Study of drimane sesquiterpenoids from the *Persicaria* genus and zingiberene from *Callitropsis nootkatensis* and their effect on the feeding behaviour of *Myzus persicae* and *Bemisia tabaci*

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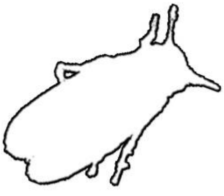
Chapter 1

General introduction

"All the pests that out of earth arise, the earth itself the antidote supplies"
– Lithica poem, c. 400 B.C. (Dayan et al., 2009)



1. Silverleaf whitefly: a global agricultural problem

Whitefly is a general name for at least 1556 species in 161 genera of insects from the order Hemiptera, family Aleyrodidae (Martin and Mound, 2007). The silverleaf whitefly, *Bemisia argentifolii* Bellows & Perring (synonym: *B. tabaci* Gennadius, strain B), the greenhouse whitefly, *Trialeurodes vaporariorum*, and the bandedwinged whitefly, *Trialeurodes abutilonea*, are the most common whitefly pests. Among these three, the silverleaf whitefly (Fig. 1.1) is usually the biggest problem (Jones, 2003). This species was first noticed on cotton in India in 1905 (Byrne and Bellows, 1991). It thrives worldwide in tropical, subtropical, and less predominantly in temperate habitats. Environments with cold temperatures often lead to mortality of both adults and larvae of the species. Nevertheless, it has spread from the Mediterranean basin to greenhouses at higher latitudes in Central and Northern Europe, claiming habitat there as well (Martin et al., 2000; Gerling et al., 2001).

1.1. Biology

Whiteflies are all plant feeders with piercing, sucking mouthparts that face backwards (opisthognathus). Adults of both sexes have four membranous wings and they undergo incomplete metamorphosis (Byrne and Bellows, 1991). These insects excrete honeydew through their anus into a depression named vasiform orifice which ‘flips’ the honeydew away (Byrne, 2003). *Bemisia* honeydew is composed to a large extent of trehalulose, a disaccharide, which is thought to aid in osmoregulation (Byrne, 2003). All life stages except the egg can produce extracuticular waxes that cover the body, and in the adults this wax forms tight curls of threads, approximately 1µm in diameter giving them their white appearance; the wax consists primarily of triacylglycerols (65-75%) with a trace of wax esters, free fatty acids, alcohols and hydrocarbons (Byrne and Hadley, 1988).

Adults have remarkable abilities to disperse (at least 7 km in a few hours). In terms of flight mechanics, *Bemisia* relies on the ‘clap and fling’ mechanism. Wingbeat frequencies vary with gender and temperature (Byrne, 2003). Sexual dimorphism in adult body dimension is usual for members of Aleyrodidae: *B. tabaci* males are smaller, measuring 1.81 ± 0.06 mm, while females measure 2.13 ± 0.06 mm (Byrne and Bellows, 1991).

Females can lay over 200 eggs and live up to 1.5 months. Tiny fusiform eggs are often laid in a circular or semi-circular pattern (**Fig. 1.1**); they hatch in about 10-12 days at temperatures between 18 and 24 °C.

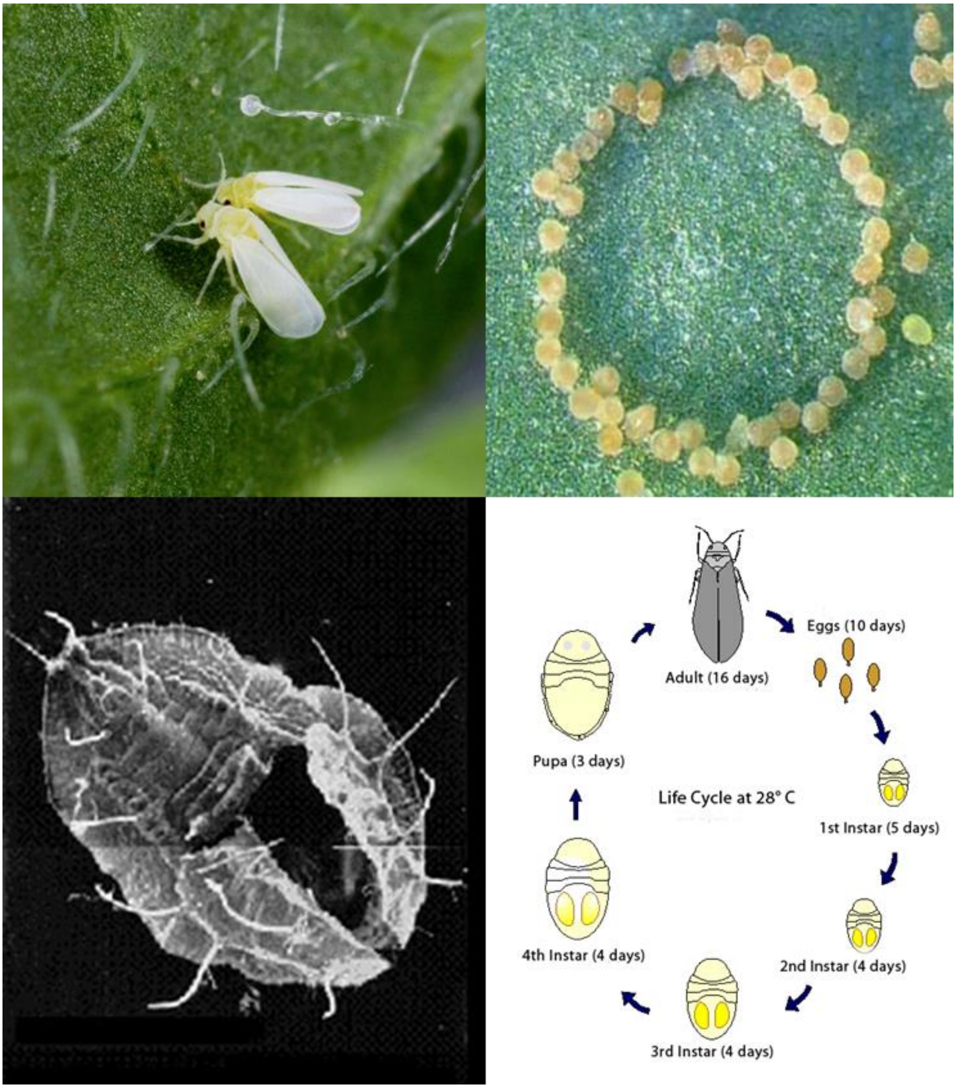


Figure 1.1. The whitefly *Bemisia tabaci*. From top to bottom and from left to right: male (smaller) and female adults feeding on a leaf; circular pattern of eggs laid by B. tabaci; exuvia of *Bemisia* that is left after adult emergence; *Bemisia* life cycle.

Sex ratios of the offspring of mated females are at parity in the egg stage, but may change with life stage and environmental circumstances (Byrne, 2003). Eggs are attached to leaves by pedicles, through which plant material is absorbed. First instar nymphs are mobile (crawlers); later nymphal instars have been only observed moving during the transitional stage between stadia. The fourth nymphal instar is unusual: the nymph acquires a red pigment in the eyes and it is during this stage that this hemimetabolous insect changes from a nymphal state into adulthood (Gerling and Mayer, 1996) (**Fig. 1.1**). As adults emerge, what is left is an exuvia with a characteristic T-shaped hole (**Fig. 1.1**). Under a constant temperature of 29.5 ± 0.6 °C and a photoperiod of 14:10 LD, 90% of *B. tabaci* adults emerged from their pupal cases between 6.00 AM and 9.30 AM (lights on occurred at 6.00 AM). Under a series of constant temperatures, a significant inverse correlation was found between the time of median emergence and temperature. No emergence was observed at temperatures below 17 ± 0.3 °C. Emergence patterns persisted under conditions of continuous light or darkness, suggesting the presence of a circadian regulator. A female can begin to lay eggs from one to four days after eclosion (Byrne and Bellows, 1991).

Most species of whiteflies reproduce by arrhenotoky (a form of parthenogenesis in which unfertilized eggs develop into males) and *Bemisia tabaci* is no exception. Unmated females, therefore, produce male offspring (XO), while mated females produce both males and females (XO and XX) (Byrne and Bellows, 1991). For mating to occur, silverleaf whitefly undergoes a very complex courtship before the actual copulation; copulation lasts 125 – 265 s. Polygyny and polyandry are common in this species (Li et al., 1989).

B. tabaci, like many other whiteflies, does not rely heavily on leaf shape, structure and odour for host plant localization, but primarily uses colour as a cue. After a host is selected, entry into the leaf by the stylet bundle is a complicated process (Byrne and Bellows, 1991). Most mouth parts enter the plant by piercing the epidermal cells; after passing through the epidermis, the stylets traverse the intercellular spaces between mesophyll parenchyma cells *en route* to the phloem tissue (Byrne, 2003).

1.2. Damage to crops

Silverleaf whiteflies not only damage plants directly, by extracting large quantities of phloem sap, which can result in greater than 50% yield reductions (Byrne and Bellows, 1991); they also cause secondary damage through honeydew excretion that enables sooty mould development, which in turn blocks sunlight and reduces photosynthesis (Inbar and Gerling, 2008). In addition, they act as a vector for more than a hundred viruses, including *Tomato yellow leaf curl virus* (**Fig. 1.2**) and *Tomato mottle virus*, which causes yield losses that cannot be controlled chemically once plants are infected (Faria and Wraight, 2001). The most threatened crops belong to the Cucurbitaceae, Leguminosae, Euphorbiaceae, Malvaceae and Solanaceae (Martin et al., 2000).

An example of economic loss due to honeydew excretion is cotton (**Fig. 1.2**): when this plant is contaminated, it becomes difficult and sometimes impossible to process the fibre at the textile mill; honeydew residues remain localized on the lint which slows down the processing and necessitates extra machinery cleaning (Henneberry and Forlow Jech, 2003).

The constitutive and induced host plant chemical defences can substantially reduce the population levels (and damage) of phytophagous insects. Insects that can avoid, overcome, or even utilize such defences gain a significant adaptive advantage. It has been found that defence responses of cotton and tomato, which were induced with elicitors, artificial wounding and insect herbivory, that usually have negative effect on various insects, have no effect on whiteflies. It has been also shown that geminivirus increases the fecundity of *Bemisia*. Thus, *Bemisia* and viruses manipulate the host plant to their own benefit suggesting mutualistic relationships (Inbar, 2003).


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Figure 1.2. Damage produced by whitefly infestation. Left: tomato plant infected by *Tomato yellow leaf curl virus*, one of the Geminiviruses vectored by whitefly; right: cotton flower developing sooty mould on the sticky honeydew excreted by whiteflies.

2. Pest-management approaches

2.1. History of agriculture

As nomadic populations of hunter-gatherers settled to form communities that are more permanent (20.000-10.000 years BC) the need for producing more food within a particular area arose (although the two phenomena likely developed in parallel rather than one being the cause of the other). The first forms of agriculture were most probably practiced in areas around dwellings and on alluvial deposits resulting from receding flood waters, that is, on ground already fertilized and requiring hardly any clearing (Mazoyer and Roudart, 2006). From that moment on and for thousands of years, agricultural practices relied heavily on crop rotation or mixed crop planting, which not only optimized soil fertility, but also natural pest control (such as predation, parasitism, and competition) (Martin and Sauerborn, 2013). Therefore, the concept of 'natural pesticides' arose early in the development of agriculture. As proof of that, there are written records by Greek, Roman and Chinese scholars ranging from the 4th century B.C. until about the 3rd century A.D. (Dayan et al., 2009).

The European agricultural revolution that followed in the 17th - 19th century (the so-called first agricultural revolution of modern times) was characterized by systems of cereal and feed grain cultivation without fallowing (Mazoyer and Roudart, 2006) and it was accompanied by more extensive and international trade that resulted in the discovery of botanical insecticides from pyrethrum (*Tanacetum cinerariifolium)* flower heads and *Derris* root which contain pyrethrins and rotenone, respectively (Dayan et al., 2009). The advent of extensive monoculture and intensive agricultural practices of the 20th century, characterized by motorization, mechanization and the use of mineral fertilizers, was accompanied by increases in yields (Glaeser, 2010). New cultivars were selected based on their higher yields, but many of these lines appeared to have lower resistance to pests, as there is often a trade-off between yield and defence (Herms and Mattson, 1992; Evenson and Gollin, 2003; Kooke and Keurentjes, 2012). This resulted in greater pest pressures, which has mostly been addressed by the use of synthetic pesticides.

2.2. Synthetic pesticides

Synthetic pesticides have been developed throughout the 20th century.

Major classes of synthetic pesticides are: organochlorides, organophosphates, carbamates, pyrethroids, neonicotinoids and ryanoids (**Fig. 1.3**).

2.2.1. Organochlorides

The insecticidal properties of the best-known representative of this class of insecticides, DDT (**Fig. 1.3E**), was made by the Swiss scientist Paul Müller. For this discovery, he was awarded the Nobel Prize for Physiology or Medicine in 1948. DDT was introduced on the market in 1944. The contemporary rise of the chemical industry facilitated the large-scale production of DDT and related chlorinated hydrocarbons. DDT functions by opening the sodium channels in the nerve cells of the insect (Green et al., 1987; Davies et al., 2007).

2.2.2. *Organophosphates and carbamates*

Another large class of synthetic insecticides are the organophosphates. These, too, target the insect's nervous system. Organophosphates interfere with the enzymes acetylcholinesterase and other cholinesterases, disrupting nerve impulses, and thereby killing or disabling the insect (Green et al., 1987). Organophosphate insecticides and chemical warfare nerve agents (such as sarin, tabun, soman and VX) work in the same way. Organophosphates have an accumulative toxic effect to wildlife, so multiple exposures to the chemicals amplify the toxicity. In humans, they cause severe acute poisoning, from which it is possible to recover with adequate therapy, and can have equally severe chronic neurological consequences (Kwong, 2002). The most prominent organophosphate, banned in over 23 countries due to its toxicity, is parathion, or E605 (**Fig. 1.3B**).

Carbamate insecticides have similar toxic mechanisms to organophosphates, but have a much shorter half-life and are thus somewhat less toxic (Kwong, 2002). An example is carbaryl (**Fig. 1.3A**).

2.2.3. *Pyrethroids*

Pyrethroid pesticides have been developed to mimic the insecticidal activity of the natural pyrethrum. Compared to their natural counterpart (described in more details in section 3.5.3 of this chapter), synthetic pyrethroids display stronger toxicity and better resistance to detoxification, oxidation and UV light. These compounds are sodium channel modulators, and are much less acutely toxic than organophosphates and carbamates (Davies et al., 2007). They are less persistent than other synthetic insecticides, but more than natural pyrethrins. Pyrethroids are amongst the most widely used synthetic pesticides in agriculture, and are often applied against household pests. Nevertheless, they can still cause acute toxicity in vertebrates, and repetitive occupational exposure produces severe poisoning symptoms in humans (Vijverberg and van den Bercken, 1990). The most salient member of this group is permethrin (**Fig. 1.3C**).

2.2.4. *Neonicotinoids*

Neonicotinoids are synthetic analogues of the natural insecticide nicotine (with a much lower acute mammalian toxicity and greater field persistence). These chemicals are nicotinic acetylcholine receptor agonists. Treated insects exhibit leg tremors, rapid wing motion, stylet withdrawal (aphids), disoriented movement, paralysis and death. These broad-spectrum insecticides have a rapid

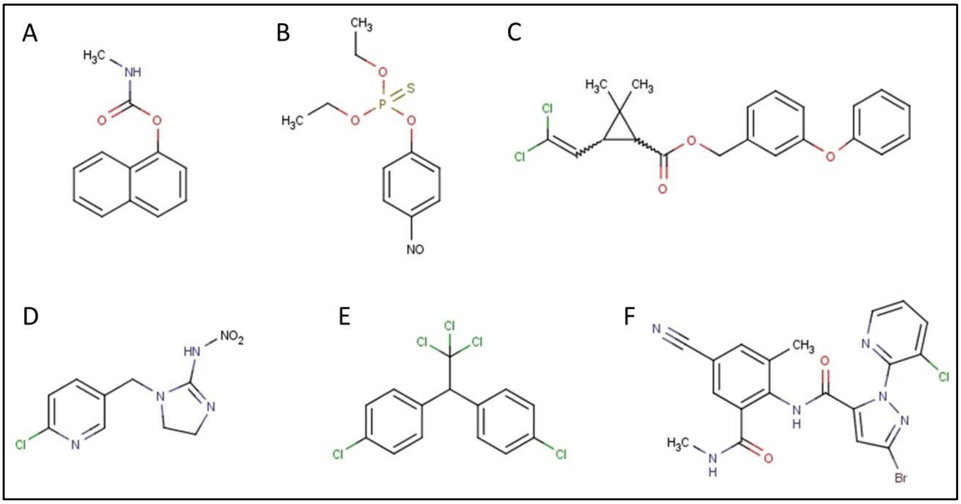


Figure 1.3. Examples of synthetic insecticides: A) carbaryl (a carbamate), B) parathion (an organophosphate), C) permethrin (a pyrethroid), D) imidacloprid (a neonicotinoid), E) DDT (an organochloride), F) cyantraniliprole (a ryanoid)

action (minutes-hours) and are usually applied as sprays, drenches, seed and soil treatments (Tomizawa and Casida, 2005). From recent literature it appears that Spanish populations of *Bemisia tabaci* developed resistance to neonicotinoids (Elbert and Nauen, 2000). Imidacloprid is the most commonly used neonicotinoid (**Fig. 1.3D**). However, it has recently come under scrutiny for its speculated deleterious effects on honeybees (*Apis mellifera*) (Decourtye et al., 2004; Iwasa et al., 2004; Yang et al., 2008), as have other neonicotinoids (Sandrock et al., 2014).

2.2.5. Ryanoids

Ryanoids are synthetic analogues with the same mode of action as ryanodine, a naturally occurring insecticide extracted from *Ryania speciosa* (Flacourtiaceae) (Rogers et al., 1948). They bind to calcium channels in the cardiac and skeletal muscle, blocking nervous transmission (Benner, 1993). One major example is cyantraniliprole (**Fig. 1.3F**) (Selby et al., 2013), successfully adopted, among others, against Bemisia tabaci (Caballero et al., 2013).

2.3. Alternatives to synthetic pesticides: integrated pest management

Use of synthetic insecticides led to numerous problems unforeseen at the time of their introduction: acute and chronic poisoning of applicators, farmworkers, and even consumers; destruction of fish, birds, and other wildlife; disruption of natural biological control and pollination; extensive groundwater contamination, potentially threatening human and environmental health; and the evolution of resistance to pesticides in pest populations. Governments responded to these problems with the introduction of new pesticide registration procedures, such as the Food Quality Protection Act in the United States. These new regulations have reduced the number of synthetic pesticides available in agriculture. Therefore, the current paradigm of relying almost exclusively on chemicals for pest control may need to be reconsidered (Dayan et al., 2009). In addition, the development of pesticide resistance in insects, due to persistent use, has rendered this need for alternatives – which include plant derived insecticides – ever more urgent (Isman, 2006).

Integrated Pest Management (IPM) is an ecosystem-approach to crop production and protection that combines different management strategies and practices to grow healthy crops and minimize the use of pesticides (Kogan, 1998). It is defined as the selection, integration and implementation of pest control based on predicted economic, ecological and sociological consequences, and as such it makes maximum use of naturally occurring control agents, including weather, disease organisms, predators and parasites (Bottrell, 1979). IPM aims to suppress pest populations below the economic injury level (EIL) (Higley and Pedigo, 1993). As defined by the Food and Agriculture Organisation (FAO) of the UN, IPM is "the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms" (FAO, 2013). IPM includes managing insects, plant pathogens and weeds.

The most effective IPM programs are based on an ecological understanding of the pests’ interactions with the crops, other organisms, and the environment (Dufour, 2001). The principles of IPM include: a) setting of action thresholds (acceptable pest levels), with emphasis on control rather than eradication; b) monitoring and identification of the pest by regular observation and record-keeping; c) prevention through the selection of best varieties for local growing conditions, as well as soil ecosystem management; d) control strategies adoption from less risky to most risky (mechanical control, followed by biological control, followed in turn by responsible use of synthetic pesticides if the less risky approaches failed to achieve the desired result) (U.S. Environmental Protection Agency, 2012).

2.3.1. Beneficial insects

Although insecticides remain the primary tactic for managing *B. tabaci* in cotton and other affected crops (Palumbo et al., 2001), considerable research has focused on the development of alternative control tactics, including the use of biological control (Gerling and Mayer, 1996; Naranjo and Ellsworth, 2001). Recent reviews have catalogued 114 species of predatory arthropods, nearly 50 species of parasitoids, and 11 species of naturally occurring fungi known to be associated with *B. tabaci* worldwide (Faria and Wraight, 2001; Gerling et al., 2001).

Beneficial insects can be used in pest control to complement other strategies. They can be predators or parasites (**Fig. 1.4**). The three strategies adopted in biological control using beneficial insects include importation, augmentation and conservation. Importation involves the introduction of a pest's natural enemies to a new area where they do not occur naturally; this is often necessary when a pest had been accidentally introduced into a new geographic area without its associated natural enemies. Augmentation involves the supplemental release of natural enemies, boosting the naturally occurring population; it can be done by inoculative or inundative release, depending on the numbers of insects released at a given time. Conservation of existing natural enemies is usually a simple and cost-effective strategy to implement enemies already adapted to the habitat of the target pest; this can be achieved by modifying the cropping systems to favour the natural enemies, like for instance providing a suitable habitat for other life stages than the one that targets the pest (Rechcigl and Rechcigl, 1999).

Figure 1.4. Left: a predator of insects such as aphids, *Coccinella septempunctata*; right: tomato leaf with whitefly nymphs (white) parasitized by *Encarsia formosa* (black)

Predators are mainly free-living species that directly consume a large number of preys during their whole lifetime. They belong to the orders Coleoptera, Diptera, Hemiptera, Hymenoptera and Neuroptera. Other useful predators include centipedes, spiders and predatory mites (Metcalf and Luckmann, 1994).

Parasitoid insects lay their eggs on or in the body of an insect host, which is then used as a food for developing larvae. All developmental stages of the host are subject to attack, though only a few species parasitize adult insects. Most parasitoids are Dipterans or Hymenopterans and usually have a very narrow host range. They are among the most widely used biological control agents. The most well-known examples are *Encarsia formosa* (**Fig. 1.4**), *Eretmocerus* spp., *Aphidius colemani*, *Gonatocerus ashmeadi* (Metcalf and Luckmann, 1994), some of them (*Encarsia* spp. and *Eretmocerus* spp.) known to parasitize *B. tabaci* as well (Gerling et al., 2001).

Beneficial insects can be attracted to the attacked plant by volatile compounds released upon herbivory (Snoeren et al., 2010). This phenomenon, which goes under the name of tritrophic interaction, has been the target of specific plant modifications that resulted in higher production of the desired compound and improved attraction of predators and parasitoids (Kappers et al., 2005; Beale et al., 2006; Houshyani et al., 2013).

The flip-side of using beneficial insects in pest control is that it can have an impact on the native biodiversity (Louda et al., 2003) when it is introduced into an area which is non-native to the insect.

2.3.2. Biopesticides

Biopesticides, a contraction of “biological pesticides”, include several types of pest management interventions. In the EU, biopesticides have been defined as “a form of pesticide based on micro-organisms or natural products” (European Commission DG Environment News Alert Service, 2008). The US EPA definition is broader and it encompasses also “pesticidal substances produced by plants containing added genetic material (Plant-incorporated protectants)” (U.S. Environmental Protection Agency, 2013). Biopesticides are typically created by growing and concentrating naturally occurring organisms (for example, bacteria, fungi, nematodes, plants, *etc.*) and/or their metabolites or proteins. They can be categorized into three major groups:

- Microbial pesticides, consisting of bacteria, entomopathogenic fungi or viruses and entomopathogenic nematodes (despite the latter being multi-cellular organisms)

- Biochemical pesticides, which encompass plant-derived pesticides and other natural substances that affect insect behaviour (pheromones, repellents, antifeedants, toxins, etc.)
- Plant-incorporated protectants (PIPs), which are the result of genetic modification of the host plant

2.3.2.1. Microbial control

Microbial pest control approaches include the use of bacteria, fungi, viruses, protozoans and nematodes, as well as chemical products (such as toxins) derived from them (Lacey and Goettel, 1995). The leading microbial pesticide, *Bacillus thuringiensis*, is a ubiquitous gram-positive, spore-forming bacterium that forms a parasporal crystal during the stationary phase of its growth cycle. This insect pathogen owes its insecticidal activity to these parasporal crystals (**Fig. 1.5**), which contain the δ-endotoxin (Cry or Bt toxin), responsible of pore formation in the insect intestinal tract. This observation led to the development of bioinsecticides based on *B. thuringiensis* for the control of certain insect species among the orders Lepidoptera, Diptera, and Coleoptera as well as Hymenoptera, Homoptera, Orthoptera, and Mallophaga (Schnepf et al., 1998). Activity has also been documented against nematodes, mites, and protozoa (Feitelson et al., 1992). Spores and crystalline insecticidal proteins produced by *B. thuringiensis* have been used to control insect pests since the 1920s and are often applied as liquid sprays (Lemaux, 2008). Besides being a useful alternative or supplement to synthetic chemical pesticide application in commercial agriculture, forest management, and mosquito control, *B. thuringiensis* is also a key source of genes for transgenic expression to provide pest resistance in plants (Ferry et al., 2004). Because of their specificity, this class of pesticides are generally regarded as environmentally friendly, with little or no effect on humans, wildlife, pollinators, and most other beneficial insects and are used in Organic farming (Wei et al., 2003). There have been, however, reported risks on non-target insects (Losey et al., 1999), as well as negative effects on mammalian liver and kidneys (Kılıç and Akay, 2008). Another example of microbial control is Spinosad, a commercial product consisting of the mixture of spinosyn A and spinosyn D, produced by the soil actynomycete *Saccharopolyspora spinosa*. These macrolides have shown activity against several orders of insects as well as mites by acting as neurotoxins (Salgado, 1998).Other microbe-derived biopesticides include products based on entomopathogenic fungi (e.g. *Beauveria bassiana* in **Figure 1.5** and *Metarhizium anisopliae*), nematodes (e.g. *Steinernema feltiae*) and viruses (e.g. *Cydia pomonella granulovirus* and *Spodoptera exigua nuclear polyhedrosis virus*) (Coombs, 2013).

Figure 1.5. Microbial insecticides. Left: bipyramidal crystals produced by *Bacillus thuringiensis*; right: hemipteran insect infested by the entomopathogenic fungus *Beauveria bassiana*.

