansy seeds were obtained from a field site in southern Germany (N 48°25'1.51"; E 11°46'1.19") and propagated in the TUM greenhouse by M. Senft. I assessed the terpenoid profiles of five different genotypes using the liquid extraction method and GC-MS analysis as described above. I split each genotype into three daughter clones twice resulting in nine daughter plants, to be used as biological replicates. M. Senft randomly collected the aphids used in this experiment from tansy plants grown near the Weihenstephan campus of TUM. I reared first instar larvae of *Spodoptera littoralis* on commercial lettuce at room temperature. For 24 h prior to the experiment I starved all larvae to ensure prompt feeding.

Using the cuvette platform designed at the Helmholtz Zentrum Munich, Research Unit Environmental Simulation (HMGU, EUS) by B. Niederbacher and W. Jud (Jud et al. 2018, Niederbacher et al. 2015), I selected five chemotypes to be treated with aphids and

caterpillars. On day 0, I randomly selected five plants and placed them into a container within the cuvette system, with one cuvette remaining empty except for a pot of soil to provide background information for statistical normalisation. Carefully, I placed a large glass bulb over each cuvette to cover each plant. After 24 hours, on day 1 of the experiment, I gently added 100 aphids to the treatment plants using a paintbrush. On day 4 of the experiment, I harvested leaf material and immediately froze it in liquid nitrogen for further analyses. I then carefully placed two second instar *S. Littoralis* larvae onto each plant. The experiment ended on day 7, and I then harvested all aboveground biomass (which was immediately frozen in liquid nitrogen for further analyses). Each experimental round lasted one week, and six rounds were repeated in total. I destroyed all *S. littoralis* larvae in accordance with Council Directive 2000/29/EC by freezing in liquid nitrogen.

In the first half of the experiment, plants that acted as control (i.e. were not treated with either aphids or caterpillars; no aphids, no caterpillars) were given the label "N". Plants that received only the aphid treatment (aphids, no caterpillars) were designated "A". In the second half of the experiment, the plants initially acting as control but were then treated with caterpillars, were labelled "C" (no aphids, caterpillars). Plants that were initially treated with aphids and subsequently treated with caterpillars were labelled "B" (with aphids and caterpillars). Plants in treatment group N reflect the original chemotypes as described above.

2.3.1 Chemotyping

2.3.1.1 Liquid extraction for GC-MS analysis

As described above in section 2.2.2.1

2.3.1.2 Headspace Sorptive Extraction for GC-MS analysis

VOCs were focused on sorbent tubes I prepared containing 40 mg Tenax TA and 10 mg Carbopack (Sigma-Aldrich, Taufkirchen, Germany) that were coupled to air drawn from each cuvette. I set the air flow to 100 ml min^{-1} and collected VOCs for a duration of two hours in experimental weeks 1 and 2, and then for three hours over weeks 3 to 6.

See manuscript III for further details.

2.3.2 Real-time measurements of VOCs

The cuvette platform (Jud, Winkler, Niederbacher, Niederbacher and Schnitzler 2018, Niederbacher, Winkler and Schnitzler 2015) enabled real-time analysis of emitted VOCs from whole tansy plants. The platform consists of six cuvettes from which air is drawn and fed to a proton transfer time of flight mass spectrometer (PTR-ToF-MS). Each cuvette contains a stainless-steel gas tight base with a large glass bulb atop it. All electrical connections are fed through the base, as well as tubing for irrigation and gas. Air is flushed into each cuvette via a series of small inlet holes in the metal base. All tubing is made of PTFE or Teflon in order to reduce potential reactions of VOCs with tubing surface areas. A T-piece allows air flow to be continuously flushed from each cuvette through individual Tenax and Carbopack GC-MS tubes, and to a multiplex system consisting of two-way solenoid valves. These valves are opened one by one and allow air from different cuvettes to be fed to the PTR-ToF-MS instrument. Instrument setup was performed by B. Niederbacher and S. Niederbacher.

I selected five tansy plants with differing VOC profiles, and used their daughter clones (described above) as biological replicates.

See manuscript III for further details.

2.3.3 RNA extraction

Using a mortar and pestle, I ground frozen leaf tissue (plant 3, all four treatment groups) into a powder under liquid nitrogen. I carried out a total RNA extraction using the innuPREP Plant RNA Kit from Analytik Jena (Jena, Germany), as per the manufacturer's instructions. Using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) I confirmed RNA quality prior to sequencing (performed by Vertis Biotechnologie AG, Freising, Germany).

See manuscript III for further details.