2.3.2.2. Semiochemicals and insect growth regulators

Smiochemicals, from the Greek *semeon* meaning signal, are chemicals that act as messengers within or between species. They can be pheromones, allomones (chemicals produced by one species that negatively affect another one), kairomones (substances produced by a species that benefit another species without benefitting the emitting one), attractants and repellents. In pest management, semiochemicals are used either to monitor pest populations to determine if control is warranted or to alter the behaviour of the pest or its enemies to the detriment of the pest. The advantages of using semiochemicals are their relative specificity, low toxicity, low environmental persistence and the difficulty for insects to develop resistance against them (Agelopoulos et al., 1999).

An insect growth regulator (IGR) may be defined, in terms of its mechanism of action, as a substance that acts within an insect to accelerate or inhibit a physiological regulatory process essential to the normal development of the insect or its progeny, in such a way that the action of the substance is necessarily dependent on the life stage of the insect. An IGR need not necessarily be toxic to its target, but may instead lead to an abnormality that impairs the survival of the insect. However, it is important to note that those IGRs which have found practical uses cause the relatively rapid death of the insect through failure in the operation of a key process such as emergence of adults from pupae (Siddall, 1976). The most notable IGR is methoprene, commercialized for the first time in the mid-1970s (Henrick, 2007).

2.3.2.3. Botanical pesticides

The practice of using plant derivatives, or botanical insecticides as we now know them, in agriculture dates back at least two millennia in ancient China, Egypt, Greece, and India. At present, there are four major types of botanical products used for insect control, namely pyrethrum, rotenone, neem, and essential oils, along with three others in limited use (ryania, nicotine, and sabadilla). Additional plant extracts and oils (e.g., garlic oil, *Capsicum* oleoresin) see limited (low volume) regional use in various countries (Isman, 2006). In Section 3 of this introduction, plant-based insecticides, as well as repellents and antifeedants will be discussed in more detail.

2.3.2.4. Plant Incorporated Protectants (PIPs)

According to the US EPA definition, Plant-incorporated protectants (PIPs) are pesticidal substances produced by plants and the genetic material necessary for the plant to produce the substance (U.S. Environmental Protection Agency, 2013). Therefore, they involve genetic modification of the crop of interest to introduce the gene(s) necessary for the pesticidal or repellent compound to be produced. The most salient example of genetically modified (GM) crops, are those producing the Bt toxin (Ferry et al., 2004). In fact, the *cry* gene, responsible for the expression of the toxin, was first successfully introduced into *Nicotiana tabacum* in 1985 (Höfte et al., 1986). Ten years later, the first commercial crop was approved safe by the US EPA, specifically potato producing the CRY 3A Bt toxin; a year later the approval for Bt maize and cotton followed (Betz et al., 2000). These crops provide highly effective control of major insect pests such as the European corn borer, southwestern corn borer, tobacco budworm, cotton bollworm, pink bollworm, and Colorado potato beetle and reduce reliance on conventional chemical pesticides. They have provided notably higher yields in cotton and corn. Other benefits of these crops include reduced levels of the fungal toxin fumonisin in corn and the opportunity for supplemental pest control by beneficial insects due to the reduced use of broad-spectrum insecticides (Betz et al., 2000).

For each new GM crop, strict evaluation is, however, necessary to assess potential undesired effects (Houshyani, 2012). Typical categories of perceived hazards arising from the introduction of transgenic crops include: possible unintended negative health effects in a susceptible subgroup of the consumer population; the development of resistance in the targeted pest/pathogen populations when the transgene confers resistance to a pest or pathogen; non-target effects on other players of the ecosystem, associated directly or indirectly with the transgenic plant or transgene product outside the plant; and those associated with the integration and subsequent expression of the transgene in a different organism or species following gene flow (Craig et al., 2008).

3. Plant-derived products with repellent, antifeedant, or insecticidal properties

Plant-based pest control agents have long been publicised as alternatives to synthetic chemicals for IPM. Such phytochemicals reputedly pose little threat to the environment or to human health. Bioactivity of plant-based compounds is well documented in literature and is a subject of increasing importance (Koul, 2008).

In the context of agricultural pest management, botanical insecticides are best suited for use in organic food production in industrialized countries, but can possibly also play a role in the production and postharvest protection of food in developing countries, if costs can be reduced, for example, by more simple extraction techniques (Isman, 2006).

Antifeedant approaches for insect control have been extensively studied, at least at laboratory level, though only a handful of plant-based compounds are currently used in agriculture. Because of the wide variety of known active plant-based antifeedants (chromenes, polyacetylenes, saponins, cyclopropanoid acids, quassinoids, cucurbitacins, phenolics, glucosinolates, terpenoids, alkaloids), and the idiosyncracy by which each insect species processes these allomones, the same compound may have very different fates and consequences in different species of insects. This implies that the mechanisms involved in antifeedant action can be very different (Koul, 2008). Insect feeding deterrents may be perceived either by stimulation of specialized deterrent receptors (Mullin et al., 1994) or by distortion of the normal function of the neurons that perceive phagostimulating compounds such as sugars. Some plant antifeedants influence the feeding activity through a combination of these two principal modes of action. Only a few highly active antifeedants have been looked into from a commercial point of view, which makes it impossible to systemize or to predict any molecular motifs in feeding inhibition (Koul, 2008). Several factors appear to limit the success of botanicals, most notably regulatory barriers and the availability of competing products (newer synthetics, fermentation products, microbials) that are more cost-effective and regarded as safe (Isman, 2006). Genetically modified plants, which could produce the active antifeedant substances in amounts high enough to protect the plants from further herbivorous damage, could be a possibility in the future (Koul, 2008).

3.1. Essential oils and vegetable oils

Steam distillation of aromatic plants yields essential oils (EOs), long used as fragrances and flavourings in the perfume and food industries, respectively, and more recently for aromatherapy and as herbal medicines (Isman, 2006). The major plant families from which EOs are extracted include Myrtaceae, Lauraceae, Lamiaceae, and Asteraceae (Regnault-Roger et al., 2012). The oils are generally composed of complex mixtures of volatile monoterpenes, biosynthetically related phenols, and sesquiterpenes (Isman, 2006). Examples include 1,8-cineole, the major constituent of oils from rosemary (*Rosmarinus officinale*) and eucalyptus (*Eucalyptus globus*); eugenol from clove oil (*Syzygium aromaticum*); thymol from garden thyme (*Thymus vulgaris*); and menthol from various species of mint (*Mentha* spp.) (Isman, 2000). In recent years, the use of EOs derived from aromatic plants as low-risk insecticides has increased considerably owing to their popularity with organic growers and environmentally conscious consumers. EOs have repellent, insecticidal, and growth-reducing effects on a variety of insects (Regnault-Roger et al., 2012). Specifically, the EO vapours from *Satureja hortensis* L., *Ocimum basilicum* L. and *Thymus vulgaris* L. (Lamiacae) have all shown to be effective insecticides against *Bemisia tabaci* under greenhouse conditions (Aslan et al., 2004). The rapid action of EOs against some pests is indicative of a neurotoxic mode of action, and there is evidence for interference with the neuromodulator octopamine by some oils (Enan, 2001; Kostyukovsky et al., 2002), with GABA-gated chloride channels by others (Priestley et al., 2003), while yet others act through the inhibition of acetylcholinesterase (Miyazawa et al., 1998). With a few exceptions, their mammalian toxicity is low and environmental persistence is short. Registration has been the main bottleneck in putting new products on the market, but more EOs have been approved for use in the United States than elsewhere owing to reduced-risk processes for these materials (Regnault-Roger et al., 2012).

Vegetable oils can also be used as a protective agent against insects. The oils of groundnuts (*Arachis hypogea*), coconuts (*Cocos nucifera*), safflower (*Arthamus tinctorius*), mustard (*Brassica* spp.), castor beans (*Ricinus communis*), hazelnuts (*Corylus avellana*), cotton seeds (*Gossypium* spp.), soy beans (*Glycine max*), neem (*Azadirachta indica*), cucumber (*Cucumis sativus*) and maize (*Zea mays*) have all been used successfully post-harvest on grains and pulses (Singh et al., 1978; Don-Pedro, 1989; Haghtalab et al., 2009; Singh et al., 2012). Though vegetable oils are mostly used on stored grains and pulses, they have been applied to living plants as well. They were effective against whitefly, green peach aphid, cabbage aphid and *Tetranychus* spp., but some of the treated crops suffered damage due to the phytotoxicity of the oils used (Butler Jr and Henneberry, 1990). The most successful vegetable oil is neem oil, obtained by cold-pressing the seeds of the Indian neem tree (Meliaceae). It has proven effective against soft-bodied insects and mites but also in the management of phytopathogens (Isman, 2006).

3.2. Plant extracts

More highly valued than neem oil are medium-polarity extracts of the seed residue after removal of the oil, as these extracts contain the complex triterpene azadirachtin (Isman, 2006). Extracts from a number of aromatic plants have also shown insecticidal and antimicrobial activities. In one study, 345 plant extracts were evaluated for their activity against *Botrytis cinerea*, a fungus known to primarily affect grapes. The outcome showed that extracts of members of the *Allium* and *Capsicum* genera were the most potent fungicides (Wilson et al., 1997). In another study, methanol extracts of *Agastache rugosa* whole plant, *Cinnamomum cassia* bark, star anise (*Illicium verum*) fruit and fennel (*Foeniculum vulgare*) fruit were effective against the tobacco beetle *Lasioderma serricorne* (Kim et al., 2003). These plant extracts, in addition to ones from other aromatic plants, were also effective against rice weevil and bean weevil (Kim et al., 2003).

3.3. Alkaloids

The term ‘alkaloid’ (=alkali-like) is applied rather loosely to denote naturally occurring organic compounds containing nitrogen (mostly in ring structures) and thus displaying basicity (Levinson, 1976). Alkaloids may also contain oxygen, sulphur and more rarely other elements such as chlorine, bromine, and phosphorus. They are characterized by a great structural diversity and there is no uniform classification of alkaloids (Hesse, 2002).

Alkaloids are secondary metabolites produced by a large variety of organisms, including bacteria, fungi, plants, and animals. Most of the alkaloids exert in various animals a more or less specific action upon different regions of their nervous system (Levinson, 1976), often with a toxic effect. They frequently have pharmacological effects and are used as medications, as recreational drugs, or in entheogenic (=“generating the divine within”) rituals. Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste (Drewnowski and Gomez-Carneros, 2000).

In insect defence, alkaloids play an equally important role. Alkaloids are rather toxic for a variety of organisms and, in fact, since ancient times man has taken advantage of such pesticidal properties to protect his cultured plants from insect attack. Several alkaloids have marked pathological effects on insects manifested by retardation of growth, development and reproduction as well as paralysis and mortality according to the dosage received. For example, nicotine poisoning in insects reveals tremors and convulsions (hyperactivity), subsequently paralysis, which is usually followed by rapid death (Levinson, 1976).

3.4. Phenolic compounds

Phenolic compounds or polyphenols are secondary metabolites of plants. Typical plant polyphenols include tannins, galloylglucoses, flavonoids and phenolic acids (Tuominen, 2013). Despite decades of research on their ecological activity, there is significant controversy over the importance of phenolic compounds. This controversy is a result of dramatic incongruities between the proposed and realized effects of phenolics on organisms and ecosystems. They may influence detritivores as well as herbivores and pathogens and affect processes occurring at the ecosystem as well as organismal levels. Their effects may be positive as well as negative, and include feeding deterrence, feeding stimulation, digestion inhibition, digestion stimulation, toxicity, toxicity amelioration, disease resistance, signal inhibition, signal transduction, and nutrient cycle regulation (Appel, 1993).

The biological activity of polyphenols is an interesting subject because of the double-edged character of reactivity. Polyphenols can act as antioxidants in their reduced form or pro-oxidants in the oxidized form, depending on the environment (Galati and O'Brien, 2004). Through their antioxidant activity, polyphenols protect plants from oxidative stress. On the other hand, the potential toxicity and harmful effects of polyphenols against herbivores and pathogens result from their pro-oxidant action (Tuominen, 2013). Summers showed that, indeed, induction of oxidative stress might be an important component of phenolic toxicity in some lepidopteran larvae (Summers and Felton, 1994).

Flavonoids have been shown to have effects on insect feeding behaviour. Major examples include rutin, quercitin and isoquercitin (Simmonds, 2001). These three compounds reduced the growth of second stadium gypsy moth (*Lymantria dispar*) larvae when tested at 0.05 and 0.1%. They also reduced growth and survivorship of the European corn borer, *Ostrina nubilalis* (Abou-Zaid et al., 1993). Depending on the dose and the developmental stage of the insect, however, the same compounds can also have phagostimulant effects (Simmonds, 2001). Even though the behaviour of insects can be modulated by quercetin-derived compounds, it is not yet known how they perceive these compounds (Simmonds, 2003).

Aside from acting directly upon the herbivore insects, phenolic compounds also influence the interactions of herbivores with their microbial pathogens, mutualists, and predators. As with direct effects, phenolics can have negative, neutral, or positive indirect effects (Appel, 1993).

3.5. Terpenoids

Terpenoids, or isoprenoids, are the largest family of natural compounds, consisting of few tens of thousands different molecules (Wink, 2003). Some of these are part of the primary metabolism of the plant, such as some phytohormones, sterols and photosynthesis-related pigments. Others are secondary metabolites, like mono-, sesqui-, di- and triterpenes, which are molecules consisting of 10, 15, 20 and 30 carbon atoms, respectively (Aharoni et al., 2005).

Monoterpenes and sesquiterpenes play important roles in interactions of the plant with its ecosystem (Cheng et al., 2007). This is especially true for volatile terpenoids, which are released into the surrounding space and may be perceived by other players in the community, both plants and animals. In the case this communication occurs between plants, it can for instance prime defence responses in conspecific plants that have not yet been attacked (Paschold et al., 2006), therefore enhancing the overall fitness of the species. When it comes to plant-insect interactions, the effect can be either attractant or repellent, and in this last case, it is either direct or indirect. Examples of attractant effects are those towards pollinators, as well as towards natural enemies of pests (indirect defence mechanisms); repellent effects are perhaps the most intuitive ones, when a plant emits a volatile compound that signals to the insect that the plant is undesirable.

Non-volatile terpenoids also unequivocally play important roles in the interaction between plants and pest: with their proven toxic, antibiotic, antifungal and antifeedant effects, these terpenoids can greatly help the plant reduce damage caused by insects, nematodes, microorganisms and even vertebrates. The modes of action are diverse. Some act on the central nervous system of insects, others on the peripheral nervous system. The most prominent symptoms are hyperactivity followed by hyper-excitation leading to rapid knock down and immobilization (Rattan, 2010). The molecular targets range from proteins (enzymes, receptors, ion-channels, structural proteins), nucleic acids, biomembranes to secondary metabolites with specific or unspecific interactions and other cellular components (Harborne, 1993). In lepidopteran larvae, drimane sesquiterpenes were shown to block the stimulatory effects of glucose and inositol on chemosensory receptor cells located on the mouthparts (Gershenzon and Dudareva, 2007). Pyrethrins and pyrethroids effect sodium channels by activation at lower threshold; which result in prolonged flow of sodium currents into neurons (Rattan, 2010). It has been reported that essential oils, often constituted by terpenoids, act by a reversible competitive inhibition of acetylcholinesterase (AChE), which causes accumulation of acetylcholine at the synapses, so that the post-synaptic membrane is in a state of permanent stimulation; this results in ataxia (lack of co-ordination in the neuromuscular system) and eventually death (Rattan, 2010). Thujone has been classified as a neurotoxic insecticide, which acts on $GABA_A$ receptors (Höld et al., 2000), thereby reducing neuronal inhibition, which leads to hyper-excitation of the central nervous system, convulsions, and death (Bloomquist, 2003). Octopamine (OA), a biogenic amine, which plays key roles as a neurotransmitter, neuromodulator and neurohormone in invertebrate system, with a physiological function as that of nornephrine in vertebrates, is another target for essential oils activity in insects (Evans, 1981; Kostyukovsky et al., 2002). The acute and sub-lethal behavioral effects of terpenoids on insects are consistent with an octopaminergic target site in insects, which acts by blocking octopamine receptors (Enan, 2005). The TRPA1 sodium channel, which mediates the heat and pain response in vertebrates (McNamara et al., 2007) and which was also found in insects (Rosenzweig et al., 2005; Al-Anzi et al., 2006), is the primary target for certain drimane sesquiterpenes (Escalera et al., 2008).

In the present thesis, the focus is primarily on three (groups of) compounds: the sesquiterpenes polygodial and zingiberene, and the terpenoid conjugates pyrethrins (**Fig. 1.6**).

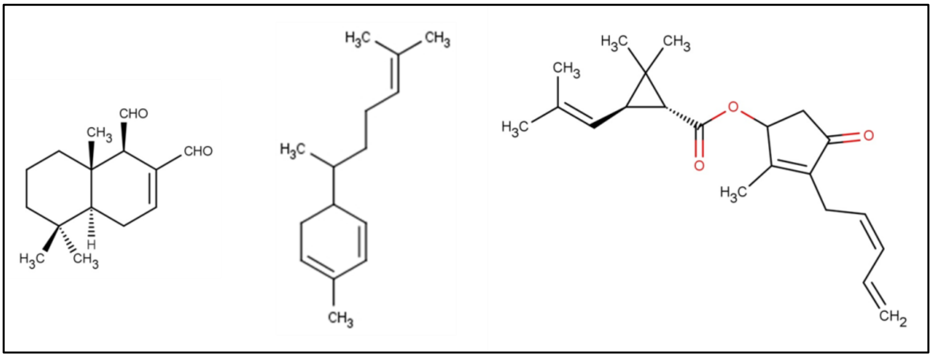


Figure 1.6. From left to right: polygodial, zingiberene and pyrethrin I

3.5.1. Polygodial

Polygodial (**Fig. 1.6**) is a sesquiterpe dialdehyde belonging to the drimane family. Purified polygodial has been shown to have many different biological activities, including (but not limited to): fungitoxic (Alves et al., 2001; Jansen and de Groot, 2004), antibacterial, antiviral, piscicidal, molluscicidal, cytotoxic, antiallergic, anti-inflammatory, and antifeedant activity (Jansen and de Groot, 2004). This compound occurs in three plant families: Polygonaceae (Starkenmann et al., 2006), Cannellaceae (genera *Drymis* (Munoz-Concha et al., 2007) and *Tasmannia* (Read and Menary, 2000)) and Winteraceae. It is also found in sponges (Paul et al., 1997) and Mediterranean nudibranchs of the genus *Dendrodoris*, where it plays a role as fish antifeedant (Cimino et al., 1985; Avila et al., 1991). In plants it is thought to act as a semiochemical, meaning that it acts by influencing insect behaviour, rather than as a toxin (Griffiths et al., 1989). Semiochemicals interact with the insect’s peripheral nervous system. They include the secondary plant metabolites involved in host-plant recognition and those that interfere with feeding behaviour (antifeedants). As an antifeedant, it has been shown to be effective against a number of herbivorous insects (Jansen and de Groot, 2004), including the aphid *Myzus persicae* (Sulzer) (Asakawa et al., 1988; Powell et al., 1995), the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Caprioli et al., 1987; Gols et al., 1996), and the larvae of the moths *Spodoptera littoralis* (Boisduval) and *Spodoptera exempta* (Walker) (Kubo and Ganjian, 1981; Caprioli et al., 1987). In a field experiment, under conditions of high pest and disease pressure, polygodial applied to barley at 50 g ha^{-1} increased the yield by over 1 ton ha^{-1}, by preventing the spreading of the insect-vectored *Barley yellow dwarf virus* (Brône et al., 2008). The field performance was similar to the conventional broad-spectrum insecticide cypermethrin, a synthetic pyrethroid (Pickett et al., 1992) . The insect-deterrent properties of polygodial have been studied by incorporation in artificial diets, as well as by coating the compound on leaves (Hardie et al., 1992; Gols et al., 1996; Moreno-Osorio et al., 2008).

3.5.2. Zingiberene

Zingiberene (**Fig. 1.6**) is a volatile sesquiterpene belonging to the bisabolane family. It is the primary constituent of ginger (*Zingiber officinale*) essential oil, hence its name. Olfactometer experiments have shown that at concentrations above 0.5% ginger oil was effective at repelling *Bemisia argentifolii*. The same study reported dose-response repellence to whitefly when leaf disks were immersed in ginger oil solutions, as well as reduced settling and oviposition when whole tomato seedlings were dipped in ginger oil solution (Zhang et al., 2004).

A stereoisomer of zingiberene, 7-*epi*-zingiberene, has also been shown to occur in glandular trichomes of a wild tomato species, *Solanum habrochaites* var. *hirsutum* Dunal (Carter et al., 1989). In this species it has been shown to help confer resistance to the Colorado potato beetle *Leptinotarsa decemlineata* Say (Carter et al., 1989) and to whitefly (Bleeker et al., 2009; Bleeker et al., 2012).

Due to the phytotoxic effects of higher doses of ginger oil (Zhang et al., 2004), there are limitations as to the potential use of this oil in pest management. A genetically engineered plant with trichome-specific production of zingiberene, however, could be a potential solution to the problem.

3.5.3. Pyrethrins

Pyrethrum oil refers to the oleroresin extracted from the dried flowers of the pyrethrum daisy *Tanacetum cinerariifolium* (Asteraceae). The flowers are ground to a powder and then extracted with hexane or a similar nonpolar solvent; removal of the solvent yields an orange-colored liquid that contains the active principles (Casida and Quistad, 1995; Glynne-Jones, 2001; Escalera et al., 2008). These are three esters of chrysanthemic acid and three esters of pyrethric acid. Among the six esters, those incorporating the alcohol pyrethrolone, namely pyrethrins I and II (**Fig. 1.6**), are the most abundant and account for most of the insecticidal activity. Technical grade pyrethrum, the resin used in formulating commercial insecticides, typically contains from 20% to 25% pyrethrins (Isman, 2006; Escalera et al., 2008).

The insecticidal properties of pyrethrins have been recognized for at least two centuries. According to an anecdote from 1840, withered *Tanacetum cinerariifolium* flowers were found surrounded by dead insects by a woman of Dubrovnik, Dalmatia, who associated the insect death with properties of the flowers. However, there are accounts of the plant being used in China and Persia in much earlier times (McLaughlin, 1973). Pyrethrins are especially labile in the presence of the UV component of sunlight, a fact that has greatly limited their use outdoors (Isman, 2006). This problem created the impetus, around the middle of the twentieth century, for the development of pyrethroids, photostable synthetic derivatives of pyrethrins, which have since been largely employed for agricultural purposes instead of natural pyrethrins (Casida, 1980).

The insecticidal action of the pyrethrins is characterized by a rapid knockdown effect, particularly in flying insects, and hyperactivity and convulsions in most insects. These symptoms are a result of the neurotoxic action of the pyrethrins, which block voltage-gated sodium channels in nerve axons (Isman, 2006). What makes pyrethrins optimal for crop protection even under strict regulatory policies, is the fact that they have low toxicity to mammals in technical grade preparations (LD_{50} ~1500 mg kg^{-1}) (Isman, 2006) and low persistency in the environment (Casida, 1980).

Studies showing the effects of commercial insecticides containing pyrethrins have been done on both *M. persicae* and *B. tabaci*. Pyronyl (a commercial preparation containing 10 g L^{-1} pyrethrins and 20 g L^{-1} piperonyl butoxide), a known synergist of pyrethrins (Bingham et al., 2007) caused 50% *M. persicae* mortality (LC50) when applied at a concentration of 180 mg L^{-1} onto cabbage leaves (Edelson et al., 2002). In the study with *B. tabaci*, the mortality after 48h from the exposure of whiteflies to sprayed *Poinsettia* leaves was 100% when a mixture of pyrethrins (6 mg L^{-1}) and piperonyl butoxide (60 mg L^{-1}) was sprayed onto the leaves (spraying quantity equivalent to 5.7 g ha^{-1}) (Price and Schuster, 1991).

Pyrethrins have also been tested against natural beneficial parasitoids of crop pests, and proved highly toxic (Price and Schuster, 1991; Jansen et al., 2010). There is a need, therefore, to find alternatives that are effective and better compatible with other Integrated Pest Management (IPM)-approaches.

4. Genetic modification to improve pest resistance of vegetable crops

Often, the cultivated varieties of the crops we consume have undergone centuries of breeding to increase yield and improve organoleptic properties. In the process, however, most of the defence mechanisms that the original wild varieties possessed were lost, leaving us with big, juicy and sometimes flavourful tomatoes, which grow on plants very susceptible to pest pressure, in contrast to the resistant wild relatives that bear unpalatable little berries. Metabolic engineering for improved plant defence is one of the possible responses to this problem. By trying to introduce parts of biosynthetic pathways from either related species or very distant taxa into the crops of interest, it becomes possible to selectively introduce or improve defence traits without sacrificing yield. For instance, through metabolic engineering, the production of volatiles that signal danger to phytophagous insects (such as (*E*)-β-farnesene) can be obtained in an otherwise non-repellent plant species (Beale et al., 2006; Unsicker et al., 2009). In another example, transgenic *Arabidopsis* plants producing linalool deterred oviposition by *Helicoverpa armigera* moths

(McCallum et al., 2011) or repelled *Myzus persicae* (Aharoni et al., 2003). Transgenic *Chrysantemum morifolium* emitting linalool was shown to be deterrent to *Frankliniella occidentalis*, albeit not immediately upon release of the insects (Yang et al., 2013). The same deterrent compounds can act as attractants for carnivorous or parasitic arthropods that prey on the herbivores (Kappers et al., 2005; Heil, 2008).

This promising approach to combat insect pests without the need for spraying either natural or synthetic pesticides has the potential to be cost-effective and relatively safe for farmers as well as consumers. If, in addition, the expression of the transgene is tightly regulated and modulated, then the costs in terms of yield reduction due to allocation of plant resources is also minimized.

From the identification of the gene of interest to the commercialization of the GM crops, several steps need to be taken. Once a compound of interest is identified in a plant species, and before it can be produced in a crop, the gene(s) responsible for its biosynthesis need to be isolated from the host plant. Usually this entails full transcriptome sequencing, which has become highly efficient and fast in recent years (Emrich et al., 2007). Sequence analysis of the ESTs and comparison with known genes of the same family (for instance terpene synthases), is the next step. Selection of the interesting sequences, molecular cloning into a bacterial expression vector, heterologous expression in *E. coli* and an enzymatic assay in which the substrate (FPP and/or GPP in the case of terpene synthases) is provided, are the further steps in the identification and characterization of the isolated genes (Colby et al., 1993; Chappell, 1995). Once the desired gene is identified and the enzyme characterized, pilot studies of expression in model plant species are usually carried out (van Herpen et al., 2010). In the experiments reported in this thesis, this was frequently done by differential subcellular targeting and in combination with genes encoding enzymes that are involved in the biosynthesis of the metabolites upstream in the pathway, in order to optimize the production (Wu et al., 2006; van Herpen et al., 2010). In the presence of a suitable transformation protocol, the crop of interest can then be transformed. The GM plant (either the model organism, or the destination crop) can then be used to test insect behaviour (Kappers et al., 2005; Beale et al., 2006; Dudareva and Pichersky, 2008), as well as other phenotypic variations, and it can be subjected to risk assessment to evaluate potential undesired effects on non-target organisms (Houshyani, 2012). After many more trials to make sure the potential risks of introducing the GM plant into the field and onto the market are kept to a minimum, potentially the plant can be patented and commercialized (Craig et al., 2008).

In reality, the GM plants cultivated worldwide nowadays are just a handful. There are potential unforeseen risks related to this relatively modern practice, which make GMOs often seen as hazards by society and overall saluted with skepticism. Currently, EU regulation precludes the growth and commercialization of virtually all GM crops within its territory (Karlsson, 2003). Although commercialized GM crops are limited in trait diversity, proof-of-concept for many other traits has been reported in laboratory experiments and small-scale field trials. These traits fit into several categories: pest resistance, agronomic performance, abiotic stress tolerance, medical applications, biofuels, and improved food, feed, and environment (Lemaux, 2008). Therefore, should the EU regulations become less stringent, an abundance of knowledge will be available to swiftly introduce these PIPs into the agricultural landscape, assuming the necessary risk assessment is carried out.

5. AIM of the thesis

The aim of the work presented in this thesis was to assess the use of the sesquiterpenoids polygodial and zingiberene to obtain crop resistance against whitefly, to isolate the genes required for the production of these compounds in transgenic plants, and to evaluate the consequences of the engineering of these compounds on whitefly behaviour.

6. Thesis layout

Chapter 2 describes the thus far unreported antifeedant effects of polygodial on whitefly. For comparison, this compound is also tested with aphids as it has been previously reported to have an antifeedant effect against this insect species. The polygodial effect is benchmarked against that of pyrethrum, a known natural pesticide.

Chapter 3 analyses the chemical similarities and differences of three *Persicaria* species to identify the most suitable tissues and/or developmental stages for the isolation of genes involved in the production of polygodial.

In **Chapter 4**, we characterized a *Persicaria hydropiper* sesquiterpene synthase and a cytochrome P450 that respectively convert FPP into drimenol and further oxidise drimenol into a number of other sesquiterpenoids, two of which were identified as cinnamolide and drimendiol. I show that cinnamolide exhibits an antifeedant effect against whitefly and aphids.

Chapter 5 describes the isolation and characterization of a zingiberene synthase from *Callitropsis nootkatensis*, and its engineering into two species of the genus

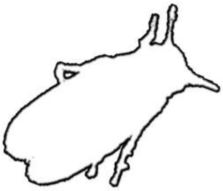
Nicotiana, with or without the concomitant expression of other genes of the biosynthetic pathway, in an attempt to confer resistance against whitefly.

In **Chapter 6**, the findings of the previous chapters are embedded within the relevant literature. Plant defence strategies, pest management approaches and ecological interactions are discussed and the advantages and disadvantages of genetic modification assessed. Finally, a shift from the intensive agricultural practices, typical of the 20th century, to a sustainable agricultural practice is brought forth as a holistic approach to tackle the many facets of modern-day agriculture.

Chapter 2

Comparative antifeedant activities of polygodial and pyrethrins against whiteflies (*Bemisia tabaci*) and aphids (*Myzus persicae*)

Neli Prota, Harro J. Bouwmeester, Maarten A. Jongsma



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1. Abstract

Polygodial, a sesquiterpene dialdehyde of the drimane family, has been shown to have deterrent and antifeedant effects on various insect species, including *Myzus persicae* (Sulzer), *Spodoptera spp*. and *Leptinotarsa decemlineata* (Say) and may have potential as a broad-spectrum biocontrol agent, similar to pyrethrins.

In this study, we report the comparative deterrent effects of polygodial and pyrethrins against the silverleaf whitefly *Bemisia tabaci* (Gennadius) and the green peach aphid *M. persicae* in dual-choice assays using compound-coated tomato leaf discs. *B. tabaci* adults were deterred by polygodial at an ED_{50} of about 25 µg gFW^{-1}, and peach aphids at about 54 µg gFW^{-1}. Bioassays were benchmarked with pyrethrins that had a 20-fold lower ED_{50} of approximately 1.4 µg gFW^{-1} against whiteflies, but only a 2-fold lower ED_{50} (about 28 µg gFW^{-1}) against peach aphids. Polygodial showed moderate phytotoxic effects on tomato leaves at concentrations above the ED_{50} concentrations (≥ 90 µg gFW^{-1}).

The sesquiterpene dialdehyde polygodial is 2-20 times less deterrent than pyrethrins, depending on the insect species, but it could provide a useful complement to pyrethrin sprays as it has a different mode of action, is food grade and has low volatility. However, a formulation that reduces the risks of phytotoxic effects should be developed.

2. Introduction

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) thrives worldwide in tropical, subtropical, and less predominantly in temperate habitats. Environments with cold temperatures often lead to mortality of both adults and larvae of the species. Nevertheless, it has spread from the Mediterranean basin to greenhouses at higher latitudes in Central and Northern Europe, claiming habitat there as well (Borg-Karlson et al., 1993; Martin et al., 2000). The phloem-feeding insect not only damages plants directly by depriving them of their sap but also causes changes in basic physiological processes such as gas exchange through the stomata, transpiration and photosynthesis rates, and chlorophyll and soluble sugar contents in the leaves (Inbar and Gerling, 2008). In addition, it also causes secondary damage through honeydew excretion that enables sooty mould development, which in turn blocks sunlight and reduces photosynthesis (Inbar and Gerling, 2008). Most importantly, however, it acts as a vector for more than a hundred viruses, including *Tomato yellow leaf curl virus* and

Tomato mottle virus, which causes yield losses that cannot be controlled chemically once plants are infected (Jones, 2003). The most threatened crops belong to the Cucurbitaceae, Leguminosae, Euphorbiaceae, Malvaceae and Solanaceae (Borg-Karlson et al., 1993).

The traditional approaches in combating whiteflies using synthetic pesticides have potential side effects on both the environment and human health (Palumbo et al., 2001). Secondary metabolites such as terpenoids can offer an alternative to synthetic insecticides and repellents, without usually harming the environment or human health to an unacceptable level (Simmonds et al., 2006).

Polygodial is a sesquiterpe dialdehyde belonging to the drimane family. Purified polygodial has been shown to have many different biological activities, including (but not limited to): fungitoxic (Alves et al., 2001; Jansen and de Groot, 2004), antibacterial, antiviral, piscicidal, molluscicidal, cytotoxic, antiallergic, anti-inflammatory, and antifeedant activity (Jansen and de Groot, 2004). This compound occurs in three plant families: the Polygonaceae (**Chapter 3**), Cannellaceae and Winteraceae (Castelli et al., 2005), and it has also been discovered in sponges (Paul et al., 1997) and sea molluscs (Gibson and Pickett, 1983; Dobson, 2006), where it most likely has a function as a feeding deterrent to natural enemies. In plants it is thought to act as a semiochemical, meaning that it acts by influencing insect behaviour, rather than as a toxin (Griffiths et al., 1989). Semiochemicals interact with the insect's peripheral nervous system. They include the secondary plant metabolites involved in host-plant recognition and those that interfere with feeding behaviour (antifeedants). As an antifeedant, it has been shown to be effective against a number of herbivorous insects (Jansen and de Groot, 2004), including the aphid *Myzus persicae* (Sulzer) (Asakawa et al., 1988; Powell et al., 1995), the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Caprioli et al., 1987; Gols et al., 1996), and the larvae of the moths *Spodoptera littoralis* (Boisduval) and *Spodoptera exempta* (Walker) (Kubo and Ganjian, 1981; Caprioli et al., 1987). In a field experiment, under conditions of high pest and disease pressure, polygodial applied to barley at 50 g ha^{-1} increased the yield by over 1 ton ha^{-1}, by preventing the spreading of the *Barley yellow dwarf virus*. The field performance was similar to the conventional broad-spectrum insecticide cypermethrin, a synthetic pyrethroid (Dawson et al., 1986 ; Brône et al., 2008). The insect-deterrent properties of polygodial have been studied by incorporation in artificial diets, as well as by coating the compound on leaves (Hardie et al., 1992; Gols et al., 1996; Moreno-Osorio et al., 2008).

Pyrethrins are extracted from the daisy-like flowerheads of pyrethrum, *Tanacetum cinerariifolium*. They represent six closely related insecticidal esters, which differ only in the terminal substituents in the side chains of the acid and alcohol components (Casida, 1980). The insecticidal properties of pyrethrins have been recognized for at least two centuries (Casida, 1980). Owing to their oxidation- and photosensitivity, since the middle of the twentieth century, synthetic derivatives of pyrethrins, called pyrethroids, have been largely employed for agricultural purposes instead of natural pyrethrins (Soderlund and Bloomquist, 1989). However, in organic agriculture, where very few plant protection agents can be used because of stricter regulations, pyrethrum-based compounds are amongst the most widely used pesticides, due to their high efficacy against invertebrates and limited persistence in the environment (Jones, 2003).

Studies showing the effects of commercial insecticides containing pyrethrins have been done on both *M. persicae* and *B. tabaci*. Pyronyl (a commercial preparation containing 10 g L^{-1} pyrethrins and 20 g L^{-1} piperonyl butoxide, a known synergist of pyrethrins (Hadaway et al., 1963)) caused 50% *M. persicae* mortality (LC_{50}) when applied at a concentration of 180 mg L^{-1} onto cabbage leaves (Edelson et al., 2002). In the study with *B. tabaci*, the mortality after 48h from the exposure of whiteflies to sprayed *Poinsettia* leaves was 100% when a mixture of pyrethrins (6 mg L^{-1}) and piperonyl butoxide (60 mg L^{-1}) was sprayed onto the leaves (spraying quantity equivalent to 5.7 g ha^{-1}) (Price and Schuster, 1991).

Pyrethrins have also been tested against natural beneficial parasitoids of crop pests, and they have proved highly toxic (Price and Schuster, 1991; Jansen et al., 2010). There is a need, therefore, to find alternatives that are effective and better compatible with other IPM-approaches.

The aim of this study was to assess the potential of polygodial in discouraging whiteflies and aphids from feeding on crop plants, and compare the effects to those of pyrethrins. The chosen compounds are natural antifeedants and insecticides, and therefore compatible with organic agriculture practices for which pesticide use is tightly regulated. Polygodial has been reported to have moderate phytotoxic effects on cabbage, but not on sugar beet, at concentrations in sprays of 0.1% yielding a residue of 250 µg g^{-1} leaf (Asakawa et al., 1988), and, therefore, this aspect was also part of this study.

3. Materials and methods

3.1. Insects

The insects used in this study were the silverleaf whitefly *Bemisia tabaci* (genotype B) and the green peach aphid *Myzus persicae*. Whiteflies were reared on tomato (*Solanum lycopersicum* L. cv. Moneymaker) in a greenhouse at 26 °C, at 60% relative humidity with a photoperiod of 16h light and 8h dark. To perform the assays, adult flies of both genders were collected from leaves with an aspirator. They were cold-anesthetized at 7 °C in a 3 cm diameter plastic cylinder covered with Parafilm® for 5 min, to ensure they would not fly out before the dishes were sealed, before being released into the Petri dish at the start of the dual-choice assay.

Apterous aphid adults were collected from Chinese cabbage (*Brassica rapa* L. subspecies *pekinensis*), on which they had been reared at room temperature in ambient light. They were collected in 2 mL test tubes and starved for about 30 min before inoculating them in the dishes.

3.2. Chemicals

Purified crystalline polygodial was kindly provided by prof. J. Pickett from the Rothamsted Research centre (United Kingdom). Pyrethrum oil, which contained 700 mg mL^{-1} pyrethrins was provided by Honghe Senju Biological Co. Ltd., Yunnan, China and had been extracted from dried, ground pyrethrum flower heads with liquid CO2 leaving no solvent residues. To the oil, 1% butylated hydroxytoluene (BHT) had been added to prevent oxidation. GC-MS analysis with a standard was used to confirm the content and composition of the sample.

3.3. Choice assays

In the dual-choice assays, insects were presented with tomato leaves coated with either 50% ethanol in water (control) or a solution of polygodial or pyrethrins in 50% ethanol in water. In order to dissolve polygodial at the highest concentration used (5 mg mL^{-1}), it needed to be firstly dissolved in 100% ethanol, to which an equal volume of water was then added. Further dilutions were made using 50% ethanol in water. Fresh ‘Moneymaker’ tomato leaves collected from 5- or 6-weeks old plants were cut in half longitudinally and placed abaxial-side up in a 9-cm-Petri dish on 45 ml of 8 g L^{-1} water agar substrate.

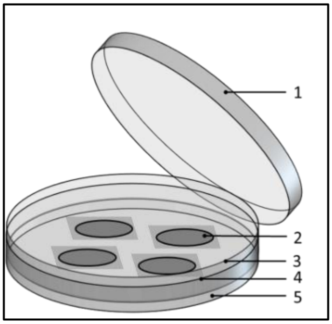


Figure 2.1. Petri dish set-up for the choice assays. 1. Petri dish lid; 2. Exposed leaf area through hole in top dish; 3. Top dish with four circular holes; 4. Bottom dish; 5. Agar substrate onto which leaves are placed

The leaves were then covered with a Petri dish bottom with four holes of 16 mm diameter, such that each half leaf had two circular arenas available for insects to feed (**Fig. 2.1**). In each arena, 15 µL of solution were applied and evenly spread out using a paintbrush. The control and treatment solutions were each spread on two diametrically opposed disks to control for potential environmental cues, such as light. The concentrations of polygodial used were 5, 1, 0.2, 0.04 and 0.008 mg mL^{-1} in 50% ethanol. For the assays with aphids, the concentrations of pyrethrum oil in 50% ethanol were 1, 0.2, 0.04, 0.008 and 0.0016 mg mL^{-1}; for the whitefly assays, the concentrations used were 0.04, 0.008, 0.0016, 0.00032 and 0.000064 mg mL^{-1} because of their higher sensitivity to pyrethrins.

The insects were introduced into the dishes after the solvent had evaporated, by placing them onto the Petri dish lid and covered with the bottom of the plates containing the leaves on agar. On average, 74 whiteflies and 21 aphids per plate were used for each assay. For every combination of insect species and compound concentration, four biological replicates (four separate dishes) were used. The Petri dishes were sealed with Parafilm® and kept upside down. The assays with aphids were carried out at room temperature on a lab bench (uncontrolled conditions). The whitefly assays were performed in a climate chamber at 25 °C, 60% humidity and 16/8h light/dark photoperiod. The assays lasted 24h and the feeding insects were counted at 30 min, 1h, 2h, 4h, 8h, 20h and 24h. Insects were considered to be feeding when they were immobile on one spot on a leaf disk.

Five leaf disks, corresponding to the size of the exposed circular areas, were punched from the leaf and weighed to determine the concentration of the insect repellent compounds in relationship to fresh weight (FW) of the leaf area treated. The average mass of each disk was found to be 34 mg and the surface area was 2 cm^{-2}. Therefore, the conversion factors are as follows: 1 mg mL^{-1} equals 441 mg gFW^{-1} or 9.375 µg cm^{-2}. The concentrations of coated polygodial correspond to approximately 3.6, 18, 90, 450 and 2250 µg gFW^{-1}.

The concentrations of pyrethrum oil used were 0.028, 0.144, 0.720, 3.6, 18, 90 and 450 µg gFW^{-1}, the five lower ones in whitefly assays, the five higher ones in assays with aphids. A concentration of 0.2 mg mL^{-1} (=90 µg gFW^{-1}=1.9 µg cm^{-2}) would then achieve a 1x coverage of 1 ha using 190 g compound.

3.4. Data analysis

To express the repellence potency of polygodial and pyrethrins towards the two insect species tested, the Antifeedant Index (AI%) was calculated according to Kutas and Nádasy, as follows: AI%=[(C-T)/(C+T)]x100, where C indicates the number of insects feeding on the control, and T the number of insects feeding on the treatment (Kutas and Nádasy, 2005). The AI% assumes positive values when the tested compound is an antifeedant, and negative values when the compound is a phagostimulant. Probit analysis was carried out using PASW statistics 18 (SPSS Inc.), in order to determine the ED_{50}, the effective dose at which 50% of the insects are deterred (i.e. the concentration at which twice as many insects feed on the control compared to the treatment).

3.5. Phytotoxicity scoring

Phytotoxicity of polygodial to tomato leaves after 48h of exposure was scored on a scale of 0 to 5 according to Asakawa: 0, no damage to leaf surface; 1, most of leaf surface normal, some slight pitting; 2, slight pitting over whole leaf surface or dry patches on leaf; 3, slight pitting over whole leaf surface with some dry patches; 4, pitting of leaf surface, large areas dry and papery; 5, leaf shrivelled (Asakawa et al., 1988).

4. Results

4.1. Bioassays with insects

The effects of polygodial and pyrethrum oil on whiteflies and aphids were tested in dual-choice assays; the parameters monitored were the fraction of insects feeding at different time points, and the preference for the treated or control areas.

4.1.1. Feeding inhibition

One of the relevant effects a pest-deterrent compound might have is to inhibit feeding even in the presence of an untreated control. In our assays we considered insects to be feeding if they were immobile on a leaf section, but this was not demonstrated directly. Based on that assumption, there were at most 80% of aphids and 75% of whiteflies feeding at any given time. The 8h- and 24h-time points were the ones for which on average the majority of whiteflies and aphids, respectively, were settling, and therefore likely feeding as mortality rates overall were very low even at the highest concentrations (**Fig. 2.2A**). To assess the inhibiting effect of polygodial and pyrethrins on the proportion of insects feeding compared to the total number of insects$\left(\frac{F}{T}\right)$, we calculated the ratios $\frac{\left(\frac{F}{T}\right)_L}{\left(\frac{F}{T}\right)_H}$ of the proportions of insects feeding in arenas with the lowest $\left(\frac{F}{T}\right)_L$ and highest $\left(\frac{F}{T}\right)_H$ concentrations of the compounds, and expressed them over time (**Fig. 2.2B**). A ratio higher than 1 (meaning that $\left(\frac{F}{T}\right)_L > \left(\frac{F}{T}\right)_H$) indicates that the higher concentration negatively affects the propensity of the insects to feed at a given time. A Student's t-test performed using as data the averages of the individual proportions $\left(\frac{F}{T}\right)_L$ and $\left(\frac{F}{T}\right)_H$ across all time points, showed that the differences between feeding at low and high dose were highly significant: polygodial on aphids ($p < 0.001$), polygodial on whiteflies ($p < 0.001$), pyrethrins on aphids ($p < 0.01$), pyrethrins on whiteflies ($p < 0.01$). In aphids, the effect of concentration was stronger at early time points, while they became habituated to the presence of the compound in time; polygodial caused a much more marked effect than pyrethrins, reducing the proportion of feeding aphids 4-fold after 2 hours and at least 2-fold at all later time points. By contrast, the whiteflies' feeding behaviour displayed little difference in time for either compound applied.

4.1.2. Feeding preference

The feeding preference was assessed using the Antifeedant Index (AI%). The 8h-time point was used as most insects were actively feeding (settled on a leaf disk) at that time, and the time for induction of potentially confounding secondary responses was kept to a minimum. There is a clear dose-dependent preference for the control disks over the treated ones, as the AI% increases with the increasing concentrations applied (**Fig. 2.3**). At higher concentrations, polygodial had a similarly strong deterrent effect on both insects tested (**Fig. 2.3**). The extrapolated effective deterrent dose for 50% feeding deterrence (ED_{50}) of polygodial was 25 µg gFW^{-1} for whiteflies and 54 µg gFW^{-1} for aphids. Whiteflies were 20-fold more susceptible to pyrethrins than aphids (**Fig. 2.3**). However, at very low concentrations (0.028 µg gFW^{-1}) pyrethrins displayed a weak, but significant attractant effect on whiteflies, which was not the case for the aphids. The ED_{50} for pyrethrins was 1.4 µg gFW^{-1} and 28 µg gFW^{-1} for whiteflies and aphids, respectively.

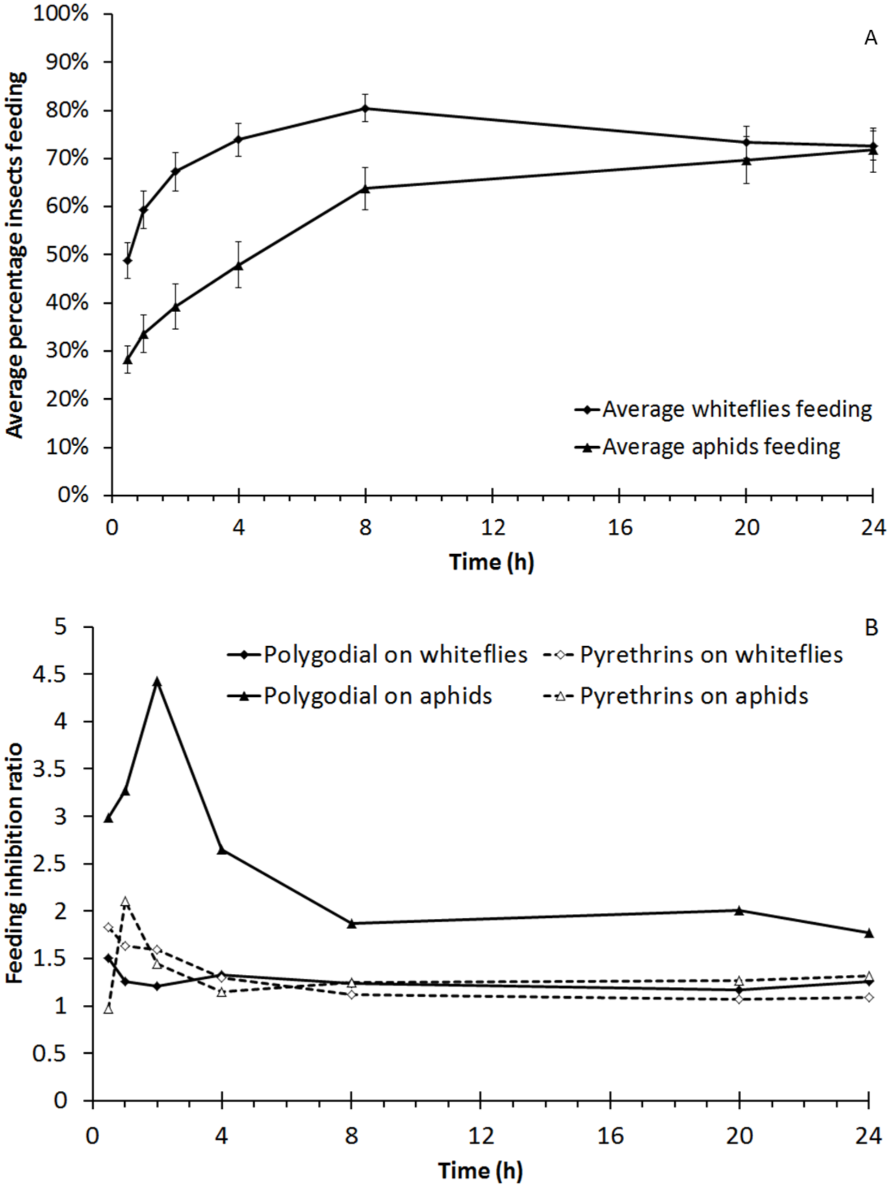


Figure 2.2. Insect feeding behaviour through time. **A)** Feeding rate (proportion of insects feeding/total insects) of the two insect species over time. The values represent the average of all treatments applied (two compounds, five concentrations, four replicates). Error bars represent SEM, *n* = 4; **B)** Feeding inhibition over time, expressed as the ratio of the proportion of insects feeding ((control + treatment)/total) on a low and high dose treatment.

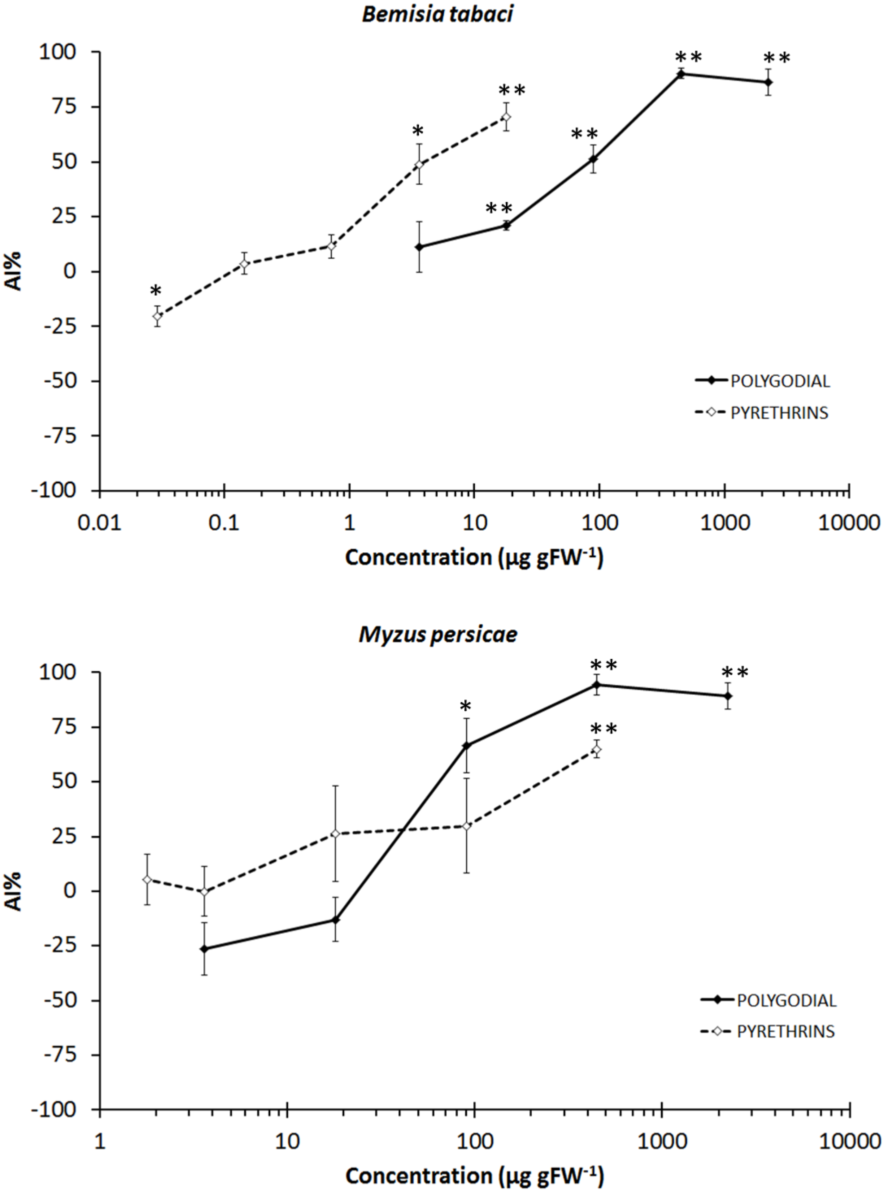


Figure 2.3. Effect of increasing concentrations of polygodial and pyrethrins on the feeding behaviour of *Bemisia tabaci* and *Myzus persicae* at 8 h from the start of the experiment. Antifeedant index (AI%) = [(C-T)/(C+T)] x 100. Positive values indicate an antifeedant effect, negative values a phagostimulant effect. Error bars indicate SEM, $n = 4$, average of 74 whiteflies and 21 aphids per replicate. Statistical significance is expressed by the asterisks: * = $p < 0.05$, ** = $p < 0.01$

The effects of exposure time on the choice that insects make between treatment and control are illustrated in **Figure 2.4**. The AI% of the highest and lowest concentrations used are plotted against time.