2.4 Chemical analysis

2.4.1 GC-MS measurements

2.4.1.1 Liquid extraction method

I placed vials containing the liquid extracts into the autosampler of the gas chromatography mass-spectrometry system. One µL of sample was injected into an empty glass cartridge containing a glass micro-vial. The samples were desorbed from 35 ºC to 240 ºC at 120 ºC min $^{-1}$ with a holding time of 2 min in the thermo-desorption unit (TDU, Gerstel, Mülheim an der Ruhr, Germany). The TDU was coupled to a GC-MS (GC type: 7890A, MS type: 5975C inert XL MSD with a triple axis detector, both Agilent Technologies, Palo Alto, CA, USA) using a 5% phenyl 95% dimethyl arlene siloxane capillary column (60 m x 250 µm x 0.25 µm DB-5MS + 10 m DG, Agilent Technologies). Following volatilisation, the compounds were refocused on a Tenax liner at -50 ºC, and desorbed to 250 ºC at a rate of 12 ºC min^{-1}. Samples were analysed splitlessly at a constant flow rate of He at 1 mL min^{-1} and the following temperature program: 40 ºC for 0 min, followed by ramping of 10 ºC min^{-1} to 150 ºC, then 80 ºC min^{-1} to 175 ºC, then 5 ºC min^{-1} to 190 ºC, then 80 ºC min^{-1} to 250 ºC, then 100 ºC min^{-1} to 300 ºC, hold for 6 min.

2.4.1.2 Stir Bar Sorptive Extraction

Samples were analysed on the same GC-MS system as described above. The temperature program was as follows: 40 ºC for 2 min, followed by ramping at 6 ºC min^{-1} to 80 ºC, hold 3 min, 3.4 ºC min^{-1} to 170 ºC, then 12 ºC min^{-1} to 300 ºC, hold 4 min.

2.4.1.3 Headspace Sorptive Extraction

Samples were desorbed using the TDU and GC-MS system described above. The CIS vaporisation inlet (cooled injection system; Gerstel) was set to -50 ºC. The temperature program was as follows: TDU heated to 290 ºC from 37 ºC at a rate of 280 ºC min^{-1}. Samples were analysed splitlessly at a constant flow rate of He at 1 mL min^{-1}. The CIS was heated to 290 ºC at a rate of 12 ºC min^{-1} after 0.2 min, and held at 290 ºC for 2 min.

2.4.1.4 Identification and quantification of VOCs

I compared collected mass spectra to those of commercially available standards (Sigma-

Aldrich, Taufkirchen, Germany), NIST 05 and Wiley library spectra, and the Kovats retention index library (Lucero et al. 2009).

2.4.2 UPLC-MS measurements

I placed vials containing liquid extracts in the auto-sampler (set at 4 ºC) of the ultra-high-performance liquid chromatography (UHPLC) system. Ultra-high-resolution mass spectra were gathered using an UltiMate 3000RS (Thermo Fisher, Bremen, Germany) coupled to an Impact II UHR-QqTOF (Bruker Daltonic GmbH, Bremen, Germany) via an electro spray ionisation source, with a 1.7 µm Waters ACQUITY BEH 2.1 x 150 mm C18 column (Waters, Milford, MA, USA). The mobile phases used were as follows: solvent A (H_2O + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid), with a constant flow rate of 0.4 ml min^{-1}.

Subsequent to injection (volume: 5 µL), the following gradient was run for 23 min at a column temperature of 40 ºC: solvent B 5% (0 - 1 min), solvent B 5% - 40% (1 - 15 min), solvent B 40% - 70% (15 - 18 min), solvent B 70% - 5% (18 - 23 min). Two technical replicates were performed in both positive and negative ionisation mode for each sample. The nebuliser was set to 2 bar, with nitrogen used as a dry gas at a flow rate of 10 L min^{-1} at 220 ºC. Capillary voltage was 3,000 and 4,000 V for negative and positive ionisation modes respectively. Data was acquired in a mass range of 50-1,300 m/z. HyStar v 3.2 (Bruker Daltonic) was used for data acquisition. Using Expressionist for MS 10.5 (Genedata, Basel, Switzerland), generated data was subjected to noise subtraction, retention time (RT) alignment, and peak picking. Data processing was performed with GeneData (Basel, Switzerland) by M. Witting (HMGU Research Unit Biogeochemistry and Analytics (BGC)). Only peaks that were present in at least 10% of mass spectra were used for isotope (^{13}C) clustering. Singletons (clusters with only one member) were removed from further analysis.

Features were putatively annotated using an in-house version of MassTRIX metabolite annotation server (Suhre and Schmitt-Kopplin 2008, Wägele et al. 2012, Witting et al. 2014). Detected m/z values were compared to a database of theoretical adducts from metabolites contained in Kyoto Encyclopaedia of Genes and Genomes (KEGG), Human

Metabolome Database, LipidMaps, and MetaCyc. In negative ionisation mode only [M - H]- were considered, in positive ionisation mode, only [M + H]+ and [M + Na]+ were considered. Matching was performed with an absolute error of 0.0005 Da.

All mass difference enrichment analysis (MDEA) was performed by F. Moritz (HMGU BGC). In brief, mass difference network (MDiN) reconstruction was carried out using m/z values, with z-corrected masses being given a 5 ppm error tolerance. Mass difference building blocks (MDBs) were manually extracted from KEGG and Moritz et. al (Moritz, Kaling, Schnitzler and Schmitt-Kopplin 2017) and were added to with further mass differences of adducts and steroids. Enrichment of MDBs was expressed as *Z* scores, which represent the distance of observed MDB frequencies with the expected frequencies with the remaining network.