In the whitefly assays, the AI% of both the lowest and the highest concentration of pyrethrins remained mostly constant over time. At the highest concentration of polygodial, the AI% increased two-fold to nearly 100% within 4 hours from the start of the experiment (**Fig. 2.4A**).

In the aphid assays, at the lowest concentration of pyrethrins, the AI% rapidly decreased (no significant effect after the first hour), while at the highest concentration it changed less intensely (**Fig. 2.4B**). The AI% of polygodial was more stable over time, with only some variation in the early time points.

At the concentrations used and during the time-span of the experiment, pyrethrins caused a negligible mortality rate of approximately 0.1% in whiteflies, and 0% in aphids; polygodial had no significant effect on mortality.

4.1. Phytotoxic effect

Polygodial-painted tomato leaves showed leaf damage at 48 h post-application for concentrations ≥ 0.004% (18 µg gFW^{-1}), as seen in **Figure 2.5**. The damage induced by a concentration of 18 µg gFW^{-1} on a scale of 0-5 was 1 (most of leaf surface normal, some slight pitting); the next two dilutions, 90 and 450 µg gFW^{-1}, caused a damage of degree 2 (slight pitting over whole leaf surface or dry patches on leaf) with larger surface areas being affected at 450 µg gFW^{-1}. The highest used concentration, 2250 µg gFW^{-1}, resulted in a damage of degree 4 (pitting of leaf surface, large areas dry and papery). No tissue damage was visible for leaves treated with pyrethrum oil at any of the concentrations used.

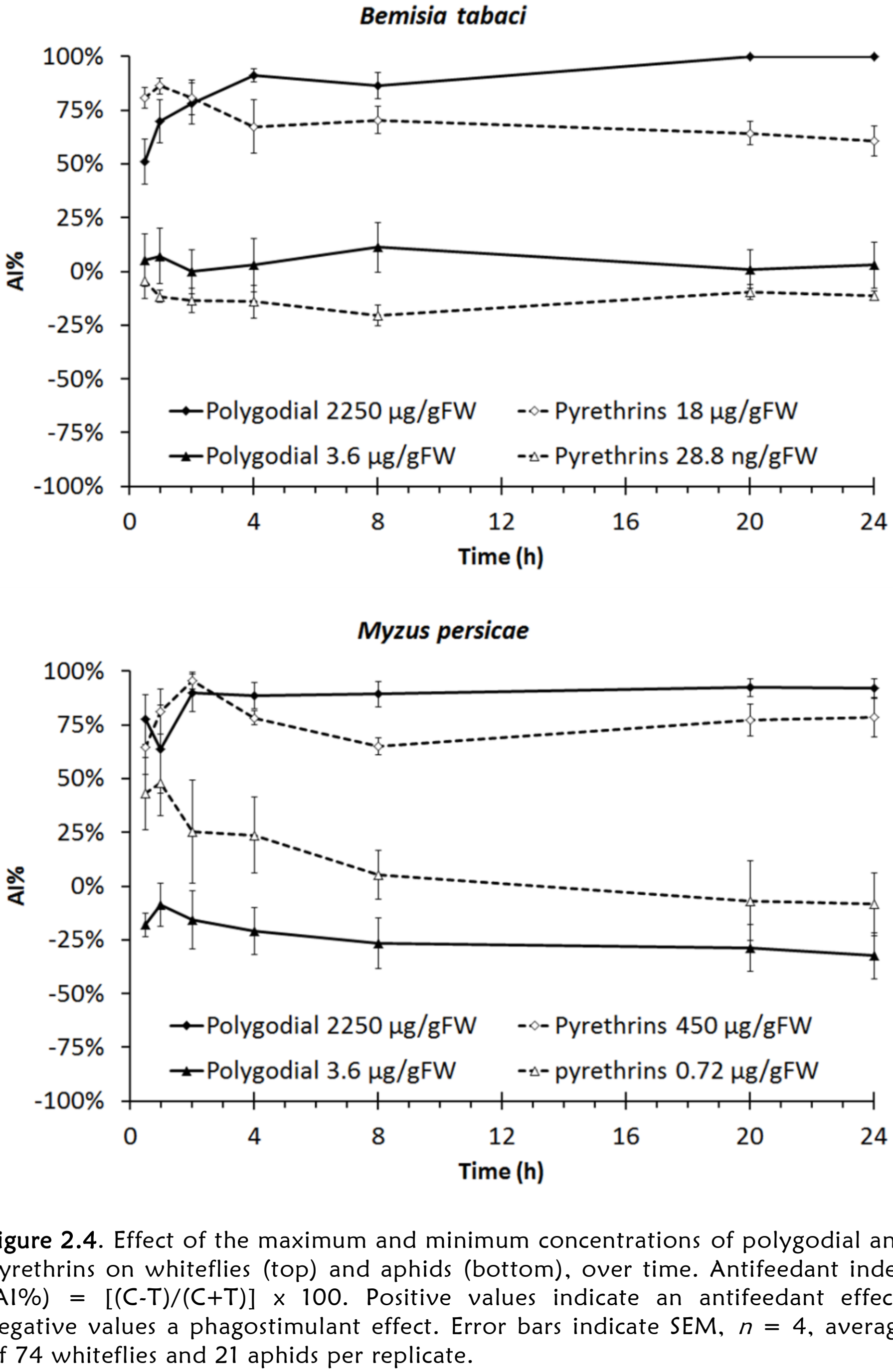


Figure 2.4. Effect of the maximum and minimum concentrations of polygodial and pyrethrins on whiteflies (top) and aphids (bottom), over time. Antifeedant index (AI%) = [(C-T)/(C+T)] x 100. Positive values indicate an antifeedant effect, negative values a phagostimulant effect. Error bars indicate SEM, *n* = 4, average of 74 whiteflies and 21 aphids per replicate.

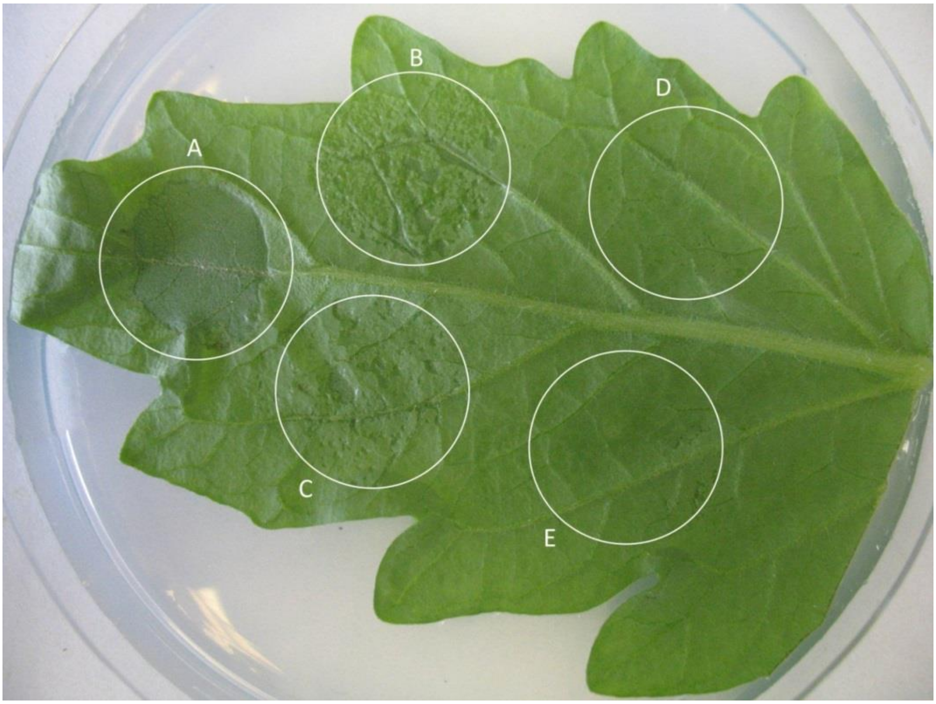


Figure 2.5. Phytotoxic effect of polygodial on tomato. White circles are the areas painted with the polygodial solution in 50% ethanol. The corresponding concentrations in µg gFW^{-1} are A) 2250 µg gFW^{-1}, B) 450 µg gFW^{-1}, C) 90 µg gFW^{-1}, D) 18 µg gFW^{-1} and E) 3.6 µg gFW^{-1}.

5. Discussion and conclusions

The concept of organic agriculture was originally introduced in 1924 by Rudolf Steiner (Paull, 2011) and has since grown to worldwide proportions, with a turnover of $US 60 billion per year in 2011 (Paull and Hennig, 2011). One of the key pillars of organic farming is the limitation of the use of synthetic pesticides in favour of biological control using natural predators, targeted breeding for resistance, and the use of natural deterrents and pesticides (Lotter, 2003). Natural deterrents and pesticides have broader relevance, however, due to the tendency for resistant insect populations to develop rapidly as a consequence of intensive use of chemical insecticides. It has stimulated investigations on integrated pest management (IPM) strategies in which biological control may play a significant role (Heinz and Zalom, 1996; Legaspi et al., 1996; Faria and Wraight, 2001; Gerling et al., 2001; Inbar and Gerling, 2008). Unlike the greenhouse whitefly *Trialeurodes vaporariorium*, for which attempts to use natural enemies as biological control were highly successful, *B. tabaci* has defied the attempts of control using natural enemies and remains problematic in most parts of the World (Gerling et al., 2001). In this light, it appears important to focus on natural pest-management strategies that do not, or not only, rely on predators or parasitoids, but also on natural compounds.

Our main goal was to study prevention of insect feeding on plants as a way to avoid the main problem of virus transmission. We, therefore, focused on the deterrent/non-settling effects of the compound polygodial and the natural insecticides pyrethrins against the polyphagous virus vectors green peach aphid and silverleaf whitefly.

Even though we assumed feeding is taking place when insects were immobile on a leaf disk, we did not perform any detailed assessment to confirm this other than noting that the settled insects were still alive at the end of the experiment. However, we consider the terms *feeding* and *antifeedant* to be rigorous enough in the light of previous studies (Powell et al., 1995) which used close-up video recordings of aphids reporting a feeding inhibition effect of polygodial similar to what we observed (**Fig. 2.2B**). We used the term *deterrence* to denote a more general property of the studied compounds that negatively affects the insect preference for the treated leaf disk in a dual-choice situation.

We found that during exposure periods of 24h pyrethrins were good deterrents of both aphids and whiteflies, with low ED_{50} values (28 µg gFW^{-1} or 62 mg L^{-1} for aphids and 1.4 µg gFW^{-1} or 3.1 mg L^{-1} for whiteflies). These ED_{50} values for pyrethrins for the two insect species observed in our experiments correlate well with reported LC_{50} (lethal dose, 50%) values. Edelson and co-workers, found an LC_{50} of 60 mg L^{-1} for *M. persicae* (Edelson et al., 2002), while in a study by Price *et al*. with *B. tabaci*, after 48h the recorded mortality of whiteflies was 100% after exposure to 6 mg L^{-1} pyrethrins (Price and Schuster, 1991). Apparently, deterrence ED_{50} concentrations are similar to the LC_{50} values that result when the insects are not offered an escape. The overall feeding inhibition of insects placed in arenas where pyrethrins had been applied tended to decrease over time, even at the higher concentrations. This might be due to adaptation, such as the documented action of detoxifying cytochrome P450 enzymes (Jao and Casida, 1974). These oxidases and esterases help in the metabolism of pyrethrins and are the reason why commercial pyrethrin-based insecticides usually contain synergists (e.g. piperonyl butoxide) that inhibit these enzymes (Yamamoto et al., 1969; Jao and Casida, 1974).

The effects of pyrethrins served to benchmark the effects of polygodial against *B. tabaci* (ED_{50} of 25 µg gFW^{-1}) and *M. persicae* (54 µg gFW^{-1}). The effects against aphids were also previously reported by Powell *et al.* (1996) in a study on the effects of polygodial on aphids' feeding behaviour, but did not include the establishment of an ED_{50}. We observed that polygodial strongly affects the proportion of aphids feeding on any substrate even when control leaves were available. This phenomenon was not observed for whiteflies or with pyrethrins (**Fig. 2.2B**). This is interesting in the light of known behavioural effects of polygodial on aphid species, such as a reduction in the number of penetrations as well as their duration (Hardie et al., 1992). In fact, close-up video recordings obtained by monitoring insect behaviour showed that aphids (*M. persicae*) were repelled following contact of antennal tips with the polygodial-treated leaf areas, while the insects became insensitive if both antennal tips were surgically removed (Powell et al., 1995), suggesting that in this species, too, polygodial may induce a noxious heat signal in the sensillum (Wang et al., 2009). Non-sprayed parts or neighbouring plants may thus also benefit from this effect, as the overall feeding propensity of aphids that have sensed polygodial appears diminished.

It is likely that these behavioural changes take place because of the mediation of the sensory stimulus by the TRPA1 ion channel, which is normally involved in heat sensing. In mammals, polygodial acts through the activation of the this cation channel, which elicits sensations such as noxious heat, pain and cold (Escalera et al., 2008). In humans these translate to a feeling of pungency, similar to that of horseradish (Escalera et al., 2008). Amongst the known agonists of this channel are a number of different chemicals such as allyl isothiocyanate, cinnamaldehyde, acrolein (Bautista et al., 2006), farnesyl thiosalicylic acid (Maher et al., 2008), nicotine (Talavera et al., 2009), formalin (McNamara et al., 2007), hydrogen peroxide (Sawada et al., 2008) and tear gasses (Brône et al., 2008). For insects it has not yet been established which receptor or channel mediates polygodial signalling, but orthologs of the vertebrate TRPA1 channel have been identified in *Drosophila melanogaster* (Meigen) (Rosenzweig et al., 2005) and *Anopheles gambiae* (Giles) (Wang et al., 2009), and in both insect species the channel has been shown to regulate thermotaxis. Furthermore, in 2006, Al-Anzi *et al.* reported that *painless*, the fruit fly homolog of the mammalian TRPA1, was mediating the perception of isothiocyanate, the pungent ingredient of wasabi, as it does in humans (Al-Anzi et al., 2006).

We propose, therefore, that polygodial represents a potentially valuable biopesticide against whiteflies and aphids and likely a host of other insect pests. Its main advantage is the overall feeding reduction in the case of aphids, as well as the ease by which it can be purified from *P. hydropiper,* as the flowers contain up to 8% of the compound on dry weight basis (Hagendoorn et al., 1994). Even though it is 2-20 times less deterrent compared to pyrethrins, it could nevertheless provide a useful complement to pyrethrin sprays as it has a different mode of action, it is food grade and with low volatility. However, just as previously reported for cabbage (Asakawa et al., 1988), polygodial displayed a phytotoxic effect when applied to tomato leaves. The degree of damage produced was proportional to the concentration used. Though it could be speculated that the deterrent effects might also be due to the damage rather than to the direct presence of polygodial, studies using artificial diets containing polygodial or other leaf-free approaches are a confirmation that the compound alone is sufficient to create a deterrent effect (Hardie et al., 1992; Gols et al., 1996; Moreno-Osorio et al., 2008). Further research is still required to find a compromise between high deterrence and low leaf damage. Potentially, the use of microencapsulated formulations of polygodial could reduce the phytotoxic effect, as was shown for other pesticides (Ohtsubo et al., 1991; Greene et al., 1992; Mulqueen, 2003).

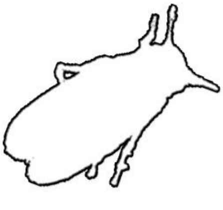
6. Acknowledgments

The authors wish to thank prof. John Pickett from Rothamsted Institute (UK) for providing us with purified polygodial, Paul Piron (PRI, Wageningen UR) for the aphids and Léon Westerd (Laboratory of Entomology, Wageningen UR) for the whiteflies used in the experiments. Alejandro Lucatti (Wageningen UR) is acknowledged for sharing the design of the arenas used in the bioassays. This project: “Molecular breeding of insect resistance in vegetable crops” (grant nr. 1C002RP) was supported by the Technological Top Institute Green Genetics (Stichting TTI Groene Genetica).

Chapter 3

Comparison of the chemical composition of three species of smartweed *(*genus *Persicaria)* with a focus on drimane sesquiterpenoids

Neli Prota, Roland Mumm, Harro J. Bouwmeester, Maarten A. Jongsma



Prota et al., *Phytochemistry* (2014), ***108***, 129-136

Abstract

The genus *Persicaria* is known to include species accumulating drimane sesquiterpenoids, but a comparative analysis highlighting the compositional differences has not been done. In this study, the secondary metabolites of both flowers and leaves of *P. hydropiper, P. maculosa* and *P. minor,* three species that occur in the same habitat, were compared. Using gas chromatography-mass spectrometry (GC-MS) analysis of extracts, overall 21/29 identified compounds in extracts were sesquiterpenoids and 5/29 were drimanes. Polygodial was detected in all species, though not in every sample of *P. maculosa*. On average, *P. hydropiper* flowers contained about 6.2 mg gFW^{-1} of polygodial, but *P. minor* flowers had 200-fold and *P. maculosa* 100,000 fold lower concentrations. Comparatively, also other sesquiterpenes were much lower in those species, suggesting the fitness benefit to depend on either investing a lot or not at all in terpenoid-based secondary defences. For *P. hydropiper,* effects of flower and leaf development and headspace volatiles were analysed as well. The flower stage immediately after fertilization was the one with the highest content of drimane sesquiterpenoids and leaves contained about 10-fold less of these compounds compared to flowers. The headspace of *P. hydropiper* contained 8 compounds: one monoterpene, one alkyl aldehyde and six sesquiterpenes, but none were drimanes. The potential ecological significance of the presence or absence of drimane sesquiterpenoids and other metabolites for these plant species are discussed.

2. Introduction

Terpenoids represent the largest class of plant secondary metabolites with over 50,000 known structures. They play important roles in the interaction of plants with their environment (Gershenzon and Dudareva, 2007). Nearly 100 sesquiterpene drimanes derived from drimenol are known; they possess a wide variety of biological activities including antibacterial, antifungal, anti-nematode, antifeedant, piscicidal, and molluscicidal properties (Jansen and de Groot, 2004). Polygodial, as best known proponent of this group of sesquiterpenoids, is present in a few plant genera, such as the Cannellaceae genera *Drymis* (Munoz-Concha et al., 2007) and *Tasmannia* (Read and Menary, 2000) as well as in some species of the *Persicaria* genus, formerly known as *Polygonum*, (Polygonaceae) (Starkenmann et al., 2006); in fact the presence or absence of polygodial in different *Persicaria* species is being proposed as a chemotaxonomic discriminant between different sub-groups of that genus (Derita and Zacchino, 2011). This dialdehyde has been also found in briophytes and pteridophytes (Asakawa et al., 2001) and it is even synthesized by Mediterranean nudibranchs of the genus *Dendrodoris*, where it plays a role as fish antifeedant (Avila et al., 1991). Polygodial has a pungent taste to mammals (Kubo and Ganjian, 1981; Escalera et al., 2008), and acts as an antifeedant for a number of herbivorous insects (Jansen and de Groot, 2004), including aphids (Asakawa et al., 1988; Powell et al., 1995), Colorado potato beetle (Caprioli et al., 1987; Gols et al., 1996), and moth larvae (Kubo and Ganjian, 1981; Caprioli et al., 1987). The neurological mode of action of polygodial is by opening TRPA1 ion channels on sensory neurons (Escalera et al., 2008).

Persicaria hydropiper L., commonly known as water-pepper, is an annual plant, native to Eurasia, widely distributed as a plant of damp places and shallow waters, such as damp rides in woods, damp meadows, ditches and sides of streams, canals and ponds (Timson, 1966). A distinguishing characteristic of the species is that its leaves, and even more its inflorescences, have a pungent taste, due to polygodial. The compound is stored in epidermal cavities, also called valvate or irritant glands, present on leaves and tepals. The latter can contain up to 8.5% of polygodial in dry weight (Hagendoorn et al., 1994). When observed with a magnification lens or binocular these cavities appear like small translucent dots of approx. 0.5 mm in diameter (Lersten and Curtis, 1992).

Persicaria minor (Hudson) Opiz, also known as small water-pepper, is an annual plant, less common than *P. hydropiper*, but with similar morphological features and habitat preferences. One discriminating feature is the number of the epidermal cavities on the flower heads, which are significantly less on *P. minor* (Eggelte, 2007). Another trait that distinguishes the two species is the colour of the flowers, which is more intensely white or pink-purple in *P. minor* compared with a light green to white in *P. hydropiper*. When chewed upon, the flowers of *P. minor* present only a slightly pungent taste, compared with the strong taste of *P. hydropiper*.

Persicaria maculosa Gray (formerly *Polygonum persicaria* L., common name lady's thumb) is also an annual weed of temperate regions, closely related to the other two and thriving in the same territory, although usually in drier habitats. The reason we included it in this study was the debated absence of cavities and pungency. According to Derita and collaborators (2008), specimens of this species in Argentina do have low numbers of cavities and produce small amounts of polygodial, while Hagendoorn *et al.* reported that the Dutch specimens analysed have no cavities, and, therefore, no polygodial at all (1994).

The aim of the present study was to analyse the distinctive differences and similarities in the chemical profiles of these three closely related species of the *Persicaria* genus occurring in geographical proximity in the Netherlands, and partly overlapping biotopes.

3. Materials and methods

3.1. Plant material

Plants of the genus *Persicaria* were collected in September and October of 2009 in the forests near Wageningen, the Netherlands (+51° 59' 8.66", +5° 43' 33.29", Supplementary Figure S3.3). The species *P. hydropiper* (water-pepper) and *P. minor* (small water-pepper) were collected in damp areas in the woods, while *P. maculosa* was collected from drier spots along roads, where it preferentially grows. Plant seeds were dried for 2-3 weeks at 28 °C. To break the dormancy, the seeds were stratified by keeping them at 4 °C for 30 days between two layers of gauze surrounded by moist soil. They were then sown in pots and covered by a transparent foil to ensure a high humidity during germination. Approximately three weeks after germination, four plants of each species were transferred to larger containers. Seven different samples were collected from each of the four plants after 6-8 weeks and frozen in liquid nitrogen: leaves of four developmental stages based on their length [a) < 1 cm, b) < 2.5 cm, c) < 5 cm, d) > 5 cm]; and flower heads of three developmental stages [a) cleistogamous flowers before the fruit set and open chasmogamous flowers, b) flowers that contain an unripe fruit, and c) flowers containing a ripe fruit].

3.2. Extraction and GC-MS analysis

The frozen tissues were ground in a mortar with liquid nitrogen. For each 50 mg of fresh tissue powder, 1 mL of dichloromethane (DCM) was used to extract the more apolar fraction of the plant constituents. The glass 4 mL vial was vortexed for ~1 min before being centrifuged at 3500 rpm for 15 min; the extract was then passed through a column containing anhydrous Na_2SO_4 to eliminate water and solid particles.

The DCM extracts were analysed by coupled gas chromatography—mass spectrometry (GC-MS) using an Agilent 7890A gas chromatograph equipped with a 30 m Zebron ZB-5 MS column (0.25 mm i.d., 0.25-µm film thickness;

Phenomenex, Torrance, CA, USA) and a quadrupole mass spectrometer (model 5975C, Agilent). A 5 m built-in Guardian™ pre-column (deactivated fused silica tubing without stationary phase; Phenomenex, Torrance, USA), was attached to the analytical column. The GC was programmed from 45 °C for 1 min, to 280 °C at 10 °C min^{-1} and held at 280 °C for 3.5 min. One microliter of sample was injected in splitless mode. The injection port and interface temperature were 250 °C and 280 °C, respectively, and the helium inlet pressure was controlled electronically to achieve a constant column flow of 1.0 mL min^{-1}. A solvent delay was set to 5.5 min. The ionization potential was set at 70 eV, and scanning was performed from 45 to 400 atomic mass units, with a scanning speed of 3.99 scans s^{-1}.

3.3. Analysis of GC-MS data

Linear retention indices were calculated using an alkane series (C9-C22) (van Den Dool and Dec. Kratz, 1963). Metabolites were identified by matching the mass spectra to authentic reference standards (polygodial and drimenol) as well as to the MassFinder 3, NIST08, Wiley, and Wageningen Natural compounds spectral libraries, and by comparison with retention indices in the literature (Strehmel et al., 2008). Compounds for which the identity could not be assigned were left out of the current study. The total peak area of a chromatogram excludes the non-assigned peaks.

The areas of the peaks of interest were obtained by manual integration using Xcalibur™ version 2.07 (Thermo scientific). Microsoft™ Office Excel was used to calculate the averages and the standard errors.

Quantification of polygodial was performed using as reference a five-point dilution series of a standard (>97% pure, Tocris). Since during GC-MS analysis of the standard, two peaks corresponding to polygodial and 9-*epi*-polygodial appear, the sum of the peak areas of the two isomers was considered as representative for each specific concentration used.

3.4. Head-space trapping and analysis

P. hydropiper spikes (flower heads attached to the stem) and leaves of several different stages were used to determine the composition of the headspace of the plant. Two spikes or three leaves were put in a glass vial filled with water (to preserve turgor) and covered with aluminium foil to limit vapour from escaping from the vial. Each vial was placed in a 1L Duran glass jar (Duran). The jar was sealed with a Viton-lined glass lid having an inlet and outlet. Inlet air was filtered by passing through a stainless steel cartridge (Markes, Llantrisant, UK) filled with 200 mg Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, USA). Volatiles were trapped by sucking air out of the jar at a rate of 100 mL min^{-1} through a similar cartridge filled with 200 mg Tenax TA for three hours. Headspace collections were made in a climate chamber at 23 ± 1 °C, 60 ± 5% RH, and 90 ± 5 µmol m^{-2} s^{-1} PAR. The experiment was carried out with biological triplicates.

Headspace samples were analysed with a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, USA) connected to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, USA) quadrupole mass spectrometer. Before desorption of the volatiles, the Tenax cartridges were dry-purged with nitrogen at 30 ml min^{-1} for 25 min at ambient temperature to remove moisture. Volatiles were desorbed from the cartridges using a thermal desorption system at 220 °C for 5 min (Model Ultra Markes Llantrisant, UK) with a helium flow of 30 mL min^{-1}. Analytes were focused at 5 °C on an electronically-cooled sorbent trap (Unity, Markes, Llantrisant, UK) and were then transferred in splitless mode to the analytical column (Rtx-5ms, 30 m, 0.25 mm i.d., 1.0 µm film thickness, Restek, Bellefonte, USA) by rapid heating of the cold trap to 250 °C. The GC was held at an initial temperature of 40 ^{o}C for 3 min followed by a linear thermal gradient of 10 ^{o}C min^{-1} to 280 ^{o}C and held for 2 min with a column flow of 1 mL min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 33-280 m/z with a scan rate of 4.22 scans s^{-1}. Compounds were identified analogously to those from DCM extracts.

3.5. Correlation and similarity analysis

The correlation analysis of the metabolites from the flower samples of *P. hydropiper* was done using R language and statistical environment (Lucent technologies, USA). To perform a Pearson correlation analysis and produce the similarity matrix, the ‘correlation’ and ‘gplots’ packages were used. The values used were the Log2-transformed peak areas of the metabolites. This similarity matrix identified groups of metabolites with the highest correlation in the production level. Subsequently, a hierarchical clustering diagram was obtained with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) hierarchical clustering method. The data used were obtained from nine samples: three biological replicates of the three flower developmental stages.

3.6. Optical microscopy and sample preparation

Fresh flower heads of the FA stage were fixed for 1 hour in 4% paraformaldehyde at room temperature under vacuum and dehydrated in graded series of ethanol (50% for 1 h two times, 80% for 1 h two times, 100% for 2 h two times) and washed three times in xylene for 2h. Samples were then infiltrated at 58 ºC with three baths of paraffin of 1, 2 and 4.5 hours, respectively. The whole procedure was carried out overnight using an automated paraffin embedding system (Shandon Excelsior). 5 µm sections were cut using a Leica 2135 paraffin microtome. Sections were floated on distilled water and collected on clean glass slides. After thorough air-drying, sections were de-waxed and observed unstained using phase contrast.

4. Results and discussion

4.1. Interspecific variation of the chemical composition of *Persicaria* spp.

Seeds of three species of the *Persicaria* genus, *P. hydropiper*, *P. minor* and *P. maculosa,* were collected in the woods in close range of each other. The three species were identified by taxonomist Ronald van den Berg (Wageningen UR) (see **Supplementary Figure S3.1** for images of the plants). Seven separate samples were taken from plants raised from the seeds: three developmental stages of flowers and four of leaves. In **Figure 1**, a cross section of a *P. hydropiper* flower clearly shows the presence of the valvate glands in the tepals of the flowers. Samples were extracted in dichloromethane (DCM) and analysed by coupled gas chromatography-mass spectrometry (GC-MS). A total of 29 major metabolites could be identified across all three species, 15 of which were present in all. Nearly all (27/29) identified metabolites were terpenoids, most were sesquiterpenoids (21/29) and a quarter of those were drimanes (5/21) (**Tables 3.1** and **3.2**, structures in **Fig 3.2**, and mass spectra in **Supplementary Figure S3.2**). **Table 3.1** shows the average relative abundance of the 29 metabolites in the flowers of the three species, while in **Table 3.2** the chemical profiles of the leaves are listed. *P. hydropiper* was the species with the widest variety of metabolites detected in our analyses; the only compound present in the other two species, but not in water-pepper, was β-selinene. In general, flower samples contained more different and higher quantities of metabolites compared to leaves, with the exception of the three neophytadiene isomers, which were more abundant in the leaves, because they are degradation products of chlorophyll (Rowland, 1957).

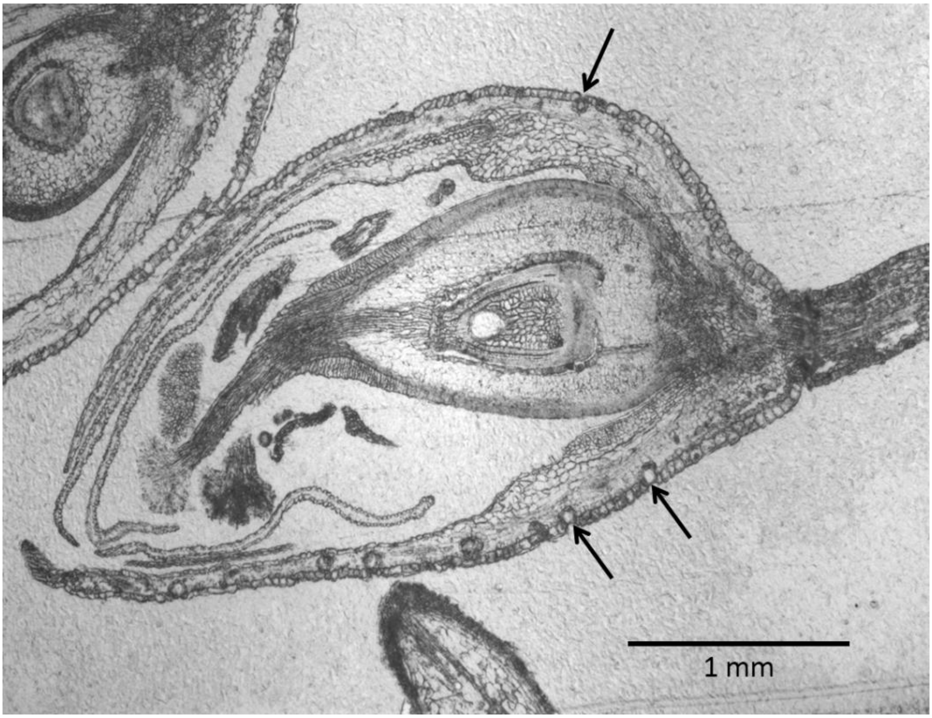
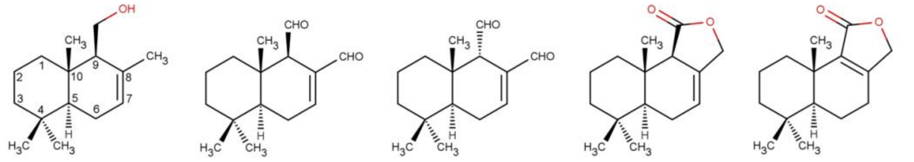


Figure 3.1. Light micrograph of a longitudinal cross section of a paraffin embedded flower of *P. hydropiper*. Arrows indicate the valvate glands (cavities) where polygodial is stored.



(1) (2) (3) (4) (5)

Figure 3.2. Structures of the five drimane sesquiterpenoids observed in the extracts of the analysed plants: (1) drimenol; (2) polygodial; (3) 9-*epi*-polygodial; (4) drimenin; (5) isodrimenin.

Table 3.1. Chemical composition of organic extracts of flowers[1] of three *Persicaria* species

Compound	R.I.	*P. hydropiper*[2]	*P. minor*[2]	*P. maculosa*[2]
Monoterpenes		0.16%	0.09%	0.08%
limonene	1034	0.14%	0.06%	0.07%
bornyl acetate	1262	0.02%	0.03%	0.01%
Sesquiterpenes		98.33%	52.76%	18.65%
Drimanes		78.57%	29.98%	0.11%
polygodial[7]	1892	76.71%	25.22%	0.1%
isodrimenin	2039	1.27%	0.88%	n.d.
drimenin	1973	0.58%	4.24%	n.d.
drimenol	1788	0.01%	n.d.	n.d.
Non-drimanes		19.76%	32.78%	18.54%
caryophyllene	1432	6.65%	12.54%	0.62%
α-humulene	1468	5.02%	1.45%	0.81%
(*E*)-β-farnesene	1454	3.87%	6.77%	4.26%
α-bisabolol	1693	1.36%	1.51%	n.d.
α-muurolene	1507	1.25%	n.d.	6.29%
aristolone	1716	0.71%	0.34%	n.d.
caryophyllene oxide	1597	0.29%	n.d.	n.d.
(*E*)-β-bergamotene	1492	0.22%	0.12%	0.02%
β-bisabolene	1513	0.12%	0.01%	n.d.
(*E*)-nerolidol	1563	0.09%	n.d.	n.d.
(*E*)-α-bergamotene	1439	0.08%	0.02%	0.01%
β-elemene[3]	1397	0.04%	0.02%	2.86%
humulene epoxide II	1625	0.03%	n.d.	n.d.
δ-cadinene	1523	0.02%	n.d.	0.03%
β-bisabolol	1677	0.01%	n.d.	n.d.
β-selinene	1500	n.d.	n.d.	3.64%
Diterpenes		1.31%	35.85%	56.14%
neophytadiene isomer I	1836	0.88%	20.67%	35.07%
neophytadiene isomer III	1880	0.3%	8.39%	14.59%
neophytadiene isomer II	1861	0.13%	6.79%	6.48%
Others		0.19%	11.32%	25.15%
heneicosane[4]	2100	0.17%	10.78%	24.74%
dihydro-α-ionone[5]	1419	0.02%	n.d.	n.d.
nonanal[6]	1105	n.d.	0.54%	0.41%
Total abundance (TIC)		100%	100%	100%
Relative abundance (TIC)		100%	1.44%	0.84%

See legend on page 58

R.I. = retention index; n.d. = not detected

[1] Average of three replicates of each of three developmental stages (N=3x3, table 3). Standard errors were on average 28% of a given value

[2] Relative content as percentage of the total content (sum of TIC peak areas) of a given species. Multiplication of the percentages in each column with the relative abundance of the species yields the relative contents between species.

[3] β-elemene is the heat-induced Cope rearrangement product formed from germacrene A in the GC-MS injection path (De Kraker *et al.*, 1998)

[4] alkane

[5] carotenoid derivative

[6] fatty acid derivative

[7] Polygodial contains a fraction of 9-*epi*-polygodial which cannot be estimated reliably by GC-MS

The five sesquiterpene drimanes observed in the gas chromatogram were drimenol, polygodial, 9-*epi*-polygodial, drimenin and isodrimenin (**Fig. 3.2**). It is known that 9-*epi*-polygodial is formed from polygodial by base-treatment (Kubo and Ganjian, 1981; Cortés et al., 1998), and there are other reports of this compound being an artifact of the GC-MS analysis (Asakawa et al., 2001). We confirmed that upon injection of a 1H-NMR verified reference standard of pure polygodial, also 9-*epi*-polygodial is observed, and that the ratio of the peaks versus 9-*epi*-polygodial varied from 1.6-0.8:1 depending on the injection temperature (**Supplementary Figure S3.3**). An earlier study, however, reports the 1H NMR spectra for both epimers in *Drymis winterii* (Rodríguez et al., 2005). As our GC-MS method does not allow the reliable estimation of the ratios, we report the combined concentrations of both epimers under the label of polygodial.

Table 3.2. Chemical composition of organic extracts of leaves1 of three *Persicaria* species

Compound	R.I.	*P. hydropiper*[2]	*P. minor*[2]	*P. maculosa*[2]
Monoterpenes		0.21%	0.02%	0.02%
bornyl acetate	1262	0.01%	n.d.	n.d.
limonene	1034	0.20%	0.02%	0.02%
Sesquiterpenes		67.85%	11.11%	3.15%
Drimanes		45.12%	4.90%	0.23%
polygodial[7]	1892	44.27%	3.96%	0.23%
isodrimenin	2039	0.65%	0.14%	n.d.
drimenin	1973	0.19%	0.80%	n.d.
drimenol	1788	n.d.	n.d.	n.d.
Non-drimanes		22.73%	6.21%	2.92%
(*E*)-β-farnesene	1454	7.78%	2.75%	1.01%
α-humulene	1468	6.20%	0.28%	0.06%
caryophyllene	1432	6.10%	2.58%	0.02%
α-bisabolol	1693	1.15%	0.41%	n.d.
(*E*)-nerolidol	1563	0.50%	0.05%	n.d.
(*E*)-β-bergamotene	1492	0.32%	0.04%	n.d.
aristolone	1716	0.31%	0.08%	n.d.
caryophyllene oxide	1597	0.13%	n.d.	n.d.
(*E*)-α-bergamotene	1439	0.11%	0.01%	n.d.
β-bisabolene	1513	0.05%	n.d.	n.d.
β-elemene[3]	1397	0.03%	0.01%	0.62%
α-muurolene	1507	0.03%	n.d.	0.88%
humulene epoxide II	1625	0.02%	n.d.	n.d.
β-bisabolol	1677	n.d.	n.d.	n.d.
δ-cadinene	1523	n.d.	n.d.	n.d.
β-selinene	1500	n.d.	n.d.	0.33%
Diterpenes		31.92%	88.80%	96.68%
neophytadiene isomer I	1836	21.01%	59.06%	62.72%
neophytadiene isomer III	1880	7.65%	21.46%	24.40%
neophytadiene isomer II	1861	3.26%	8.28%	9.56%
Others		0.05%	0.09%	0.15%
heneicosane[4]	2100	0.03%	0.06%	0.12%
dihydro-α-ionone[5]	1419	0.01%	n.d.	n.d.
nonanal[6]	1105	0.01%	0.03%	0.03%
Total abundance (TIC)		100%	100%	100%
Relative abundance (TIC)		100%	34.4%	22.3 %

See legend on page 60

R.I. = retention index; n.d. = not detected

[1] Average of three replicates of each of four developmental stages (N=3x4, table 3). Standard errors were on average 28% of a given value

[2] Relative content as percentage of the total content (sum of TIC peak areas) of a given species. Multiplication of the percentages in each column with the relative abundance of the species yields the relative contents between species.

[3] β-elemene is the heat-induced Cope rearrangement product formed from germacrene A in the GC-MS injection path (De Kraker *et al.*, 1998)

[4] alkane

[5] carotenoid derivative

[6] fatty acid derivative

[7] Polygodial contains a fraction of 9-*epi*-polygodial, which cannot be estimated reliably by GC-MS

Table 3.3. Species-specific content of polygodial in different tissues

Species	Flowers[a] μg gFW^{-1}	Leaves[a] μg gFW^{-1}
Persicaria hydropiper	6198 ± 820	500 ± 38
Persicaria minor	32 ± 6	17 ± 6
Persicaria maculosa	0.07 ± 0.03	0.7 ± 0.5

[a] values with standard error (*n* = 3)

[b] Polygodial contains a fraction of 9-*epi*-polygodial which cannot be estimated reliably by GC-MS

Polygodial was the most abundant compound in *P. hydropiper* flowers, making up for 77% of the extract, equivalent to 6.2 mg g FW^{-1} (**Tables 3.1** and **3.3**). It was 200 times less abundant in *P. minor* flowers (0.032 mg g FW^{-1}), representing 25% of the total compounds, and 100,000 times less abundant in *P. maculosa* flowers (70 ng g FW^{-1}), representing 0.1% of total compounds (**Tables 3.1** and **3.3**). The drimane sesquiterpene lactones drimenin and isodrimenin were minor compounds when compared to polygodial, accounting for only 2% of the *P. hydropiper* flower extract. Relative to *P. hydropiper*, they were 10- and 100-fold less abundant in *P. minor* flowers, respectively, and not detected in *P. maculosa* flowers. Drimenol, the putative precursor of polygodial (Pickett, 1985) was only detected at low concentrations in *P. hydropiper* and not in the other species, presumably because it serves as an intermediate and it is promptly converted. Comparing leaves to flowers, polygodial was 10x more abundant in flowers of *P. hydropiper*, equally abundant in *P. minor* leaves and flowers and 10x less abundant in flowers than leaves of *P. maculosa.*

The low levels of polygodial in *P. maculosa* were earlier not found by Hagendoorn *et al.* (1994) for specimens collected in the Netherlands, but they are in line with the results of Derita *et al.* (2008) who detected low amounts of polygodial (0.54 mg gDW^{-1}) in leaves of *P. maculosa* from Argentina. Yet, by comparison, our specimen contains only 70 ng g FW^{-1}. Taking into account the FW/DW comparison, this is still 100 times less than accessions from Argentina.

4.2. Accumulation pattern of secondary metabolites during flower and leaf development in *P. hydropiper*

To investigate the relative abundance of the secondary metabolites possibly involved in defence throughout development, three stages of flowers and four of leaves were analysed for their chemical composition. Table 4 shows how all drimane sesquiterpenoids were most abundant in Flower stage B. The leaf content of these chemicals was 13% or less, compared to flowers, and the trend was that smaller leaves contained more drimanes. This is possibly due to a relative decrease in cavity density per surface area as leaves expand, as is the case with trichomes of some plant species (Ascensao and Pais, 1987). By MANOVA, differences between flowers and leaves were significant for all compounds, but generally not between stages. However, a paired t-test across all compounds per stage did reveal some significant differences between stages (**Table 3.4**).

Table 3.4. Abundance of GC-MS detectable compounds during the development of flowers and leaves in *P. hydropiper.*

Compound	Rank[1]	FA[2,3]	FB[3]	FC[3]	LA[3]	LB[3]	LC[3]	LD[3]
Monoterpenes								
limonene	15	6.86x10^{4}	109%	82%	16%	26%	12%	9%
bornyl acetate	22	1.05x10^{4}	121%	87%	5%	7%	3%	1%
Sesquiterpenes								
Drimanes								
polygodial[8]	1	2.45x10^{7}	202%	158%	12%	12%	9%	6%
isodrimenin	7	3.92x10^{5}	213%	165%	12%	11%	8%	5%
drimenin	10	2.19x10^{5}	180%	101%	6%	6%	3%	3%
drimenol	25	3.69x10^{3}	341%	131%	6%	0%	0%	0%
Non-drimanes								
caryophyllene	2	3.63x10^{6}	94%	61%	10%	14%	6%	5%
α-humulene	3	2.70x10^{6}	95%	64%	15%	20%	8%	5%
(*E*)-β-farnesene	4	1.94x10^{6}	101%	80%	22%	37%	11%	15%
α-bisabolol	5	6.41x10^{5}	112%	88%	14%	15%	5%	3%
α-muurolene	6	5.44x10^{5}	138%	92%	1%	3%	0%	0%
aristolone	9	2.55x10^{5}	190%	117%	8%	8%	5%	10%
caryophyllene oxide	12	1.40x10^{5}	115%	81%	4%	9%	3%	3%
(*E*)-β-bergamotene	13	1.15x10^{5}	95%	69%	23%	21%	9%	5%
(*E*)-nerolidol	16	5.47x10^{4}	79%	50%	53%	93%	20%	10%
β-bisabolene	17	5.04x10^{4}	134%	100%	5%	9%	2%	1%
(*E*)-α-bergamotene	19	4.32x10^{4}	96%	70%	20%	21%	8%	5%
β-elemene[4]	20	2.16x10^{4}	100%	55%	6%	13%	2%	2%
humulene epoxide II	21	1.60x10^{4}	114%	90%	6%	14%	7%	8%
δ-cadinene	23	8.41x10^{3}	169%	102%	0%	3%	0%	0%
β-bisabolol	26	3.09x10^{3}	268%	135%	0%	0%	0%	0%
Diterpenes								
neophytadiene I	8	3.49x10^{5}	108%	154%	392%	315%	409%	298%
neophytadiene III	14	1.13x10^{5}	110%	171%	410%	362%	465%	383%
neophytadiene II	18	4.91x10^{4}	111%	169%	400%	361%	450%	371%
Others								
heneicosane[5]	11	1.88x10^{5}	5%	6%	1%	1%	1%	0%
dihydro-α-ionone[6]	24	4.92x10^{3}	292%	193%	11%	14%	7%	5%
nonanal[7]	27	4.11x10^{2}	208%	445%	244%	71%	249%	231%
Average		100%	171%	133%	18%	19%	15%	10%
$p < 0.05$[9]		ac	b	bc	e	e	f	-

See legend on page 63

[1] The "Rank" column gives a value according to the abundance of each compound: the lower the value the more abundant the metabolite.

[2] For flower stage 1 (FA) all TIC areas are given expressed in arbitrary units. Injections of 1 µL of a 50 mg mL^{-1} DCM extract of fresh tissue was used in all samples.

[3] All other flower stages (FB and FC) and all leaf stages (LA to LD) are expressed as percentages relative to FA.

[4] β-elemene is the heat-induced Cope rearrangement product formed from germacrene A in the GC-MS injection path (De Kraker *et al.*, 1998)

[5] alkane

[6] carotenoid derivative

[7] fatty acid derivative

[8] Polygodial contains a fraction of 9-*epi*-polygodial, which cannot be estimated reliably by GC-MS

[9] Significant differences between tissues based on overall contents by paired t-test. Tissue LD was only done in duplicate and therefore not part of the analysis

The accumulation patterns of different compounds throughout flower development were grouped together by means of a similarity analysis in order to point to common biosynthetic pathways, common storage tissues like cavities or common biological roles or physical properties. The similarity matrix in **Fig. 3.3** shows how flower derived compounds of *P. hydropiper* cluster. The phylogenetic tree, obtained with the Unweighted Pair Group Method with Arithmetic mean (UPGMA) hierarchical clustering method, shows four distinct groups when half of the clustering distance is considered as a relevant cut-off point. The two minor groups seen at the left hand side and bottom of the matrix represent non-terpenoids (others) and diterpene neophytadiene isomers, with the exception of drimenol. Drimenol is an intermediate in the biosynthesis of polygodial and would not be expected to be stored in the cavities, which are not biosynthetically active, but rather in surrounding cells, which may produce the contents of the cavities. Therefore, an accumulation pattern of drimenol different from polygodial is expected. The three major drimane sesquiterpenes cluster together and belong to the largest group, which, next to bornyl acetate and dihydro-α-ionone, contains 12 sesquiterpenes. This could indicate that all these compounds are stored in the cavities (**Figure 3.1**). The other large cluster contains the monoterpene limonene with five other sesquiterpenes. They are characterized by lower levels in later flower stages (**Table 3.4**). Possibly these are regulated differently, more volatile and/or not localized in the valvate glands.

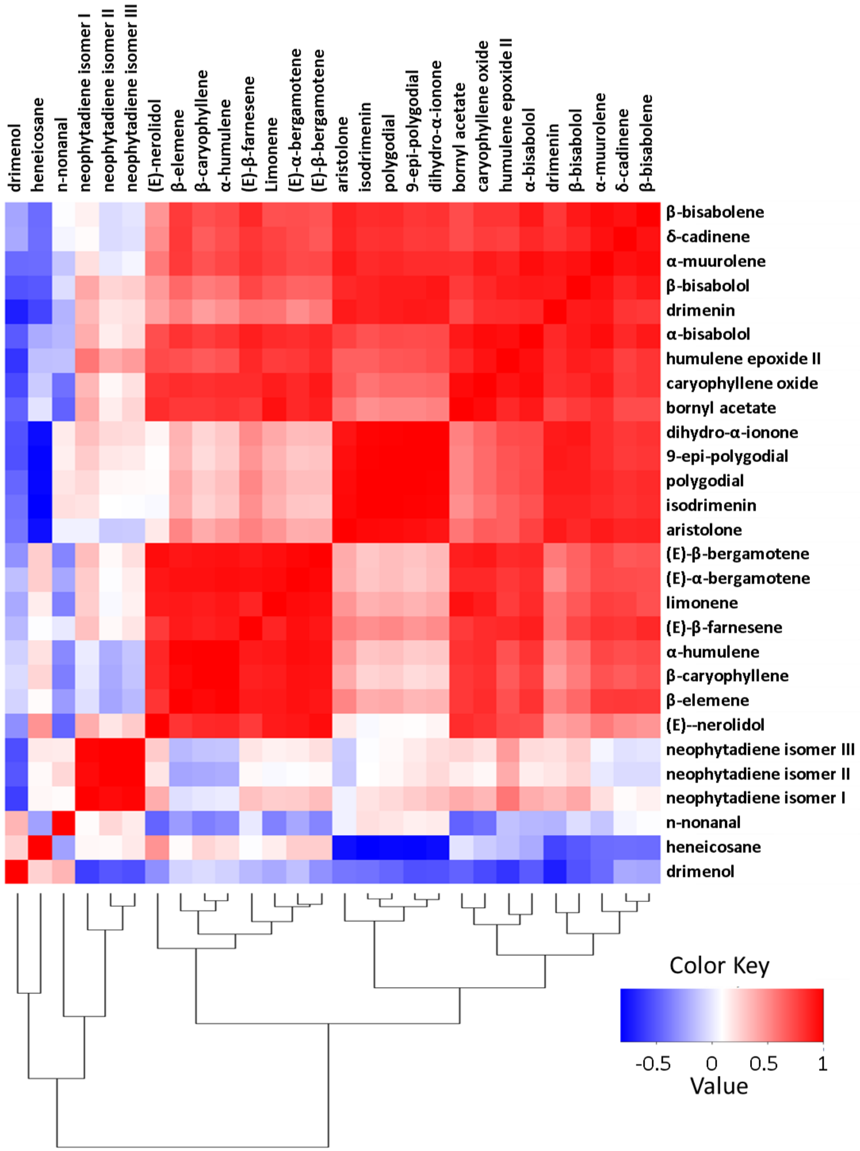


Figure 3.3. Similarity matrix of the compounds, obtained using the Log2-transformed peak areas of the different *P. hydropiper* flower samples throughout development. The similarity of the abundance pattern between two given compounds assumes values between -1 and 1, with 1 meaning a 100% identical pattern. The cells at the diagonal are always intensely red as they represent the comparison of the abundance pattern of one compound with itself.

4.3. Volatile emissions from *P. hydropiper*

In our investigation, we also analysed the headspace of *P. hydropiper*. We focussed only on this species, as it had a far higher content of secondary metabolites in the extracts. The volatile blend emitted by leaves and flowers of *P. hydropiper* contained eight major compounds which were, identified like the compounds in the extracts, by matching the library hits with the known and observed retention indices (**Table 3.5**). The six sesquiterpenes in the headspace more or less reflect the relative contents in the extracts of flowers and leaves. No drimanes were detected in the headspace, presumably because their predicted boiling point (~323 °C for polygodial) is at least 50 degrees higher compared to that of other sesquiterpenes (e.g. 261 ºC for zingiberene), which results in a 228-fold lower vapour pressure and thus volatility (0.000107 vs. 0.0245 mm Hg at 25 °C for polygodial and zingiberene, respectively) (Values were generated using the US Environmental Protection Agency's EPISuite™; information retrieved at www.chemspider.com). The monoterpene limonene was much more dominant in the headspace of flowers than in the extract, presumably due to its high volatility.

Table 3.5. Volatile compounds identified in the headspace of flowers and leaves of *P. hydropiper*.