2.4.3 PTR-ToF-MS measurements

I placed five tansy plants of differing VOC profiles into each cuvette, with the sixth one left blank (pot and soil, no plant) for later background normalisation. Three biological replicates were performed over the entire time period of the experiment. The cuvettes were flushed with air drawn from the chamber using a rotary vane compressor (DLT 40, Gardner Denver Schopfheim GmbH, Schopfheim, Germany). Air flow was set to 17 L min^{-1} for weeks 1 - 3, and reduced to 10 L min^{-1} from weeks 4 - 6. Air flow to the PTR-ToF-MS alternated between cuvettes; switching time was set to five minutes. Mass spectra were set to record up to a range of 318 m/z. The operational parameters for the drift tube were set to: pressure 2.2 mbar, voltage 500 V, temperature 60 ºC, E/N value 115 Td. Data processing was performed by W. Jud (HMGU EUS) as described in Jud et. al (Jud, Winkler, Niederbacher, Niederbacher and Schnitzler 2018), and Guo et. al (Guo et al. 2020). In brief, the raw signals in counts per second (cps) were normalised to 10^6 reagent ion counts (sum of H_3O^+ signals and its cluster, $H_3O^+ \cdot H_2O$, divided by 10^6) to account for the differences in absolute humidity in each cuvette; giving the signals in normalised counts per second (ncps).

2.5 Statistical analysis

All experiments were performed using two technical replicates and three biological replicates where applicable. I performed all statistical analyses (clustering, PCA, PLS, OPLS, t-tests) using MetaboAnalyst (Xia and Wishart 2016), SIMCA-P (Umetrics, Sweden), SigmaPlot (Systat Software), and R (R_Core_Team 2013).

3 Manuscript overview

This thesis contains three first author/co-first author manuscripts. This section contains a brief summary of each manuscript and includes detailed descriptions of author contributions.

Manuscript I

Chemotypic variation in terpenes emitted from storage pools influences early aphid colonisation on tansy

Mary V. Clancy, Sharon E. Zytynska, Matthias Senft, Wolfgang W. Weisser, and Jörg-Peter Schnitzler

Published in 2016 in Scientific Reports, **6**, 38087, DOI: 10.1038/srep38087

Summary

Tansy (*Tanacetum vulgare* L.) is an aromatic plant known for exhibiting high levels of intraspecific chemical variation, particularly of mono- and sesquiterpenes. These volatile organic compounds (VOCs) play an important role in plant-insect interactions. It is host to *Metopeurum fuscoviride* Stroyan, an aphid species that is specialised to tansy, that is also obligately tended by mutualistic ants (*Lasius niger* and *Myrmica rubra*).

In this study, I assessed 176 tansy plants from a single field site (less than 1 km^2) on the basis of their terpenoid chemical phenotype (chemotype) in order to determine how tansy chemotypes relate to colonisation by *M. fuscoviride*. I proposed a novel method of chemotyping, based on GC-MS analysis of two extraction procedures: leaf hexane extracts and SBSE analysis of headspace. Using an overlap of compounds, termed "likely emitted from storage", tansy plants were categorised into four chemotype classes according to the relative abundance of these compounds in the overall blend.

M. fuscoviride has a main dispersal event in late May/early June, where winged morphs are able to move relatively freely between plants before selecting a host, whereas later in the season (from late June on), aphids are only able to move between plants by walking. The results show that under field conditions, tansy chemotypes significantly affected aphid colonisation during the main dispersal event (when winged morphs are produced), but no longer had an effect on aphid colonisation later in the season. Almost half of plants belonging to chemotypes 1 and 2 were colonised by aphids, with less than 20 % of plants belonging to chemotypes 3 and 4 being colonised. We found that chemotype did not have

an effect on either of the tending ant species, however some individual minor terpenes were associated with ant presence.

The findings presented in this manuscript provide a novel method for chemotyping, and highlight that it is not always the dominant compound in a blend that exerts an effect on aphid colonisation.

Author contributions

M. Clancy, S. Zytynska, M. Senft, W.W. Weisser, and J.P. Schnitzler designed the study. M. Clancy, S. Zytynska, and M. Senft performed the experiments together. M. Clancy and M. Senft did the sampling, and M. Clancy performed GC-MS identification and quantification. M. Senft analysed the invertebrate community and carried out weekly field surveys. S. Zytynska performed the statistical modelling. M. Clancy and S. Zytynska wrote the first draft of the manuscript and prepared the figures. All authors contributed to interpretation of the findings and edited and approved the manuscript.

Manuscript II

Metabotype variation in a field population of tansy plants influences aphid host selection

Mary V. Clancy, Sharon E. Zytynska, Franco Moritz, Michael Witting, Philippe Schmitt-Kopplin, Wolfgang W. Weisser, and Jörg-Peter Schnitzler

Published 2018 in Plant, Cell & Environment, 41: 2791 – 2805, DOI: 10.1111/pce.13407

Summary

While it is well known that plant volatiles are involved in mediating the relationship between a plant and its herbivores, there is less information available on how the non-volatile metabolome of a plant influences these interactions.