Compound	R.I.	Flowers[1]	Leaves[1]
β -caryophyllene	1446	32.3%	43.6%
α-humulene	1477	18.0%	19.6%
(*E*)- β -farnesene	1450	17.0%	21.8%
limonene	1040	15.4%	1.2%
α-bisabolol	1650	7.1%	1.7%
decanal	1211	6.6%	9.6%
α-muurolene	1506	2.8%	1.6%
humulene epoxide II	1611	0.9%	0.9%

[1] The composition is expressed as percentage of the total peak area of the volatile blend (*n* = 3)

5. Conclusions

In this study, we compared the chemical profiles of three species of smartweed. In all three species, the flowers contained higher amounts and more types of compounds compared with leaves of the same plant. The vast majority of the compounds identified were sesquiterpenes, five of which were drimanes. We observed that *P. hydropiper* produced the highest amounts of sesquiterpene secondary metabolites, while its congeners, by comparison, accumulated at least 100-500 times less in flowers, and 15-100 times less in leaves. This differential investment in making secondary metabolites might reflect differences in defence strategies or needs between the three species of smartweed. Interesting, in that respect, is the observation that in *P. maculosa*, in both leaves and flowers, there seems to be selective loss (or lack of selected gain) of drimanes and most sesquiterpenes except for (*E*)-β-farnesene and germacrene A. According to the Optimal Defence Theory (ODT), the chemical-defence needs of any part of the plant are determined by value and vulnerability (McCall and Fordyce, 2010). In this frame, young developing tissues are very vulnerable to predation by herbivores. This could explain the higher quantity of polygodial found in young leaves as well as in the tepals of those flower heads with a young developing fruit, although it does not explain why three closely related species, occurring in the same habitats have diverged so much with respect to drimane-based defences. Quite possibly the vulnerability of the other two species to whatever attacks *P. hydropiper* is less, so that there has been not sufficient value to drimane accumulation to balance the cost associated with producing it. A pattern similar to that of *P. hydropiper* accumulation of polygodial (much higher in flowerheads than in leaves) has been observed in the unrelated shrub *Pseudowintera colorata* (Raoul), where berries contained roughly 100 times more polygodial and 9-deoxymuzigadial compared to leaves (Larsen et al., 2007). Those authors speculate that the presence of those two pungent sesquiterpene dialdehydes might exert a protective function against non-specialist herbivores. Considering the broad action of polygodial against mammals, insect pests and microbial pathogens, we would argue that ecological studies with genotypes that are differential in the accumulation of drimanes could provide the necessary evidence for this.

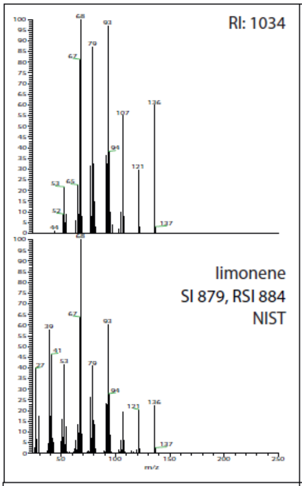
6. Acknowledgments

The authors thank Geert Stoopen, Francel Verstappen, Franck Lhuissier, Desalegn Etalo and Benyamin Houshyani for their help with technical aspects and data analysis. Prof. John Pickett from Rothamsted Research (UK) is acknowledged for the polygodial standard provided as a reference. The research (grant number 1C002RP) was supported by the Technological Top Institute Green Genetics (Stichting TTI Groene Genetica). R.M. was supported by the Netherlands Genomics Initiative via the Centre for BioSystems Genomics.

7. Supplementary material


Persicaria hydropiper

Supplementary Figure S3.1. Images of the three species: *Persicaria maculosa* (p.68), *P. hydropiper* (p.69) and *P. minor* (p.70), collected around Wageningen, NL.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SI 879, RSI 884
NIST
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93
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m/z
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200
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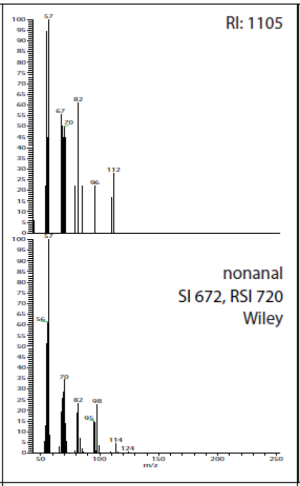
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nonanal
SI 672, RSI 720
Wiley
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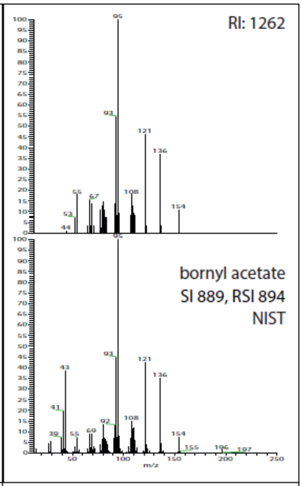
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SI 889, RSI 894
NIST
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197
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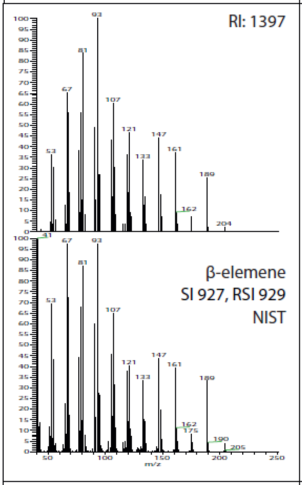
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SI 927, RSI 929
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93
81
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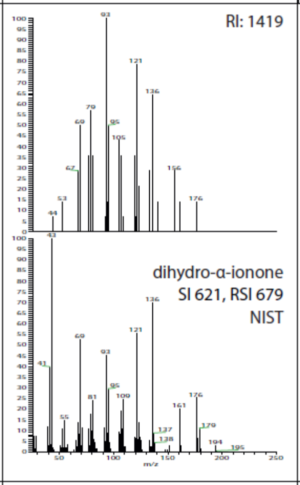
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dihydro-α-ionone
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NIST
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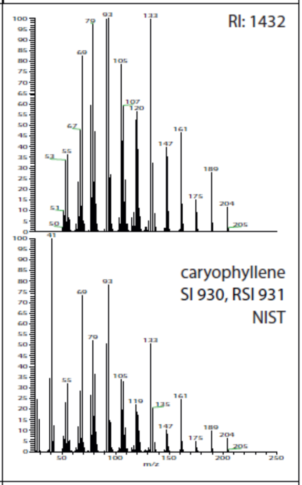
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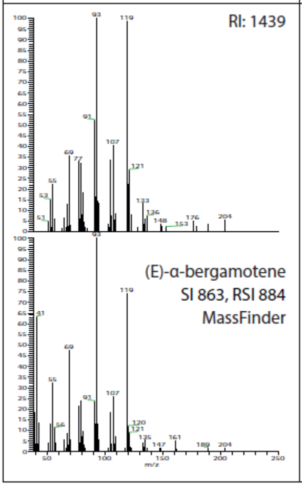
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(E)-α-bergamotene
SI 863, RSI 884
MassFinder
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119
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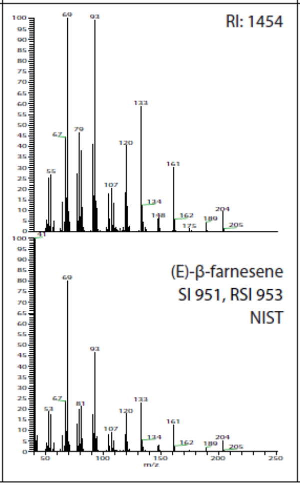
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m/z
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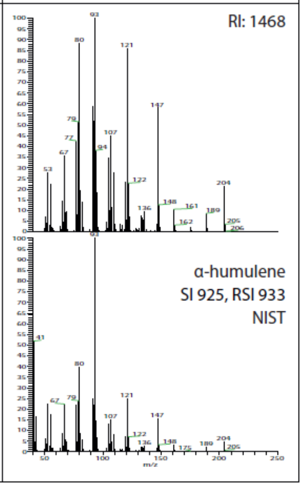
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α-humulene
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NIST
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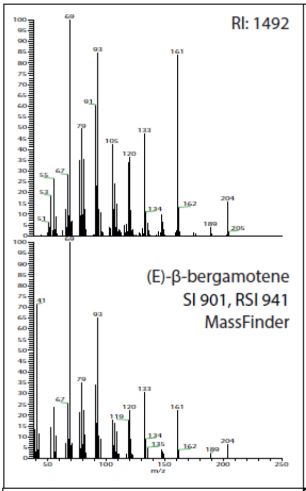
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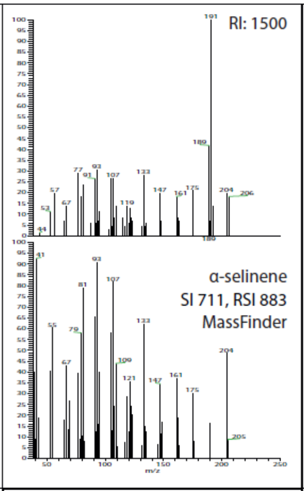
MassFinder
m/z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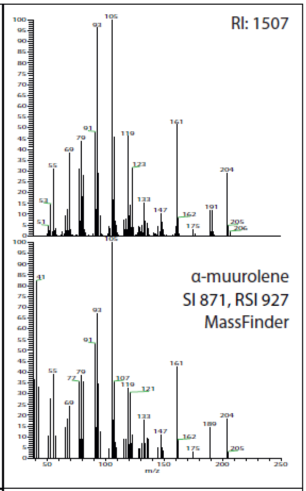
MassFinder
m/z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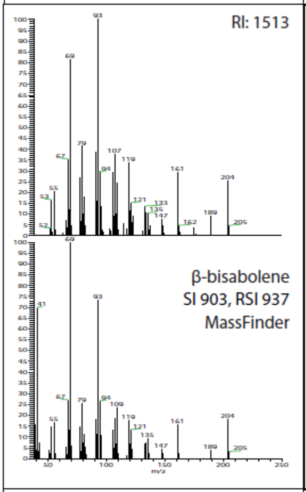
MassFinder
m/z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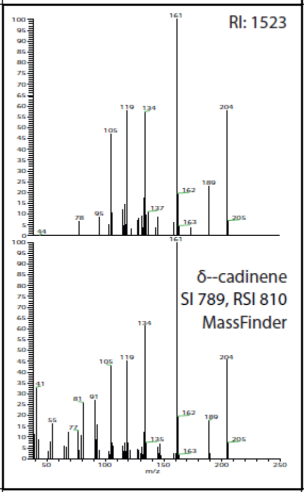
MassFinder
m/z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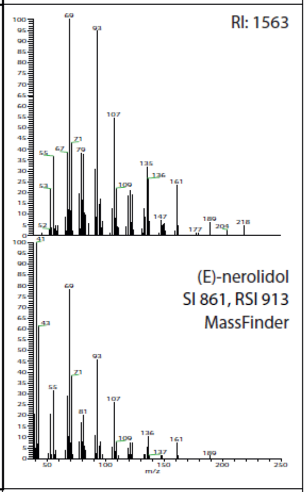
MassFinder
m/z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(E)-nerolidol
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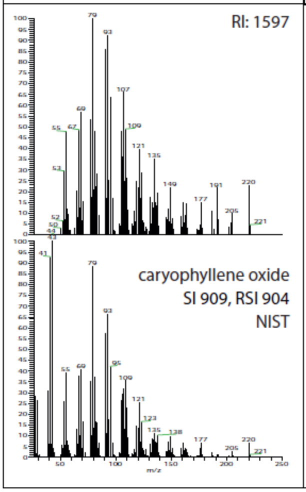
m/z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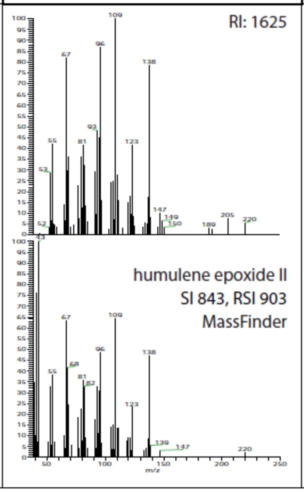
NIST
m/z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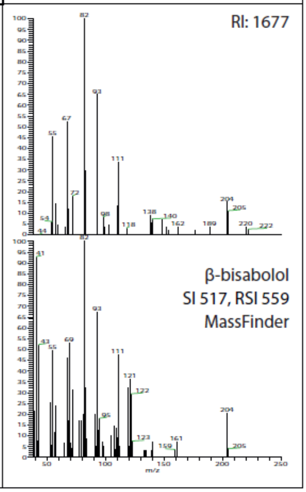
MassFinder
m/z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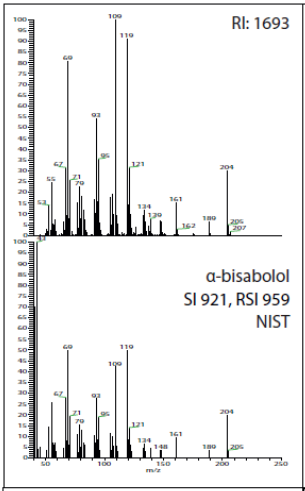
m/z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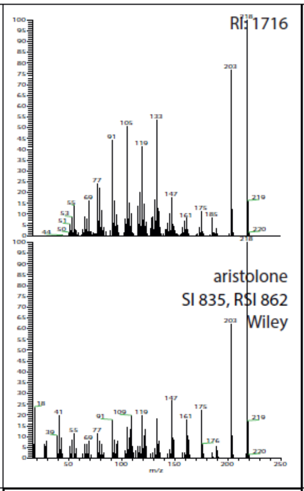
m/z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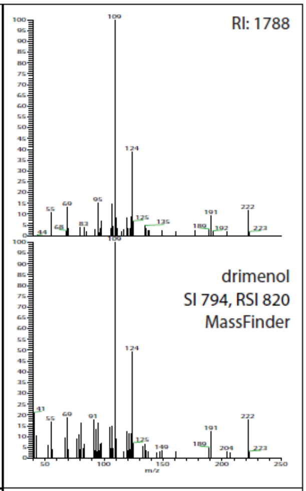
Wiley
m/z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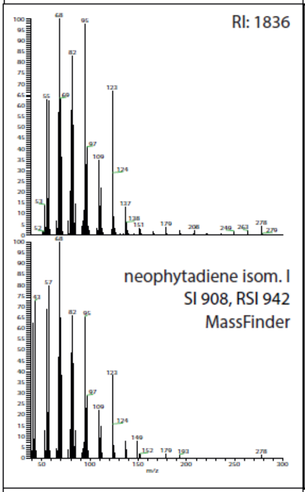
MassFinder
m/z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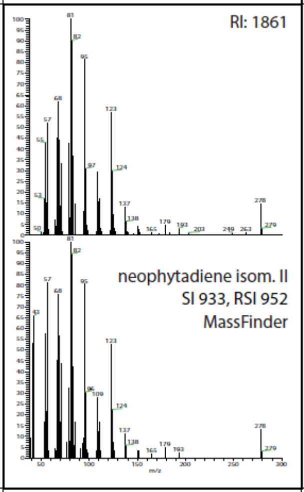
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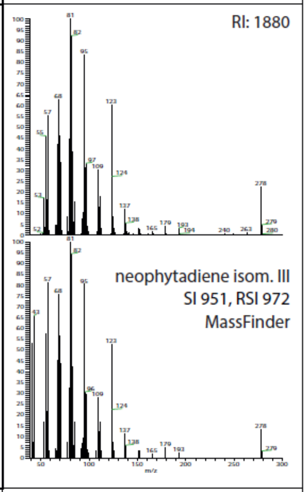
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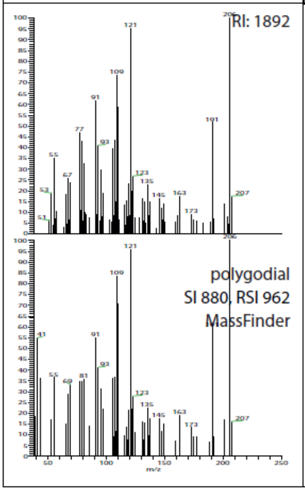
m/z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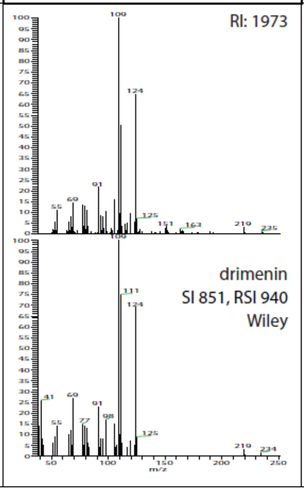
m/z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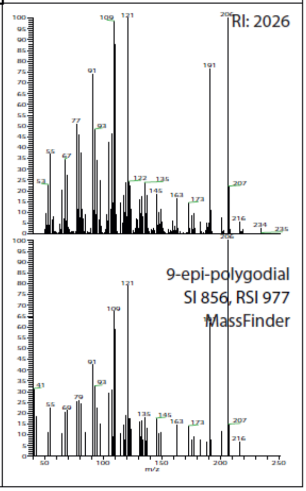
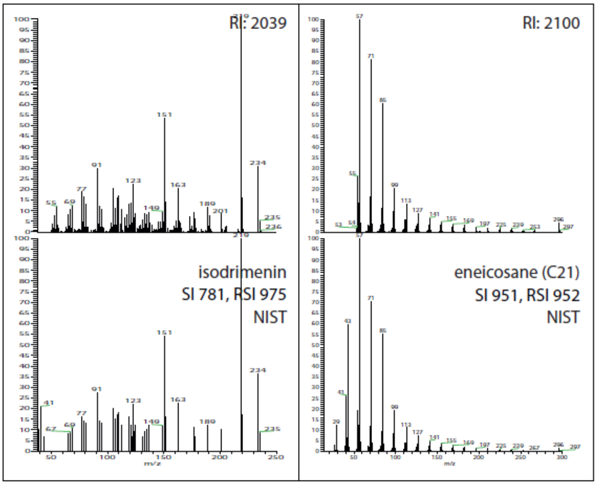
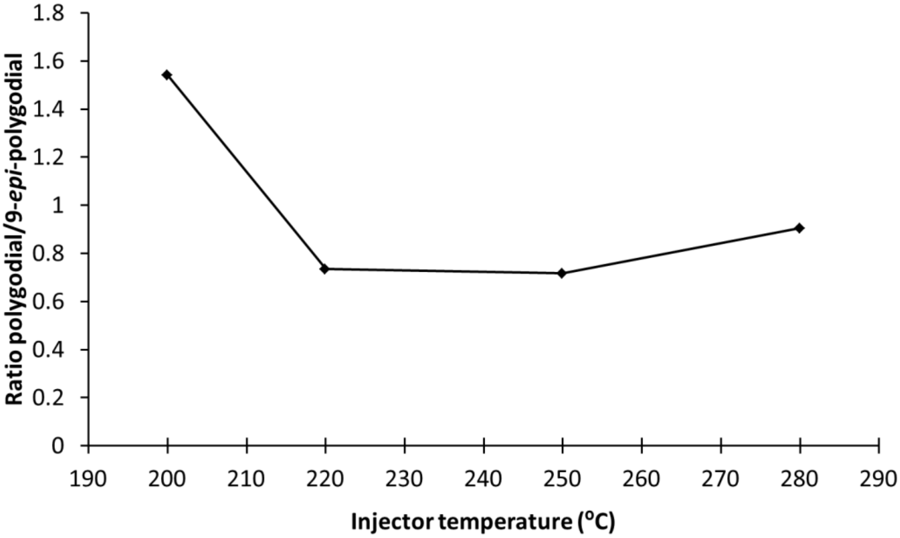
Wiley
m/z


RI: 2026
9-epi-polygodial
SI 856, RSI 977
MassFinder
m/z



Supplementary Figure S3.2. Comparison of the mass spectra of the identified compounds with those from library entries.

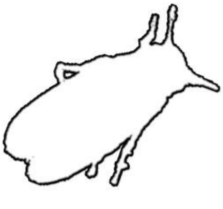


Supplementary Figure S3.3. Ratio between the peak areas of polygodial and 9-*epi*-polygodial at different temperatures of the injection port of the GC (n = 1).

Chapter 4

Identification of a drimenol synthase and drimenol oxidase from *Persicaria hydropiper*, involved in the biosynthesis of insect deterrent drimanes

Maurice G.L. Henquet, Neli Prota, Justin J.J. van der Hooft, Marina Varbanova, Raymond J.M. Hulzink, Martin de Vos, Marcel Prins, Michiel T.J. de Both, Harro J. Bouwmeester and Maarten A. Jongsma



In preparation for publication

1. Abstract

The sesquiterpenoid polygodial, belonging to the drimane family, has been shown to be an antifeedant for a number of herbivorous insects. It is presumed to be synthesized from farnesyl diphosphate via drimenol, subsequent C-12 hydroxylation, and further oxidations at both C-11 and C-12 to form a dialdehyde. Here, we have identified a drimenol synthase (*PhDS*) and a cytochrome P450 drimenol oxidase (*PhDOX1*) from *Persicaria hydropiper*. PhDOX1 oxidizes the C-12 position of drimenol into an aldehyde, which is subsequently converted to cinnamolide, presumably by spontaneous lactone formation with the C-11 hydroxyl group. Expression of *PhDS* in yeast and plants resulted in production of drimenol only. Co-expression of *PhDS* with *PhDOX1* in yeast yielded drimendiol, the 12-hydroxylation product of drimenol, as a major product, and cinnamolide. When *PhDS* and *PhDOX1* were transiently expressed by agro-infiltration in *Nicotiana benthamiana* leaves, drimenol was almost completely converted into cinnamolide. Both in yeast and *N. benthamiana* several additional drimenol derivatives were observed, which were partly unique to the host organism, suggesting complex conversions resulting from PhDOX1 and endogenous enzymatic activities. Purified cinnamolide was shown to be a deterrent with an effective deterrent dose (ED_{50}) of ~200-300 µg gFW^{-1} against both whiteflies and aphids. The putative additional physiological and biochemical requirements for polygodial biosynthesis and stable storage in plant tissues are discussed.

2. Introduction

Sesquiterpenes are a family of C_{15} isoprenoids that are found in both higher and lower plants, microbes and some marine organisms. They are predominantly synthesized from farnesyl diphosphate through the mevalonate pathway in the cytosol and many have biological activities, including antimicrobial, anti-tumor, and cytotoxic properties. In plants, they play important roles in the interaction with insects and microbes and act as attractants, deterrents or antifeedants (Dudareva et al., 2005; Sallaud et al., 2009). Sesquiterpenes may be acyclic or contain rings in many unique stereo-specific combinations. Biochemical modifications such as oxidation may form alcohols, aldehydes, ketones, acids and lactones.

Drimanes are sesquiterpenes that have a bicyclic structure and are widespread in plants, liverworts, fungi and certain marine organisms (sponges and slugs) and are also reported to have antibacterial and antifungal properties (Jansen and de Groot, 2004). Well-known examples of drimane sesquiterpenes are the dialdehydes warburganal, ugandensidial and muzigadial, which all display insect antifeedant activity against armyworms (Kubo and Ganjian, 1981). The best known is polygodial with a pungent taste to mammals (Kubo and Ganjian, 1981; Escalera et al., 2008). In plants, polygodial has been shown to act as antifeedant against a number of herbivorous insects (Jansen and de Groot, 2004). The most common plant in which polygodial occurs is water-pepper, *Persicaria hydropiper* (formerly *Polygonum hydropiper*) (Starkenmann et al., 2006). It was reported that *P. hydropiper* produces and stores polygodial especially in the leaves and flowerheads, in specialized epidermal cavities named valvate or irritant glands (Hagendoorn et al., 1994; Derita et al., 2008).

Pickett proposed a biosynthetic pathway for the synthesis of polygodial, in which the sesquiterpene alcohol drimenol is a precursor (Pickett, 1985) (**Figure 4.1**). Drimenol was proposed to be synthesized directly from farnesyl diphosphate (FPP) by a sesquiterpene synthase. The conversion to polygodial was presumed to occur through one or more oxidases belonging to the cytochrome P450 family, but neither the drimenol synthase nor the oxidases have so far been identified.

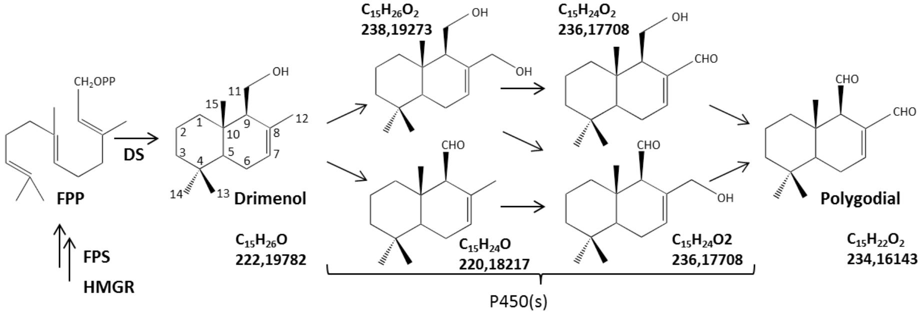


Figure 4.1. Proposed biosynthetic pathway from farnesyl diphosphate (FPP) to drimenol and polygodial (adapted from Pickett, 1985). Carbon atoms are numbered in the drimenol structure. Enzymes relevant for this study have been indicated. FPS: farnesyl diphosphate synthase; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase; DS: drimenol synthase. The biosynthesis of polygodial is believed to involve the oxidation of drimenol, which may be obtained in one step by the cyclization of FPP, and enzymatically controlled oxidation steps by one or more P450s.

The aim of this study was to identify genes from *P. hydropiper* involved in the biosynthesis of drimenol and polygodial and characterize the compounds they produce in heterologous expression systems such as *Saccharomyces cerevisiae* and *Nicotiana benthamiana*. Enzyme products were evaluated by

GC-MS, as well as by untargeted metabolomics using LC-QTOF-MS. The purified major products were identified by NMR and tested against two phloem-sucking insect pests.

3. Materials and methods

3.1. cDNA synthesis and GS FLX Titanium General Library Preparation

cDNA synthesis was performed using the SMART PCR cDNA Synthesis kit (Clontech, USA) according to manufacturer's instructions with minor modifications. An aliquot of 1 µg of total RNA was used as input for the reverse transcriptase reaction using SuperScript II reverse transcriptase (Life Technologies, USA). To facilitate the removal of the poly(A)-tail prior to sequencing, first-strand cDNA synthesis was carried out with a modified 3' SMART CDS primer IIA containing a rare cutter restriction endonuclease site in the 5' region of the oligo(dT) sequence. cDNA amplification was performed using the Advantage 2 PCR kit (Clontech, USA) following the instructions in the SMART PCR cDNA Synthesis protocol. Double-stranded cDNA was purified using the QiaQuick PCR purification kit (Qiagen, Germany). cDNA quality was determined on a BioAnalyzer using a DNA 7500 chip (Agilent Technologies, Germany). cDNA normalization was carried out using the TRIMMER cDNA Normalization kit (Evrogen, Russia) as per manufacturer's instructions. Amplification of normalized cDNA was performed using the Advantage 2 PCR kit (Clontech Inc., USA) following the instructions in the TRIMMER cDNA Normalization manual. Instead of using the Evrogen's PCR primer M2, a modified biotinylated primer was used for second amplification of normalized cDNA. The normalization efficiency was determined with a DNA 7500 chip (Agilent Technologies, Germany).

Random cDNA shearing prior FLX Titanium library was performed by nebulization of 15 to 20 µg of normalized cDNA according the instructions in the GS FLX Titanium General Library Preparation manual (Roche Applied Science, Germany). After nebulization, the randomly sheared cDNA was purified using the QiaQuick PCR purification kit (Qiagen, Germany) and the size distribution was determined with a DNA 1000 chip (Agilent Technologies, Germany). Removal of biotinylated 3' cDNA ends comprising the poly(A)-tail was performed as follows. First, the nebulized cDNA was digested with a restriction endonuclease that specifically recognizes the restriction site that was previously introduced in the 5' region of the poly(A) tail. After digestion, the biotinylated ends were bound to M-270 streptavidin Dynabeads according to manufacturer's instructions (Life Technologies, USA) and the supernatant containing the digested cDNA without biotinylated ends was collected and purified using the MinElute PCR purification kit (Qiagen, Germany). Exclusion of smaller-sized fragments was performed using the double SPRI method as described in the Roche GS FLX Titanium General Library Preparation protocol (Roche Applied Science, Germany). Further library preparation was performed according to the standard GS FLX Titanium General Library Preparation Method protocol as supplied by Roche Applied Science (Germany). The cDNA samples were analyzed by massive parallel pyrosequencing using a Genome Sequencer FLX Titanium sequencing platform (Roche Applied Science, Germany).

3.2. Isolation and cloning of drimenol synthase and cytochrome P450 genes from *P. hydropiper*

The identification of polygodial biosynthetic genes relied first on obtaining a 454 EST library from both *P. hydropiper* (water pepper) and *P. maculosa* (lady's thumb). Good quality mRNA was obtained from young flowers from both species and the normalized cDNA sequenced. Comparative screening of the libraries allowed the identification of a sesquiterpene synthase with a much higher apparent abundance in *P. hydropiper* compared to *P. maculosa*. The full-length putative drimenol synthase (*PhDS*) gene was amplified using high fidelity Phusion polymerase (Finnzymes) and the primers PhDS-F (BsaI) 5'GTGACGGTCTCCCATGTCTACTGCCGTTAACGTCC'3 and PhDS-R (NotI) 5' GTGACGCGGCCGCCTAAATCGGAATGGGATCGGTGA'3 with the addition of BsaI restriction site (with NcoI overhang) and NotI restriction sites which allowed subsequent cloning into the pYES3/CT yeast expression vector (Invitrogen), containing the TRP1 auxotrophic selection marker. The *PhDS* gene was transformed into yeast strain WAT11, expressing the *Arabidopsis thaliana ATR1* NADPH-cytochrome P450 reductase (Urban et al., 1997). The sequence of the gene was deposited in the NCBI GenBank Nucleotide database under accession number KC754968.

A BLAST search using already known sesquiterpene cytochrome P450 sequences from literature, revealed several potential terpene hydroxylases with, once again, a much higher abundance in *P. hydropiper* compared to *P. maculosa*. Seven selected full-length *CytP450* genes were cloned, using the *NotI/PacI* restriction sites, into the yeast expression vector pYEDP60 (Pompon et al., 1996) modified to contain the *Pac*I and *Not*I sites at the polylinker. The candidate CytP450s were co-transformed with the *PhDS* from *P. hydropiper* into the yeast strain WAT11. After transformation, yeast clones expressing the *PhDS* alone or *PhDS* combined with each *CytP450* were selected on Synthetic Dextrose minimal medium (0.67% Difco yeast nitrogen base medium without amino acids, 2% D-glucose, 2% agar) supplemented with amino acids, but omitting L-tryptophane or uracil, adenine sulphate and L-tryptophane, respectively, for auxotrophic selection of transformants. Only one P450 was able to convert drimenol in yeast. The sequence of this drimenol oxidase (PhDOX1) gene was deposited in the NCBI GenBank Nucleotide database under accession number KC754969. The sequence was also submitted to David Nelson's cytochrome P450 homepage (http://drnelson.uthsc.edu/cytochromeP450.html) and was assigned the name CYP76AJ1 (Nelson, 2009).

3.3. Co-expression of *PhDS* with putative P450s in yeast

A starter yeast culture was grown overnight at 30 °C in 5 mL of Synthetic Galactose minimal medium (0.67% Difco yeast nitrogen base medium without amino acids, 2% D-galactose, amino acids, but omitting either L-tryptophane or uracil, adenine sulphate and L-tryptophane). The starter culture was diluted to OD_{600} of 0.05 in 50 mL of Synthetic Galactose minimal medium and incubated at 200 rpm at 30 °C. The culture was either grown for 3 days or overlaid with 5 mL of n-dodecane when the OD_{600} was in the range from 0.8 to 1 and cultivation continued for 3 days. The n-dodecane layer was collected and centrifuged at 1200 rpm for 10 min, diluted threefold in ethyl acetate, dried using anhydrous Na_2SO_4 and then used for GC-MS analysis. In expression experiments without n-dodecane, the metabolites from the medium were extracted with 10 mL ethyl acetate. From this, a sample was analyzed by GC-MS.

3.4. Plasmid construction for expression in *Nicotiana benthamiana*

The *PhDS* and *PhDOX1* genes were cloned into ImpactVector1.1 (www.impactvector.com) to express them under the control of the Rubisco Small Subunit (RbcS) promoter for expression in *N. benthamiana* (Outchkourov et al., 2003). The *PhDS* gene was also cloned into ImpactVector 1.4 and 1.5 to fuse it with the RbcS promoter and the plastid or CoxIV mitochondrial targeting sequence, respectively. To clone each gene into the pBinPlus binary vector between the right and left borders of the T-DNA for plant transformation, a recombination reaction was carried out, using the Gateway-LR Clonase TM II (Invitrogen).

3.5. Transient expression in leaves of *Nicotiana benthamiana*

Agrobacterium tumefaciens-mediated infiltration (agroinfiltration) was performed as described previously (van Herpen et al., 2010). Briefly, *A. tumefaciens* strains were grown at 28 °C at 220 rpm for 24 hours in LB media with kanamycin (50 mg L^{-1}) and rifampicin (100 mg L^{-1}). Cells were harvested by centrifugation for 20 min at 4000 g and 20 °C, and then resuspended in 10 mM MES buffer containing 10 mM $MgCl_2$ and 100 µM acetosyringone (4′-hydroxy-3′,5′-dimethoxyacetophenone, Sigma) to a final OD_{600} of 0.5, followed by incubation at room temperature with shaking at 50 rpm for 3 hours. For co-infiltration, equal volumes of the *A. tumefaciens* strains were mixed. *N. benthamiana* plants were grown from seeds on soil in the greenhouse with a minimum of 16 hour light. Day temperatures were approximately 28 °C, night temperatures 25 °C. Strain mixtures were infiltrated into leaves of four-weeks-old *N. benthamiana* plants using a 1 mL syringe without needle. The bacteria were slowly injected into the intercellular space through the abaxial side of the leaf. The plants were grown and the infiltrated leaves were collected 4 or 5 days after infiltration.

To analyze the compounds accumulated in the leaves, the harvested plant material was snap-frozen and ground in liquid nitrogen. 500 mg of powder was extracted with 2 mL dichloromethane. The extracts were briefly vortexed and submerged in a sonication bath for 5 min. They were then centrifuged for 10 min at 1200 rpm and the clear part of the solution was transferred to a fresh vial. Finally, the extracts were dehydrated using anhydrous Na_2SO_4 and concentrated by evaporating the solvent to a volume of about 0.5 mL. Analysis of the samples was performed by GC-MS (Agilent GC-MS, Agilent technologies).

3.6. GC-MS analysis

Analytes from 1 µL samples were separated using a gas chromatograph (5890 series II, Hewlett-Packard) equipped with a 30 m × 0.25 mm, 0.25 mm film thickness column (ZB-5, Phenomenex) using helium as carrier gas at flow rate of 1 mL min^{-1}. The injector was used in splitless mode with the inlet temperature set to 250 °C. The initial oven temperature of 45 °C was increased after 1 min to 300 °C at a rate of 10 °C min^{-1} and held for 5 min at 300 °C. The GC was coupled to a mass-selective detector (model 5972A, Hewlett-Packard). Compounds were identified by comparison of mass spectra and retention times (Rt) with those of the authentic standards, when available, or with known retention indeces (R.I.) from literature. Drimenol and polygodial were kindly provided by John Pickett (Rothamsted Research, Uk). Putative identification was achieved by comparing mass spectra with the NIST mass spectra library.

3.7. LC-Orbitrap-FTMS analysis

High-resolution mass spectrometry was performed on an LC-LTQ-Orbitrap FTMS system (Thermo Scientific) operating in positive ionization mode. The instrument consisted of an Accela HPLC, an Accela photodiode array detector, connected to an LTQ/Orbitrap hybrid mass spectrometer equipped with an ESI source. Chromatographic separation took place on a Phenomenex Luna C18 analytical column (150 × 2.0 mm, 3 µm particle size), using H_2O and acetonitrile, both containing 0.1% v/v formic acid, at a flow rate of 0.19 mL min^{-1} and a column temperature of 40 °C. A linear gradient from 5 to 75% acetonitrile in 45 min was applied, which was followed by 15 min of washing and equilibration before the next injection. The injection volume was 5 µL.

3.8. Drimendiol purification

Ethyl acetate extract of yeast expressing *PhDS* and *PhDOX1* was evaporated with N_2 flow and redissolved and concentrated in 100% MeOH. The HPLC was performed using a water:acetonitrile gradient from 40% to 75% acetonitrile. All solutions contained 0.1% v/v formic acid (FA). The flow rate 1mL min^{-1} and 1-min fractions were automatically collected. The fractions of interest were either extracted with DCM and analyzed by GC-MS or freezedried to analyze by NMR.

3.9. HLB column concentration

To remove impurities and concentrate the HPLC-purified fractions, HLB solid phase extraction (SPE) cartridges (3cc, OASIS, Waters) were used prior to NMR analysis. The columns were activated under vacuum with 4 mL of methanol + 0.1% FA and washed with 6 ml 0.1% FA. The samples dissolved in 25% methanol and 0.1% FA were loaded onto the SPE cartridges, and the columns then washed with 4 ml of 0.1% FA. Elution was performed with 4 mL of 100% methanol + 0.1%FA. Subsequently, the eluates were dried and redissolved in 100% methanol and analyzed by LC-MS and NMR.

3.10. NMR spectroscopy

NMR analysis of the plant extract was performed at Spinnovation Analytical BV (Nijmegen, the Netherlands) on a Bruker Avance III 500 MHz spectrometer equipped with a 5-mm CPTCI cryo probe (1H-13C/15N/2H + Z-gradients) operating at 303 K. The structure identification of polygodial, used as a reference compound is based on a 1D 1H, 1H-1H-DQF-COSY, 1H-1H-TOCSY, 1H-1H-NOESY, 1H-13C-HSQC and 1H-13CHMBC spectra. The structure identification of the purified compound is based on the same selection of NMR experiments with the exception of the 1H-13C-HMBC spectrum due to the limited amount of material. The proton and carbon chemical shifts were referenced to the internal reference TMS (proton, d = 0.00 ppm; carbon, d = 0.00 ppm). The data were processed using Topspin 2.1 pl5.

NMR analysis of the isolated yeast compound was performed at Bisqualis/Wageningen UR (Wageningen, the Netherlands) on a Bruker Advance III 600 MHz spectrometer equipped with a cryo-probe. 1D-1H NMR measurements of 128 scans and a d1 of 4 s (i.e., almost 15 min) were obtained at a receiver gain of 128 in MeOD using standard pulse sequences. Twodimensional (2D) 1H measurements (COSY, HSQC, HMBC; using standard pulse sequences) were conducted for structural confirmation.

3.11. Cinnamolide purification

Cinnamolide was purified from a plant methanolic extract, obtained from transiently transformed *N. benthamiana* plants expressing the *AtHMGR*, *AtFPS2*, *PhDS* and the *PhDOX1* genes. The extract was evaporated and injected onto a C18 analytical column. The HPLC was performed using a water:acetonitrile gradient from 40% to 75% acetonitrile. After identification of the desired fraction by UV absorbance, it was collected multiple times until a sufficient amount was obtained. The resulting fractions were pooled and evaporated using a SpeedVac and the crystalline compound was weighed and finally dissolved in 96% ethanol.

3.12. Insects

The insects used in this study were the silverleaf whitefly *Bemisia tabaci* (genotype B) and the green peach aphid *Myzus persicae*. Whiteflies were reared on tomato (cv. Moneymaker) in a greenhouse at 26 °C and 60% relative humidity with a photoperiod of 16 h light and 8 h dark. To perform the assays, adult flies of both genders were collected from leaves with an aspirator. They were cold-anesthetized for 5 min at 7 °C in a plastic cylinder (3 cm Ø) covered with Parafilm®, before being released into the Petri dish at the start of the dual-choice assay, to ensure they would not fly out before the dishes were sealed. Apterous aphid adults (*Myzus persicae*) were collected from Chinese cabbage (*Brassica rapa* L. subspecies *pekinensis*), on which they had been reared at room temperature in ambient light. They were placed in

2-mL-test tubes and starved for about 30 min before inoculating them in the dishes.

3.13. Insect choice assays

In the dual-choice assays, insects were presented with four circular tomato leaf areas coated with either 50% ethanol in water (control) or a solution of cinnamolide in 50% ethanol in water (treatment; two disks with each solution). Three cinnamolide concentrations were used; 1 mg mL^{-1}, 500 µg mL^{-1} and 250 µg mL^{-1} in 50% ethanol. Fresh Moneymaker tomato leaves collected from 5 or 6 weeks old plants were cut in half longitudinally and placed abaxial-side up in a 9-cm-Petri dish on 45 mL of 8 g L^{-1} water agar substrate. The leaves were then covered with a Petri dish bottom through which four holes of 16 mm diameter were drilled, making sure that each half leaf had two circular areas available for insects to feed upon. On each circular area (disc), 15 µL of solution were applied and spread using a size 3-watercolour brush. The control and treatment solutions were each spread on two diametrically opposed disks to control for potential environmental cues, such as light. The insects were introduced into the dishes after the solvent had evaporated. Insects were put onto the lid of the dishes and then covered with the bottom of the Petri dish containing the leaves. On average, 98 whiteflies and 20 aphids per plate were used for each assay. For each combination of insect species and compound concentration, eight biological replicates (eight separate arenas) were used. The plates were sealed with Parafilm® and kept upside down. The assays with aphids were carried out at room temperature on a lab bench (uncontrolled conditions). The whiteflies assays were performed in a climate chamber at 25 °C, 60% humidity and 16/8 hours light/dark photoperiod. The aphid assays were carried out in a laboratory under uncontrolled climatic conditions. The assays were carried on for either 18 or 24 hours and the feeding insects counted at 30 min, 1 h, 2 h, 6 h, and 18 h for whiteflies and at 30 min, 1 h, 2 h, 6 h, and 24 h for aphids. Insects were considered to be feeding when they were immobile on one spot on a leaf disk. Five leaf disks were weighed to be able to determine the concentration of the insect repellent compounds in relationship to fresh weight (FW) of the leaf area treated. The average mass of each disk was found to be 34 mg. It was estimated that the concentrations of coated cinnamolide correspond to approximately 450, 225 and 113 µg gFW^{-1}.

3.14. Data analysis

To express the repellence potency of cinnamolide towards the two insect species tested, the Antifeedant Index (AI%) was calculated according to Kutas and Nádasy, as follows: AI%=[(C-T)/(C+T)]x100, where C indicates the number of insects feeding on the control, and T the number of insects feeding on the treatment (Kutas and Nádasy, 2005). The AI% assumes positive values when the tested compound is an antifeedant, and negative values when the compound is a phagostimulant. To determine the ED_{50}, the effective dose at which 50% of the insects are deterred (i.e. the concentration at which twice as many insects feed on the control compared to the treatment), a Probit analysis was carried out using the PASW statistics 18 (SPSS Inc.) package.

4. Results

4.1. Identification of the drimenol synthase gene *PhDS*

In a previous study we analyzed the chemical profile of three *Persicaria* spp., *Persicaria hydropiper*, *Persicaria maculosa* and *Persicaria minor* (Prota et al., 2014). *P. hydropiper* was shown to produce by far the highest amounts of polygodial (6.2 mg gFW^{-1} in flowers), while in *P. maculosa* only traces of polygodial were detected. To identify genes involved in the biosynthesis of polygodial, we made a 454 EST library using both *P. hydropiper* and *P. maculosa* flower material. By sequence assembly, 28,800 and 23,480 contigs were formed for *P. hydropiper* and *P. maculosa*, respectively. Initial comparative screening allowed the identification of a dominant sesquiterpene synthase with ~30 times higher abundance in *P. hydropiper* compared with *P. maculosa*. The complete ORF was cloned and expressed in yeast. GC–MS analysis of the dodecane overlay of the yeast culture showed a novel product which was identified as drimenol, confirming that this gene encodes a drimenol synthase (*PhDS*) (**Figure 4.2**). The full-length cDNA sequence of *PhDS* (GenBank accession no. KC754968) contained an open reading frame of 1677 bp, encoding a protein of 559 amino acid residues and a predicted molecular weight of 64,729 Daltons. Analysis of the deduced amino acid sequence, using SignalP version 4.0 server software, indicated that no signal peptide was apparent in the N-terminal region of the protein (Bendtsen et al., 2004). A similarity search in Genbank using BlastP revealed the highest similarity with a putative sesquiterpene synthase from *Santalum murrayanum*, followed by germacrene A synthase from *Vitis vinifera* (both 52% identity).

4.2. Identification of the drimenol oxidase, *PhDOX1*

The *P. hydropiper* cDNA database was also used to search for homologs of the cytochrome P450 sequences of amorpha-4,11-diene oxidase from *Artemisia annua* (van Herpen et al., 2010), an elicitor-inducible cytochrome P450 from

Nicotiana tabacum (Ralston et al., 2001), a cytP450 *hydroxylase* from *Hyoscyamus muticus* (Takahashi et al., 2007) and GA3 ent-kaurene oxidase from *Arabidopsis thaliana* (Helliwell et al., 1999).

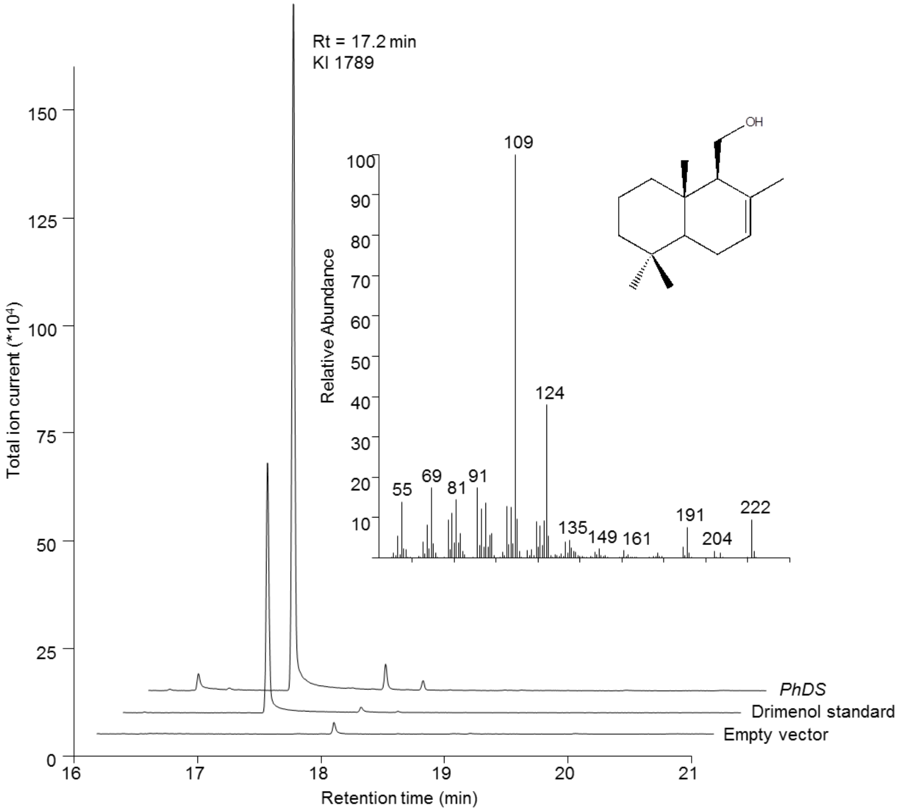


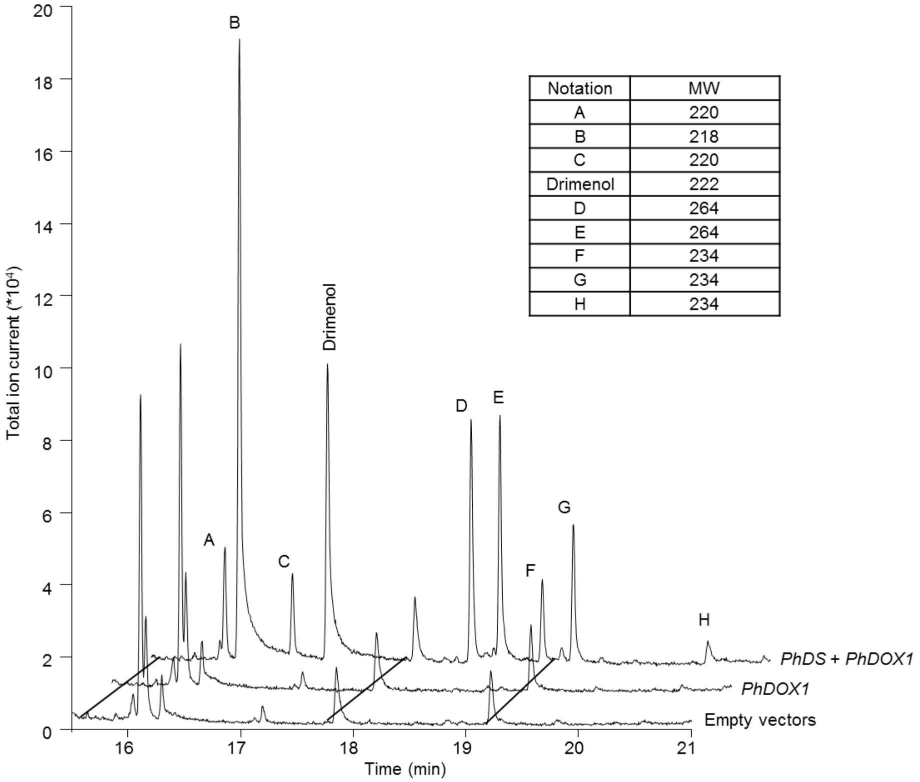
Figure 4.2. GC-MS chromatogram (total ion current) of the n-dodecane layer from the yeast strain WAT11 expressing the *Persicaria hydropiper* drimenol synthase (*PhDS*) and an empty vector as control. The predominant peak was identified as drimenol (Rt = 17.17 min). Mass fragmentation pattern and the structure of drimenol are also shown. KI = Kovats Index

The search revealed several potential terpene oxidases with higher abundance in *P. hydropiper* compared with *P. maculosa*. Also a “P450” keyword search in the *P. hydropiper* database resulted in a cytochrome P450 which was ~150 times less abundant in *P.maculosa*. Seven of these P450 sequences were cloned into a yeast expression vector and co-expressed with the *P. hydropiper* drimenol synthase. Yeast cultures were overlaid with dodecane. After 3 days the organic solvent was extracted and analyzed by GC-MS analysis. Only one of the P450s, the most abundantly expressed one, encoded an enzymatic activity that converted ~94% of drimenol into several new sesquiterpene derivatives, but not into polygodial, and was therefore called drimenol oxidase *PhDOX1* (**Fig. 4.3**, **supplementary Figure S4.1**). No activity was detected for the other candidate P450s. *PhDOX1* (GenBank accession no. KC754969) encodes a 503-residue protein with a predicted mass of 56,149 Da. The deduced amino acid sequence contains the PFGAGRRICPG motif that corresponds to the highly conserved heme-binding domain PFGxGRRxCxG found in most plant P450s (Durst and O'Keefe, 1995). Comparison of the deduced amino acid sequence showed only up to 52% sequence similarity with the CYP76F and CYP76B subfamily. Therefore, the enzyme was classified by Dr. David Nelson into a new subfamily and designated CYP76AJ1 (Nelson, 2009).

4.3. Characterization of *PhDOX1* products in yeast

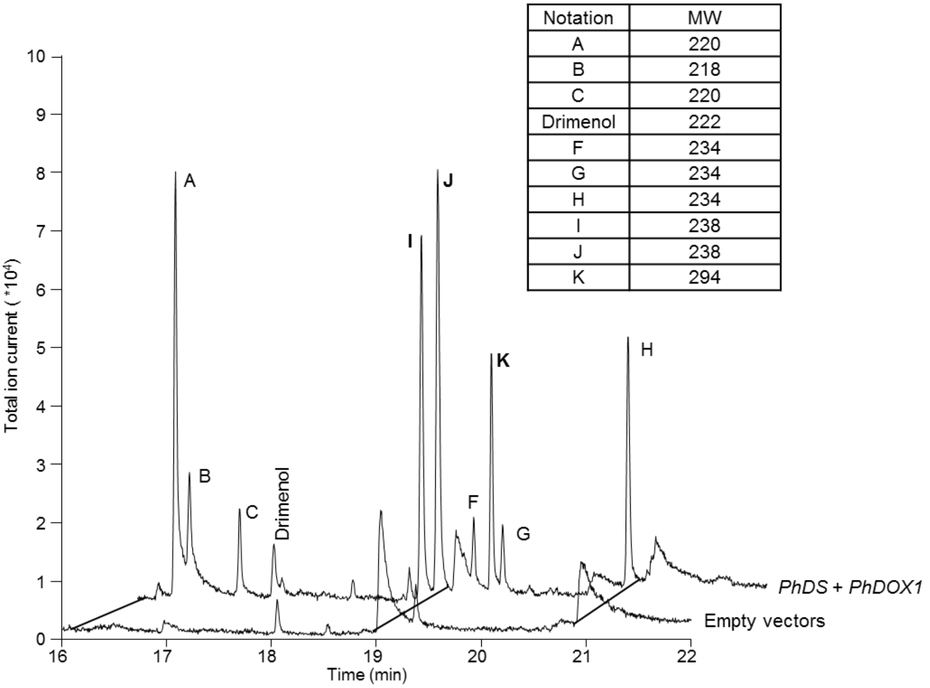
PhDOX1 expression in drimenol-producing yeast did not yield polygodial, but eight other drimenol derivatives (A-H, **Figure 4.3**). Comparison of the spectra with those of published reference spectra of terpenoids in the National Institute of Standards and Technology (NIST) MS database did not show significant homology to any compounds in the database. Compound D and E (*m/z* 264) and F and G (*m/z* 234) have highly similar mass spectra, suggesting they have a similar structure. To test the effect of the dodecane overlay, which extracts compounds from the water phase upon their formation, yeast co-transformed with *PhDS* and *PhDOX1* was also grown without a dodecane overlay. Yeast medium was subsequently extracted with ethyl acetate and analysed by GC-MS. The chromatogram showed that almost all drimenol was converted by PhDOX1 and that nine peaks appeared (**Fig. 4.4**, **Supplementary Figure S4.1**). Six of those were also detected among the eight compounds of the dodecane yeast extract, with D and E missing. The three novel compounds (I, J and K) could also not be reliably identified through a NIST library search. The mass fragmentation spectra of compounds I and J (*m/z* 238) were very similar suggesting they have a similar structure.