In this study I assessed the non-volatile chemical profile of 122 tansy (*Tanacetum vulgare* L.) plants from a single field site. Using non-targeted Ultra-High-Performance Liquid Chromatography Qq-Time-of-Flight Mass Spectrometry (UPLC-MS). I showed that the tansy plants could be grouped, forming five metabotype classes. These classes were not found to be associated with the volatile chemotype of the same plants, however they influenced the colonisation of a tansy-specialist aphid (*Metopeurum fuscoviride* Stroyan). All five metabotypes were identified within each volatile chemotype. Irrespective of chemotype, plants belonging to metabotypes C and E were the most likely to be successfully colonised by aphids; almost all plants belonging to these two metabotypes were colonised by the end of the season.

Plants in the field were visited on a weekly basis to ascertain colonisation status as well as colony size. The data presented in this manuscript show that there was no structured difference in the non-volatile metabolic profiles of plants that were currently infested at the time of sampling, and plants that had been infested a number of weeks prior (but were aphid-free at the time of sampling). This suggests a constitutive difference between plants, however it is possible that this might be a result of a long-lasting induction caused by earlier

aphid infestation. Compounds related to the salicylic acid (SA) defence pathway (and systemic acquired resistance (SAR) were found to drive the separation between metabotypes C and E (the most colonised metabotypes) and A, B, and D, including compounds such as SA, methyl salicylate (MeSA), isoleucic acid, and pipecolic acid. Interestingly, plants that contained these SAR-related compounds were more attractive to aphids.

This paper shows that non-targeted mass spectrometry can be a useful tool in chemical ecology by ascertaining that the large diversity of non-volatile metabolites within a local plant population can lead to the formation of distinct subgroups that are linked to aphid colonisation.

Author contributions

S. Zytynska, W.W. Weisser, and J.P. Schnitzler designed the study. M. Clancy did the sampling and performed the LC–MS analysis. F. Moritz (HMGU BGC) performed the mass difference network analysis . M. Witting (HMGU BGC) carried out data normalisation and structural elucidations. P. Schmitt-Kopplin (HMGU BGC) supervised the LC–MS and mass difference network analyses. M. Clancy analysed the data and together with S. Zytynska performed the statistical modelling, wrote the first draft of the manuscript, and prepared the figures. All authors contributed to interpretation of the findings and edited and approved the manuscript.

Manuscript III

Under fire -simultaneous volatilome and transcriptome analysis unravels fine-scale responses of tansy chemotypes to dual herbivore attack

Mary V. Clancy, Georg Haberer, Werner Jud, Bishu Niederbacher, Simon Niederbacher, Matthias Senft, Sharon E. Zytynska, Wolfgang W. Weisser, and Jörg-Peter Schnitzler

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Summary

In nature, plants are rarely predated on by a single species of herbivore. One method of defence plants utilise are volatile organic compounds (VOCs). VOCs are intimately involved in plant-herbivore interactions, and have been used to classify plants into subgroups known as chemotypes. These chemotypes have been found to mediate plant-aphid interactions in tansy (*Tanacetum vulgare* L.), a highly aromatic plant.

This manuscript represents the first study to date on how different tansy chemotypes respond to herbivory by more than one insect species. I investigated the responses of five tansy chemotypes to herbivory by two different insect species using a specially designed cuvette system. Sucking and/or chewing herbivores (*Metopeurum fuscoviride* Stroyan, a tansy-specialised aphid, and *Spodoptera littoralis* Boisduval, a generalist caterpillar, respectively) were sequentially applied to the tansy chemotypes to feed. Various analytical techniques were then used to measure changes in VOC emissions.

Minimal changes in volatile emissions were observed following aphid feeding, however herbivory by caterpillars strongly increased VOC emissions across each chemotype. Caterpillar feeding subsequent to aphid infestation resulted in chemotype-specific changes in VOC emissions, indicating that tansy chemotypes do not all respond to herbivory in the same way. Transcriptome analysis of one chemotype is the first *de novo* assembly of a tansy transcriptome to date, and shows evidence of a priming effect of aphid feeding on later herbivory.

Overall this manuscript demonstrates that different tansy chemotypes react to double herbivory in various ways, indicating that some chemotypes may respond more efficiently when attacked by herbivores. This may be of importance in natural settings; if some individuals are “louder” than others in a field population, they might have an effect on local insect communities, e.g. via recruitment of natural enemies, or inducing defences in neighbouring plants.

Author contributions

M. Clancy, S. Zytynska, M. Senft, W.W. Weisser, and J.P. Schnitzler designed the study. M. Clancy performed the experiment. M. Senft reared and provided the aphid populations. W. Jud, B. Niederbacher, S. Niederbacher (all HMGU EUS) and M. Clancy ran and analysed PTR-ToF-MS measurements, while M. Clancy performed all GC-MS identification and quantification. G. Haberer (HMGU Research Unit Plant Genome Systems Biology (PGSB)) processed and analysed transcriptome data. M. Clancy wrote the first draft of the manuscript, M. Clancy and W. Jud and prepared the figures. All authors contributed to the manuscript drafts and approved the manuscript.

4 Discussion

This section summarises the main findings of this thesis.

Using several mass spectrometric technologies and developing fit-for-purpose methods of sample collection and processing, I provide evidence in this thesis that tansy plants vary significantly in their volatile and non-volatile chemical profiles on a small scale, allowing for classification according to their chemical phenotypes (chemotype and metabotype respectively). I show that these groupings are associated with observable effects on a specialist insect population. I also demonstrate the variability of tansy chemotypes in their volatile response following herbivory by two species of insect from different feeding guilds.

4.1 Field study: chemotyping

The results presented in manuscript I show that tansy plants could be grouped into chemotypes on the basis of compounds "likely emitted from storage", and that early in the season these chemotypes influenced colonisation by a specialist aphid species, *Metopeurum fuscoviride*. This manuscript describes a new approach to chemotyping using a subset of compounds that are constitutively stored but are volatile enough to be emitted to the headspace. These volatile compounds can be stored in specialised storage glands and passively emitted, or they can be synthesised and released in response to varied stressors, including herbivory, pathogen infection, climate extremes, etc. Preliminary experiments using the SBSE and hexane methods to assess whether there is a difference between the profiles of volatiles that are released to the headspace, and those that are stored in glandular trichomes showed that a wide range of compounds, including stress-related compounds and LOX pathway products could be detected. Using these two methods I determined that the overlapping compounds satisfied two key criteria in relation to their potential effect on insect populations. The overlapping compounds were deemed to be (I) compounds that were stored in the leaf and not synthesised in response to stress (and therefore thought of as the intrinsic chemotype), and (II) compounds that were volatile enough to be released into the headspace. Of these 22 compounds, the majority were mono- and sesquiterpenoids. I only considered compounds that were 'likely emitted from storage' as they form the unstressed constitutive emitted blend, and are therefore more likely to have an effect on insects specialised to tansy.

There are several advantages to the chemotyping methods presented in this work. The above criteria were defined so as to exclude compounds that are ubiquitous across all plant

species, and to include those that are constitutively stored by unstressed plants. The chemical profile of a plant freshly elicited by aphid feeding might be more variable and less useful. In practical terms this means that compounds that provide little information to specialised herbivores, such as LOX products, were disregarded. Previously, tansy chemotypes have been functionally investigated using only a single dominant compound as the defined chemotype. This oversimplification may have resulted in information being lost, as it is now known that the overall blend of compounds is important to herbivores, not just the main dominant compound. The chemotype groups in the present body of work were formed on the basis of the overall pattern of VOCs detected in each plant, both qualitatively and quantitatively.

In order to determine whether the volatile profile found in hexane extracts were stable across clones, I propagated seven tansy plants in the greenhouse for investigation. I split each plant into five daughter clones, and qualitatively and quantitatively assessed their chemical profiles using GC-MS. All of the same compounds were found in each set of the daughter clones, with variation in the concentrations of each terpenoid averaging 15% within clones of the same genotype. This finding indicates that, allowing for handling and technical variation, there is a high level of chemotypic stability. The indicated stability of the stored chemotype means that tansy plants can be manipulated for experiments investigating chemotype, and that each plant measured in the field experiments could be assigned a chemotype. As tansy exhibits rhizome growth, it is a very suitable plant for propagating via splitting; only rarely did daughter clones fail to establish after splitting.

Manuscript I shows, for the first time, the extent of chemotypic variation among tansy plants in a small field site. It is well documented (Benedek, Bálint, Salamon, Kovács, Ábrahám, Fazakas, Loxdale and Balog 2015, Holopainen, Hiltunen and von Schantz 1987, Keskitalo, Pehu and Simon 2001) that tansy plants exhibit high levels of intraspecific chemical variation, however the majority of studies so far have investigated tansy plants separated over larger geographic scales. The field site used in this body of work was less than 1 km^2. In the field, plants were not clustered geographically according to their chemical profiles; rather individuals with similar chemotypes were randomly distributed (analyses performed by S. Zytynska).

Using only compounds that were likely emitted from storage, 176 plants were clustered into four groups. Three of the four assigned groups had profiles that were dominated by one compound; their profile contained one compound that constituted over 40% of the whole. Correlation analysis of all compounds likely emitted from storage showed that there is a high correlation of specific terpenoids to one another. Mono- and sesquiterpenoids that are closely linked biochemically were highly correlated, for example α- and γ- terpinene, camphene and camphor, and β-cubebene and α-amorphene, indicating tightly linked biosynthetic pathways.

The main dispersal event for *M. fuscoviride* is at the beginning of June/end of May, when aphids produce winged morphs. During this time period most plants are uncolonised and aphids are free to select whichever host plant they choose. Late in the season when unwinged morphs are produced, chemotype no longer had an effect on host selection. It is likely that plant chemotypes are useful for aphid orientation during this dispersal phase as it has been shown the sensory systems of winged aphids are more developed than unwinged aphids (Braendle et al. 2006).

4.2 Field study: metabotyping

Once aphids have selected an initial tansy plant using the volatile chemotype as an olfactory cue, they will perform test probes to finally decide if the plant is an acceptable host or not (Pettersson et al. 2007). Aphids can reject a plant as a host for a variety of reasons, from unacceptable nutrient levels to high amounts of secondary metabolites (Züst and Agrawal 2016). Using non-targeted metabolomic analysis of non-volatile compounds in tansy plants, I was able to classify 122 plants into five metabotypes. The results show that, in contrast to volatile chemotype, metabotype is associated with aphid colonisation late in the season, when winged morphs are no longer produced. Further, the data indicates that metabolic differences between plants that were colonised or not were not caused by induction following aphid infestation, rather they indicate constitutive differences. This gives credence to the idea that specialised insects use volatile cues to find their host species, and select their ideal host plant based on the non-volatile metabolic profile.

Using the same leaf material that was already processed for chemotyping, which removed the majority of lipid compounds, I extracted and analysed 122 plant samples. The list of exact masses was processed by M. Witting (HMGU BGC) using an in-house version of MassTRIX to obtain a list of putative annotations. In total (including non-discriminant features), 851 (13%) and 2,340 (10%) features were tentatively annotated in negative and positive ionisation mode respectively. This small percentage of annotations is a common issue when working with metabolomics data (Böttcher et al. 2008), however the mass difference enrichment analysis (MDEA) performed by F. Moritz (HMGU BGC) (Forcisi et al. 2015, Moritz, Kaling, Schnitzler and Schmitt-Kopplin 2017) supported the annotations as, for example, reactions involving amino acids and phenolic compounds were strongly upregulated in plants that were and were not colonised by aphids. I categorised the tentatively annotated features into 14 groups based on their chemical super classes according to HMBD and KEGG. A large number of flavonoid and phenylpropanoid compounds were tentatively annotated in negative ionisation mode, as was expected, whereas lipid compounds (including amino acids) dominated in positive ionisation mode. Following statistical reduction, I removed a large portion of non-significant features, culminating in a total (both modes of ionisation) of 1,020 discriminant mass features.

Analysis of similarities, performed by S. Zytynska, revealed that the metabolic profiles of plants that were colonised at some stage during the growth season clearly differed from those that were never colonised by aphids. Using multivariate analyses (PCA and OPLS) I was able to show that the metabolic profiles of colonised plants did not differ depending on how long the plant had been colonised prior to harvesting (whether they had been colonised already for several weeks or only one week), indicating that the differences between colonised and uncolonised plants was unlikely to be driven by the induction of compounds following aphid feeding. Interestingly, this suggests that aphids respond to rather than cause the observed differences between the metabolomes of colonised and non-colonised tansy plants.

A number of compounds important in plant defence were tentatively annotated in the dataset prior to statistical reduction. Salicylic acid, isoleucic acid, and pipecolic acid, were found to be present in the dataset, but were not found to be statistically significant (VIP <

1; $p > 0.05$). Although these compounds were not included in the working dataset, they contributed to the grouping seen between plants that were colonised by aphids and those that were never colonised in a PLS biplot. It is possible that aphids select plants because of constitutive differences, or perhaps a long-lasting induction of defence compounds, which could reflect the fitness of the plant. That said, it cannot be discounted that these differences are simply induced by aphid feeding, as these compounds have previously been shown to be induced by aphid feeding (Morkunas et al. 2011).

Manuscript II describes for the first time the scope of non-volatile metabolic variation among tansy plants in a small field site. I performed cluster analysis on a combined dataset of the discriminant mass features from both the negative and positive ionisation modes of all plants (irrespective of colonisation status). The analysis showed that although each plant is highly specific in its metabolic composition, a grouping based on metabolic similarities could be performed, leading to the classification of five metabotypes. The five metabotype classes were designated as A, B, C, D, and E. Minimal evidence for an association between chemotypes and metabotypes was found (analyses performed by S. Zytynska). All metabotypes (A-E) were found within each chemotype (1-4). Chemotypes 1 and 2 are more likely to be colonised early in the season when winged aphid morphs are found. We found that late in the season (when winged morphs are no longer produced) aphids were more likely to colonise plants belonging to metabotypes C and E, irrespective of the volatile chemotype. Almost every plant belonging to metabotypes C and E was colonised by the end of the season. I found a strong separation between metabotypes C and E and metabotypes A, B, and D. From the statistically discriminant masses (VIP > 1, p-value < 0.05 in t-test), the majority of compounds that drive this separation are flavonoids and phenolics. Metabotypes C and E, which were most frequently visited and colonised by aphids, exhibited metabolic enrichments of these compounds.

Plant-sap feeders such as aphids typically induce the salicylic acid (SA) pathway of defence (Moran and Thompson 2001, Morkunas, Mai and Gabryś 2011), whereas chewing herbivores generally induce production of jasmonic acid (JA) and ethylene (Onkokesung et al. 2010). Aphid performance is negatively affected by JA-induced defences, but is less reliably negatively affected by SA-induced defences (Zhang et al. 2017). These two defence

pathways participate in extensive crosstalk, which is often antagonistic (Schweiger, Heise, Persicke and Müller 2014). Pipecolic acid (a positive regulator of SA production and defence amplifications (Bernsdorff et al. 2016)), isoleucic acid (triggers production of SA (Maksym et al. 2018)), SA, and methyl salicylate are compounds associated with the SA defence pathway that were tentatively annotated in the complete tansy dataset. These compounds did not pass statistical reductions for significance; however, they are among the compounds driving the separation between metabotypes C and E and metabotypes A, B, D. The occurrence of these compounds in plants that had been colonised by aphids at any stage suggests that SAR was activated. It could not be determined from this study if these plants were exhibiting a long-lasting induction of systemic acquired resistance (SAR) due to aphid infestation or if they simply expressed higher levels of constitutive defences. As the leaf material came from plants that were part of a natural field environment, it's possible that SAR activation could have occurred due to other biotic stresses such as pathogen infection. Field studies have shown that plants often exhibit activation of defence compounds, when compared to plants grown under laboratory conditions (Kigathi et al. 2009).

4.3 Variability in volatile response of different chemotypes to dual herbivory

Plants growing under natural field conditions constantly have to cope with a variety of stresses, both biotic and abiotic. While the previous sections discussed chemotypes in relation to herbivory by a single insect species, here I investigated how different tansy chemotypes respond to attack by both sucking and chewing herbivores. By combining a number of analytical methods and techniques I observed the effects of feeding by *Metopeurum fuscoviride* Stroyan and/or *Spodoptera littoralis* Boisduval on the volatile emissions of five tansy chemotypes.

In this experiment, aphid feeding did not have a strong measured effect on tansy VOC emissions or production, which is likely due to the feeding mechanism of aphids; as they are sap suckers they do not cause mechanical damage to the plant, nor do they disrupt oil-filled glandular trichomes. Previous studies have shown aphid feeding to have variable effects on plant VOC emissions (Holopainen and Gershenzon 2010, Unsicker et al. 2009). A study by Schwarzberg and colleagues (Schwartzberg and Tumlinson 2014) found that

feeding by the pea aphid (*Acyrthosiphon pisum*) on its host plant *Vicia faba* did not induce any measurable changes in VOC emissions, while Staudt and colleagues (Staudt et al. 2010) found that in peach cultivars, exposure to the green peach aphid (*Myzus persicae*) resulted in quantitative and qualitative differences in VOC emissions, and that these differences were genotype-specific. Another reason that feeding by *M. fuscoviride* had such minimal effects on tansy VOC emissions might be related to the specialised relationship between plant and aphid. As *M. fuscoviride* is a tansy specialist, they might have co-evolved to lessen negative effects on each other. Manuscripts I and II show that aphid selection of host plants is non-random, that aphids actively choose their host early in the season, when winged morphs with heightened olfactory systems are produced.

Caterpillars are chewing herbivores and can cause severe damage to plants by consuming large quantities of plant tissue. After the caterpillars were applied, an immediate increase of VOC emissions was recorded, which was in line with expectations as when glandular trichomes are ruptured they cause an instantaneous release of stored VOCs. I found evidence of defence priming; overall, following caterpillar feeding, plants that had previously been treated with aphids exhibited higher concentrations of VOCs than plants that did not receive aphid treatment, indicating that aphid feeding had an effect on terpene biosynthesis.

Feeding by aphids did not result in strong chemical changes, however caterpillar feeding increased both emitted and stored volatile compounds in each chemotype. Caterpillar feeding following aphid infestation had more varied effects on the stored and emitted compounds of each chemotype in comparison to plants that had not been previously exposed aphid feeding. Interestingly, I found that the responses to dual herbivory were chemotype-specific; feeding by aphids precedent to caterpillars further increased concentrations of stored VOCs in plant chemotypes 1, 4, and 5, and increased concentrations of VOCs emitted to the headspace in plant chemotypes 3, 4, and 5. Thus, each chemotype exhibited either reduced or increased emissions depending on whether they had previously been exposed to aphid feeding.

Real-time measurements of VOCs emitted to the headspace confirm that aphid feeding did

not have a strong effect. Immediately following application of caterpillars, the emission rates of all measured masses increased. Transient increases in emissions were also observed; the caterpillars consumed leaf tissue resulting in random bursts of volatiles (monoterpenes and green leaf volatiles) during both day and night. These fluctuations indicate that the immediate dispersion of volatile compounds following disruption of trichomes was a major cause of emissions. Plants that had been pre-treated with aphids displayed a global trend towards higher emissions of monoterpenes (*m/z* 137.133), oxygenated monoterpenoids (*m/z* 135.116, *m/z* 151.112, *m/z* 153.128), and DMNT (a C11 homoterpene; *m/z* 151.149). Across all chemotypes, closely related compounds tended to increase simultaneously, indicating a tightly linked biochemistry. Using the hexane extraction method, I was able to measure the local response to herbivory. The increase in concentration of compounds found in the lipophilic extracts indicates an increased level of synthesis as well as storage of compounds, which contributed to the increase of emissions to the headspace.

Transcriptome analysis performed by G. Haberer (HMGU PGSB) represents the first *de novo* assembly of a tansy transcriptome. Using RNA-Seq we were able to examine transcriptional changes following dual herbivory; statistical analysis performed by G. Haberer found 592 differentially expressed genes (DEGS: $p < 0.05$). I found that in treatment group (a) that NAC transcription factor 56 was upregulated. NAC56 is involved in plant responses to abiotic / biotic stress (Zhang et al. 2020), and is activated by the JA signalling pathway. A study performed by Chen and colleagues showed that in *Brassica napus* the NAC transcription factor BnNAC56 induced hypersensitive response (HR) like cell death (Chen et al. 2017). Plants initiate a similar defence response to aphids as they do to pathogens (Morkunas, Mai and Gabryś 2011), and it is interesting that DEGs commonly associated with pathogen infection were upregulated in plants that received the aphid treatment. I found that in treatment group (*c*) the receptor-like protein kinase FERONIA was downregulated while a lipase was strongly upregulated. As caterpillar feeding induces the JA defence pathway it is not unexpected that FERONIA was downregulated as it is an antagonistic regulator of JA signalling (Guo et al. 2018). In treatment group (*ca*) I found the highest number of DEGs of all treatment groups. These plants were treated with both caterpillars and aphids, and as such transcripts associated with defence were upregulated.

FERONIA was upregulated as were other DEGs involved in cell wall processes (laccase-7 and endochitinase EP3). Indole-3-acetic acid-amido synthetase GH3.5, which is responsible for the synthesis of indole-3-acetic acid (IAA) was downregulated. IAA aids plants in coping with an excess of auxin, and is involved in a mechanism that inhibits cell wall loosening and cell growth, thereby conferring an enhanced resistance to pathogens. Aphid feeding significantly modifies cell wall metabolism (Foyer et al. 2015). In treatment group (*d*) I did not find many DEGs, however FERONIA was very highly upregulated. Due to fluctuations in gene expression, it cannot be ruled out that some possible synergistic interactions between aphid and caterpillar attack were overseen.

As tansy exhibits such diversity in its terpenoid content, transcripts with high similarity to terpene synthases (TPS) found in the *de novo* assembled transcriptome were examined. Using known sequences from closely related plant species (such as other Asteracea species, *Helianthus annuus* and *Artemisia annua*) and the putatively annotated tansy TPS genes, I performed a phylogenetic analysis. This showed that the putative tansy TPS genes had high similarity to the known TPS subgroups. Putative TPS genes were found in the TPS-b (i.e. monoterpene synthases), TPS-a (sesquiterpene synthases), and TPS-f (linanool synthases) (Chen, Tholl, Bohlmann and Pichersky 2011, Jiang et al. 2019). Overall I was able to show that the putative tansy TPSs likely belong to the predicted TPS subfamilies as seen in manuscript III.

5 Conclusion

The chemical ecology of tansy is complex and requires the combination of several areas of study to attempt to untangle it. The results presented in this thesis aim to bridge a gap in our understanding of how tansy interacts with specialist herbivores on a small geographical scale, and how different chemotypes respond to herbivory by multiple species of attackers. As shown in this body of work, tansy plants can be classified according to their chemotype, and that these chemotypes play an important role in how the plant interacts with a specialist aphid. Tansy plants can also be classified according to their non-volatile metabolic profiles, showing that untargeted metabolomics analysis can be applied to plants in the field and give important information about how plants interact with their local community. Furthermore, as well as showing that tansy plants can be meaningfully grouped according to both their volatile and non-volatile chemical profiles, this thesis shows that different chemotypes respond to dual herbivory in different manners. The method of VOC chemotyping presented here can be adapted for other plant species, thereby allowing for the *in situ* chemical profiling of multiple populations at different geographic locations. Some tansy chemotypes respond more "loudly" to herbivory stress than others, a phenomenon that might also occur in other species and could potentially be used to exploit plant-plant communication in pest biocontrol. By providing the first report to date of a tansy transcriptome, the results presented here could act as a scaffold for future studies on terpene synthase genes in tansy and other closely related plant species. The chemotype and metabotype of an individual plant gives valuable information to herbivores, enabling them to first locate their host species and then to hone in on the most suitable individual plant; factors that are important when considering how plants interact with their local environment and the metacommunity dynamics of a population. The results presented in this thesis give us a greater understanding of the chemical ecology of tansy and provides the groundwork for future investigations into the chemical ecology of other plant systems.

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