Untargeted LC-Orbitrap-FTMS analysis was used to investigate whether any other metabolic products were formed by expression of *PhDS* and *PhDOX1* in yeast and to determine the accurate mass of the compounds produced (**Fig. 4.5**). The analysis revealed a major new compound with a mass of MH+ 203.1780. This compound was isolated by HPLC. GC-MS analysis of the isolated compound suggested it consists of a mixture of compound B (*m/z* 218), C (*m/z* 220) and predominantly K (*m/z* 294). However, after concentrating the isolated compound by HLB SPE column, the most abundant compound K was almost completely converted into compound M (*m/z* 238) and a minor compound with mass *m/z* 220 (**Supplementary Figure S4.2**). Both the HLB concentrated and a freeze-dried sample of the HPLC purified compound were subjected to ^{1}H and ^{13}C NMR. The NMR data identified the compound as drimendiol (~95% pure) in both the HLB and freeze-dried concentrated sample (**Supplementary Figure S4.3**). Upon ionization in the LC-MS drimendiol likely loses two water molecules from the C-11 and C12 hydroxyl groups , thereby explaining the apparent mass difference (calculated mass MH+ 239.1927), since the NMR data corresponded completely to the previously published drimendiol identification (Brown, 1994; Kuchkova et al., 2004).



Notation	MW
A	220
B	218
C	220
Drimenol	222
D	264
E	264
F	234
G	234
H	234

Figure 4.3. GC-MS chromatograms of the n-dodecane overlay of yeast strain WAT11 expressing *PhDOX1* with or without drimenol synthase (*PhDS*) from *Persicaria hydropiper* including empty vectors as controls. Eight novel peaks were detected (A-H). The compounds D/E and F/G are likely structurally similar. Mass fragmentation spectra of these peaks are shown in Supplementary Figure S4.1.



Notation	MW
A	220
B	218
C	220
Drimenol	222
F	234
G	234
H	234
I	238
J	238
K	294

Figure 4.4. GC-MS chromatograms of ethyl acetate extracts of medium of yeast strain WAT11 expressing drimenol synthase from *PhDS* and *PhDOX1* from *Persicaria hydropiper* or control empty vectors. Nine novel peaks (A-K minus D,E) were detected. F/G and I/J are likely structurally similar. Mass fragmentation spectra of these peaks are shown in Supplementary Figure S4.1.

4.1. Characterization of *PhDOX1* products in plants

For analysis of *in planta* activity, *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* strains harboring plasmids containing *PhDS* and/or *PhDOX1*. *PhDS* agrobacteria were mixed with strains carrying Arabidopsis farnesyl diphosphate synthase (*AtFPS2*) and truncated Arabidopsis 3-hydroxy-3-methylglutaryl-CoA reductase (*AtHMGR*) to increase terpene production (van Herpen et al., 2010). Infiltrated leaves yielded ~392 µg drimenol per gram fresh weight. In contrast to our expectation, targeting AtFPS2 and PhDS both to the plastids or mitochondria reduced the production of drimenol by 40- and 400-fold, respectively (data not shown).

Also in *N. benthamiana*, co-expression of *PhDOX1*with *PhDS* resulted in several novel compounds as determined by GC-MS analysis (**Fig. 4.6, Supplementary Figure S4.1**).

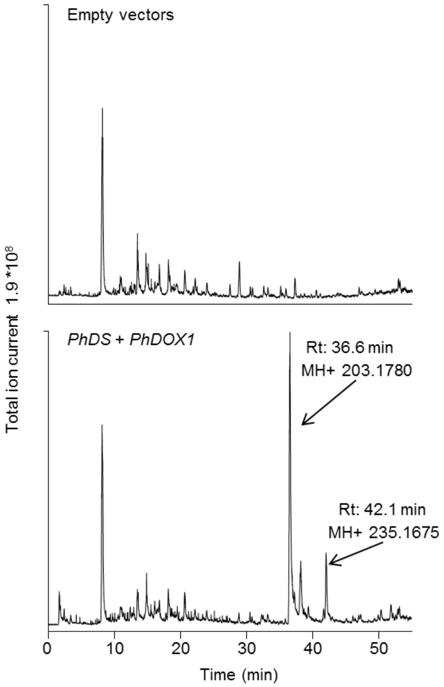
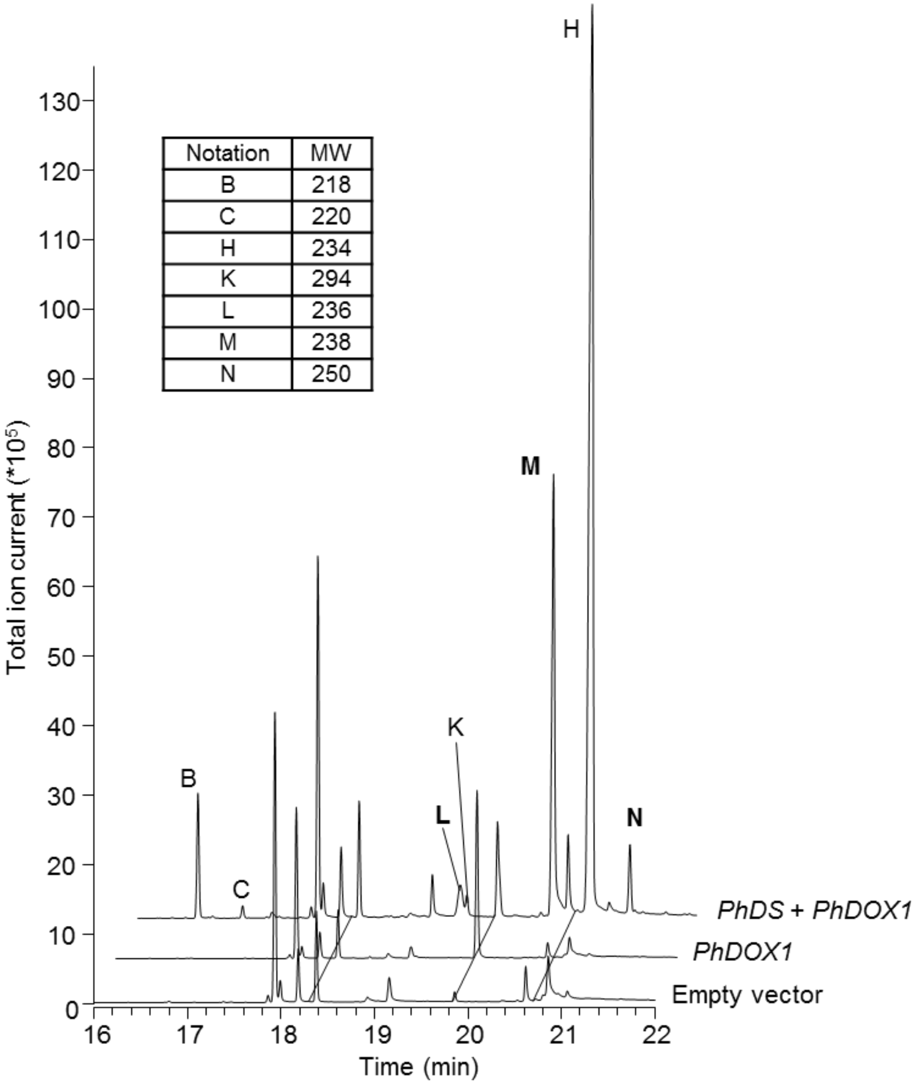


Figure 4.5. LC-MS chromatogram of yeast extract co-transformed with *PhDS* and *PhDOX1* compared with the empty vector control. The scale of the Y-axis is identical in both chromatograms.

The chromatogram showed seven peaks (B, C, H, K, L, M, N), five of which were also detected in the GC-MS analysis of the yeast dodecane overlay (B, C, H) and medium (K, M) extract. Except for compound H, all these compounds (B, C, K, M) were also detected upon GC-MS analysis of the drimendiol purified from yeast (**Supplementary Figure S4.2**) . Two novel compounds (L and N) have a similar base peak (*m/z* 109) as drimenol but could not be reliably identified through a NIST library search. The major compound H with a parent ion of *m/z* of 234, was also detected in the yeast analysis and showed the highest homology (40.3%) to the sesquiterpene lactone drimenin in a NIST library search but with a different retention index KI 2137 vs 1807.

N. benthamiana leaves transiently expressing *PhDS* and *PhDOX1* were also extracted with methanol and analysed by LC-MS (**Fig. 4.7**). A compound with a similar mass and retention time as drimendiol (MH+ 203.1798; so after double water loss, see above) and a major compound with a mass of MH+ 235.1691 were detected. A compound with a similar mass and retention time as the latter was also detected in extracts of yeast expressing *PhDS* and *PhDOX1* (**Fig. 4.5**).



Notation	MW
B	218
C	220
H	234
K	294
L	236
M	238
N	250

Figure 4.6. GC-MS chromatograms of dichloromethane extracts of *N. benthamiana* leaves transiently expressing *PhDS* and *PhDOX1* and an empty vector pBin+ control construct. Seven novel peaks (B, C, H, K, L, M and N) were detected. Mass fragmentation spectra of these peaks are shown in supplementary Figure S4.1.

In order to identify this compound it was purified by HPLC and subjected to NMR analysis. The compound was identified as cinnamolide (**Supplementary Figure S4.4**). The NMR data corresponded completely to what was reported by Hollinshead and co-workers (Hollinshead et al., 1983). GC-MS analysis of the purified cinnamolide identified it as compound H previously detected in both yeast and plant extracts co-expressing *PhDS* and *PhDOX1*.

4.2. Effects of PhDOX1-generated cinnamolide against whiteflies and aphids

The cinnamolide produced by transient co-expression of *PhDS* and *PhDOX1* in *N. benthamiana* represented the major compound produced *in planta*, and we were interested to test its antifeedant activity against two major insect pests. Cinnamolide was tested in dual-choice assays in three different concentrations (450, 225 and 113 µg gFW^{-1}). Dose-dependent responses in feeding preference were recorded at regular intervals during a period of 18 or 24 hours post inoculation with aphids or whiteflies, respectively. The 6h-time point was selected as the most reliable as most insects were actively feeding at that time, and the time for induction of potentially confounding secondary responses resulting from the applied compound were kept to a minimum. The feeding preference was assessed using the Antifeedant Index (AI%) (Kutas and Nádasy, 2005; Prota et al., 2013). The AI% of cinnamolide increased with the increasing concentrations applied, as seen in **Figure 4.8**, meaning that there was a clear dose-dependent preference for the control disks over the treated ones. The extrapolated effective deterrent dose for 50% feeding deterrence (ED$_{50}$) was 195 µg gFW^{-1} for whiteflies and 423 µg gFW^{-1} for aphids.

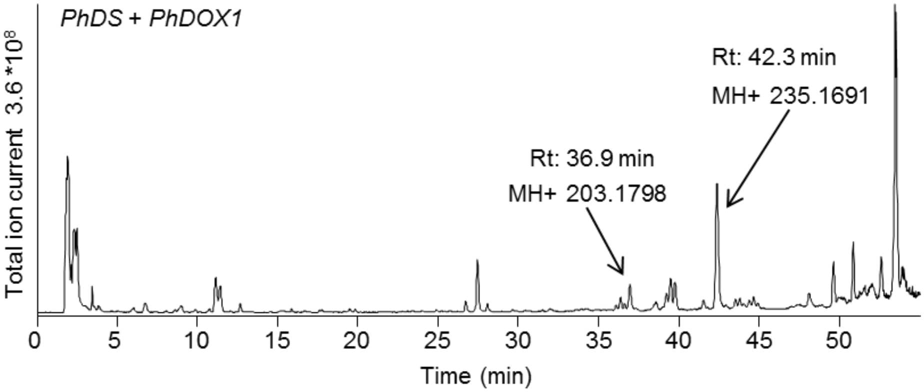


Figure 4.7. LC-MS chromatogram of a methanolic extract of *N. benthamiana* leaves transiently expressing *PhDS* and *PhDOX1*.

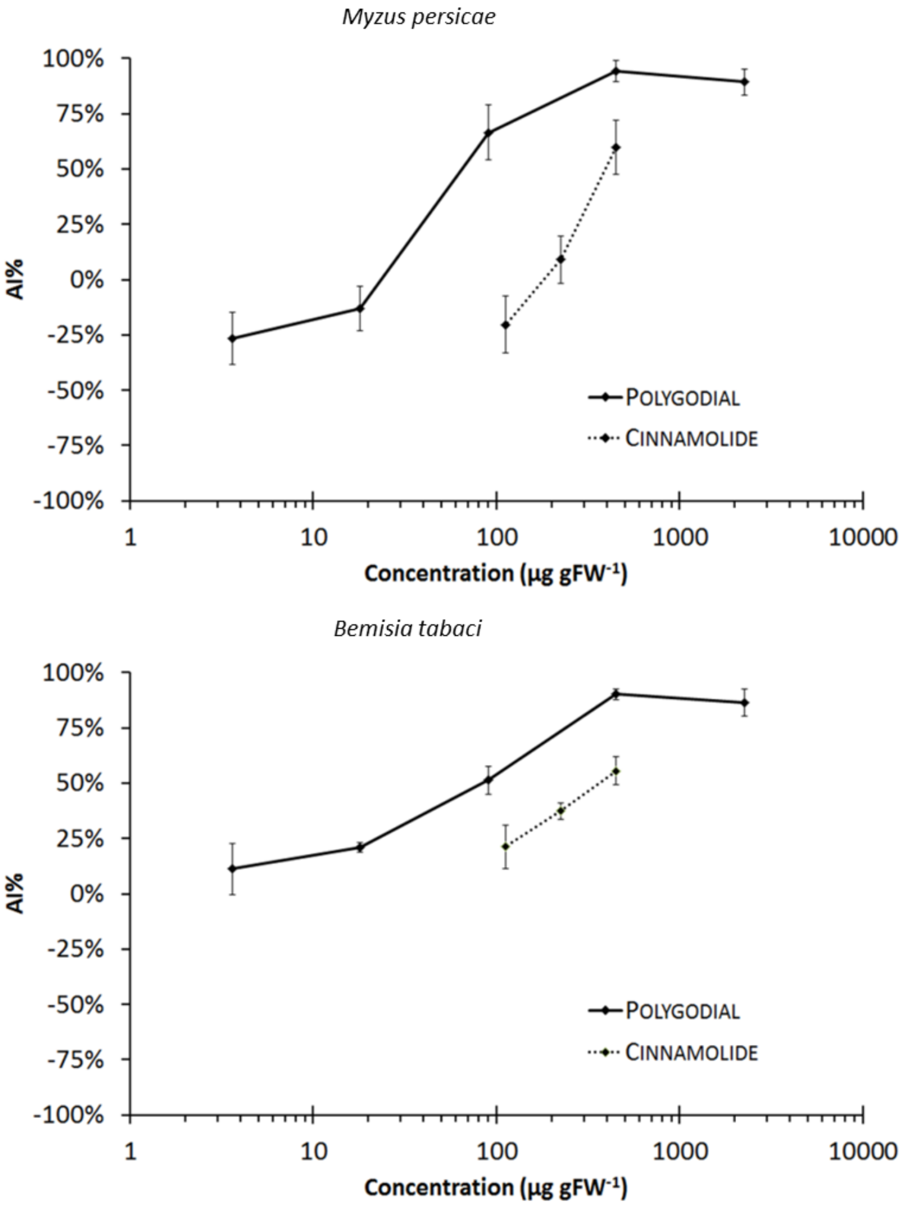


Figure 4.8. Effect of increasing concentrations of cinnamolide on the feeding behaviour of *Bemisia tabaci* and *Myzus persicae* at 6 h from the start of the experiment (dotted lines). Antifeedant index (AI%) = [(C-T)/(C+T)] x 100. Positive values indicate an antifeedant effect, negative values a phagostimulant effect. Error bars indicate the standard error, Six replicates for aphids and eight for whiteflies, average of 98 whiteflies and 20 aphids per replicate. As reference, curves pertaining to the effect of polygodial (Chapter 2) are also included.

5. Discussion

In the present study we identified and characterized a drimenol synthase and drimenol oxidase from *P. hydropiper*, which are involved in the formation and conversion of drimenol. Expression of the drimenol synthase (*PhDS*) showed production of drimenol, while co-expression with the cytP450 *oxidase* (*PhDOX1*) resulted in production of mainly drimendiol (B,C,K,M) and cinnamolide (H) in both *S. cerevisiae* and *N. benthamiana*.

Production of high levels of sesquiterpenes through the expression of sesquiterpene synthases in *Arabidopsis* or tobacco has generally been difficult when the proteins were targeted to their native cytosolic compartment (Aharoni et al., 2005). It was shown for several genes that targeting to the mitochondria or plastids resulted in higher production, presumably because of higher substrate availability in those compartments (Kappers et al., 2005; van Herpen et al., 2010; Liu et al., 2011; Wu et al., 2012). Similar to the expression of (*E*)-β-farnesene synthase in *Arabidopsis* (Beale et al., 2006), however, for *PhDS* we observed that under the same promoter the native cytosolic enzyme was 40-400 fold more active compared with targeting to the plastids or mitochondria. Although we cannot rule out that the enzymes were poorly imported into those compartments, the high activity in the cytosol at least suggests that factors other than substrate availability may limit or promote the enzymatic activity of sesquiterpene synthases in plants.

The cytochrome P450 that is oxidizing drimenol, PhDOX1, was active in both yeast and *N. benthamiana* and almost fully converted drimenol into several new compounds. Eleven novel compounds were detected by GC-MS in yeast and seven in plants. Five compounds, (B, C, K, M, and H) were detected in both yeast and plants. Compound H, (*m/z* 234) was identified as cinnamolide by all analyses. The other four compounds (B, C, K and M with *m/z* 218, 220, 294 and 238, respectively) were detected by GC-MS in HPLC-purified drimendiol, where M has the molecular weight of *m/z* 238 expected for drimendiol. The NMR analysis showed almost pure drimendiol both in the freeze-dried and HLB-purified fractions and the LC-MS analysis of purified drimendiol also showed only one compound of MH+ 203. In LC-MS, drimendiol apparently loses two water molecules during ionization. Possibly, some of the other compounds detected in the GC-MS are impurities which are not detected by LC-MS or NMR. Based on drimenol, a base peak of *m/z* 109 in the GC-MS mass spectrum seems characteristic for drimane type sesquiterpenes. Therefore, we expect that compounds C-H and L-N are also drimane type sesquiterpenes. Lack of standards prevented us from further identifying the GC-MS detected compounds. However, some of the potential drimane type sesquiterpenes detected in *S. cerevisiae* and *N. benthamiana* (compounds C, I, J, L,M) have masses similar to the calculated masses of intermediates in the putative pathway towards cinnamolide and/or polygodial (**Fig. 4.1**). Compounds A, B, I, J and K do not show any similarity to drimenol but could be compounds formed by unspecific activities of PhDOX1 or modifications by endogenous enzymes in yeast or *N. benthamiana* like previously described by others (van Herpen et al., 2010; Liu et al., 2011; Hofer et al., 2013).

In yeast grown with a dodecane overlay, approximately 6% of the drimenol was not converted when *PhDOX1* was co-expressed with *PhDS* while in yeast grown without dodecane nearly all drimenol was converted, as was the case in plants. Apparently, the active shaking of the medium with dodecane promoted extraction of drimenol from the yeast before it could be further oxidized by DOX1. In that sense the dodecane fraction may represent an early snapshot of the conversion process whereas extract from the yeast medium without dodecane overlay identifies final steady-state products. This is further supported by the amount of cinnamolide and drimendiol detected in both yeast extracts. Only traces of cinnamolide (Compound H) and drimendiol (Compound B, C and K) were detected in the yeast dodecane overlay (**Fig. 4.3**), while they are major products in the yeast medium (**Fig. 4.4**).

The newly identified *PhDOX1* gene has been classified to belong to the CYP76 P450 family. PhDOX1 at least performs the first hydroxylation step to drimendiol and possibly also the subsequent oxidation to the corresponding aldehyde. Other members of this family were also shown to be involved in terpenoid biosynthesis (Guo et al., 2013; Hofer et al., 2013). Many of them are reported to catalyse regio-selective hydoxylation reactions, and some members also perform multiple oxidation reactions. For instance, CYP76C4 from *Arabidopsis thaliana* has been reported to be a geraniol hydroxylase, oxidizing geraniol in two positions, and CYP76B6 from *Catharanthus roseus* has been reported to catalyze the regio-selective geraniol 8-hydroxylation and the further oxidation to 8-oxogeraniol (Hofer et al., 2013). Therefore, *PhDOX1* could also potentially catalyze multiple reactions.

The proposed pathway to cinnamolide starts with the formation of drimenol from farnesyl diphosphate (FPP) by *PhDS*. In the next step, PhDOX1 hydroxylates drimenol at C-12 to form drimendiol. The resulting C-12 hydroxyl group could subsequently be oxidized into an aldehyde catalyzed by PhDOX1. Alternatively, an endogenous plant or yeast enzyme performs the oxidation to the aldehyde. The aldehyde can spontaneously react with the C-11 hydroxyl group into a hemiacetal as depicted in **Figure 4.9**, and then be oxidized into cinnamolide. Cyclization through hemiacetal formation has been described before for diterpenes (Yong et al., 2008). Sugars are also known to rapidly interconvert between straight-chain and cyclic hemiacetal forms. These sugar cyclization reactions occur spontaneously and reversibly in solution (Alves and Pio, 2005). Alternatively, lactone ring formation occurs as reported for the sesquiterpene lactone costunolide through further oxidation of the aldehyde to an acid followed by spontaneous formation of the lactone ring (de Kraker et al., 2002; Ikezawa et al., 2011; Ramirez et al., 2013).

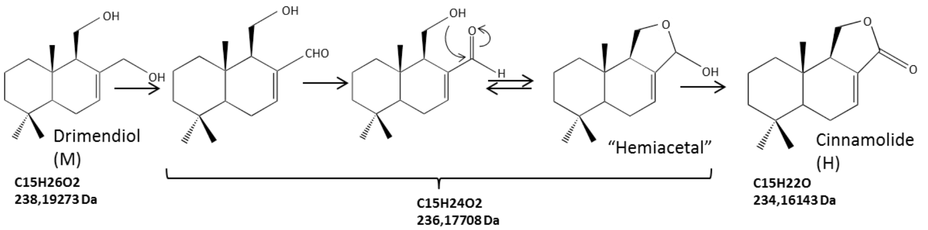


Figure 4.9. Putative pathway from drimendiol (M) to cinnamolide (H). PhDOX1 hydroxylates drimenol to form drimendiol. In the next step, this hydroxyl group is oxidized into an aldehyde catalyzed by PhDOX1 or by an endogenous plant- or yeast enzyme. This aldehyde reacts with the other hydroxyl group to form a hemiacetal that is further oxidized to cinnamolide.

Interestingly, cinnamolide is not found in *Persicaria hydropiper* despite the fact that we used mRNA of the two most dominant enzymes of that plant to reconstruct this pathway. Apparently, there are conditions in *P. hydropiper* which promote the formation of the dialdehyde (producing polygodial) over the formation of the lactone. Two important elements in this puzzle are that, firstly, polygodial is a very reactive compound in the presence of proteins. In previous reports it has been shown that polygodial forms a pyrrole derivative with compounds possessing a primary amine group (Caprioli et al., 1987; Kubo et al., 2005). In that sense polygodial, if formed, may immediately react inside the cells with a variety of intracellular compounds possessing an amine group, which makes it very difficult to prove production of polygodial in a heterologous expression system like yeast or tobacco. Secondly, polygodial accumulates in cavities devoid of other cell content (Hagendoorn et al., 1994; Derita et al., 2008). This suggests that the production of the dialdehyde in plants may require a suit of additional factors and secretory structures to prevent the dialdehyde to spontaneously react to amino-groups of proteins and to prevent that the aldehyde is further converted to form cinnamolide. Possibly, in *P.hydropiper* the enzymes form a metabolon such that drimenol is converted into a dialdehyde in a protected environment, thereby preventing lactone ring formation. Interestingly, polygodial and cinnamolide have been detected together in the fern *Blechnum fluviatile* (Asakawa et al., 2001) which supports their close biosynthetic relationship.

Drimendiol has been isolated from *Drimys winteri* and was reported to have anti-feedant activity against the Egyptian cotton leafworm, *Spodoptera littoralis* (Brown, 1994; Rodríguez et al., 2005; Zapata et al., 2009). Furthermore, drimendiol has recently been shown to inhibit quorum sensing in *Chromobacterium violaceum* and *Pseudomonas syringae* (Paza et al., 2013). Interestingly, polygodial can be synthesized very efficiently from drimendiol using Swern oxidant as described by Hollinshead and coworkers so that biological production of polygodial, a food ingredient in Japan, would be a possibility using our enzymes to produce drimendiol (Hollinshead et al., 1983). Cinnamolide has been identified in *Cinnamosma fragrans* and in the stem bark of *Warburgia ugandensis* and was reported to exhibit antifungal activity (Wube et al., 2005). Here we demonstrate that cinnamolide can also act as an anti-feedant for both silverleaf whitefly *Bemisia tabaci* and the green peach aphid *Myzus persicae* (**Fig 4.8**), although not as strong as polygodial which has ED_{50} values of 25 vs 195 µg gFW^{-1} and 54 vs 423 µg gFW^{-1}, respectively (Prota et al., 2013).

In conclusion, we have isolated and characterized two genes (*PhDS* and *PhDOX1*) involved in the biosynthesis of drimenol and conversion to drimendiol and the sesquiterpene lactone cinnamolide. The identified drimenol oxidase at least performs the first hydroxylation step in the biosynthesis pathway of cinnamolide and polygodial. The subsequent oxidation to its aldehyde is catalysed by PhDOX1 or endogenous enzymes in yeast and tobacco. We propose that cinnamolide is formed by spontaneous formation of a sesquiterpene hemiacetal which is further oxidized to cinnamolide by PhDOX1 or endogenous enzymes. In both yeast and *in planta*, several additional drimenol derivatives were observed when *PhDS* and *PhDOX1*were co-expressed, suggesting complex conversions resulting from PhDOX1 and endogenous enzyme activities.

6. Acknowledgments

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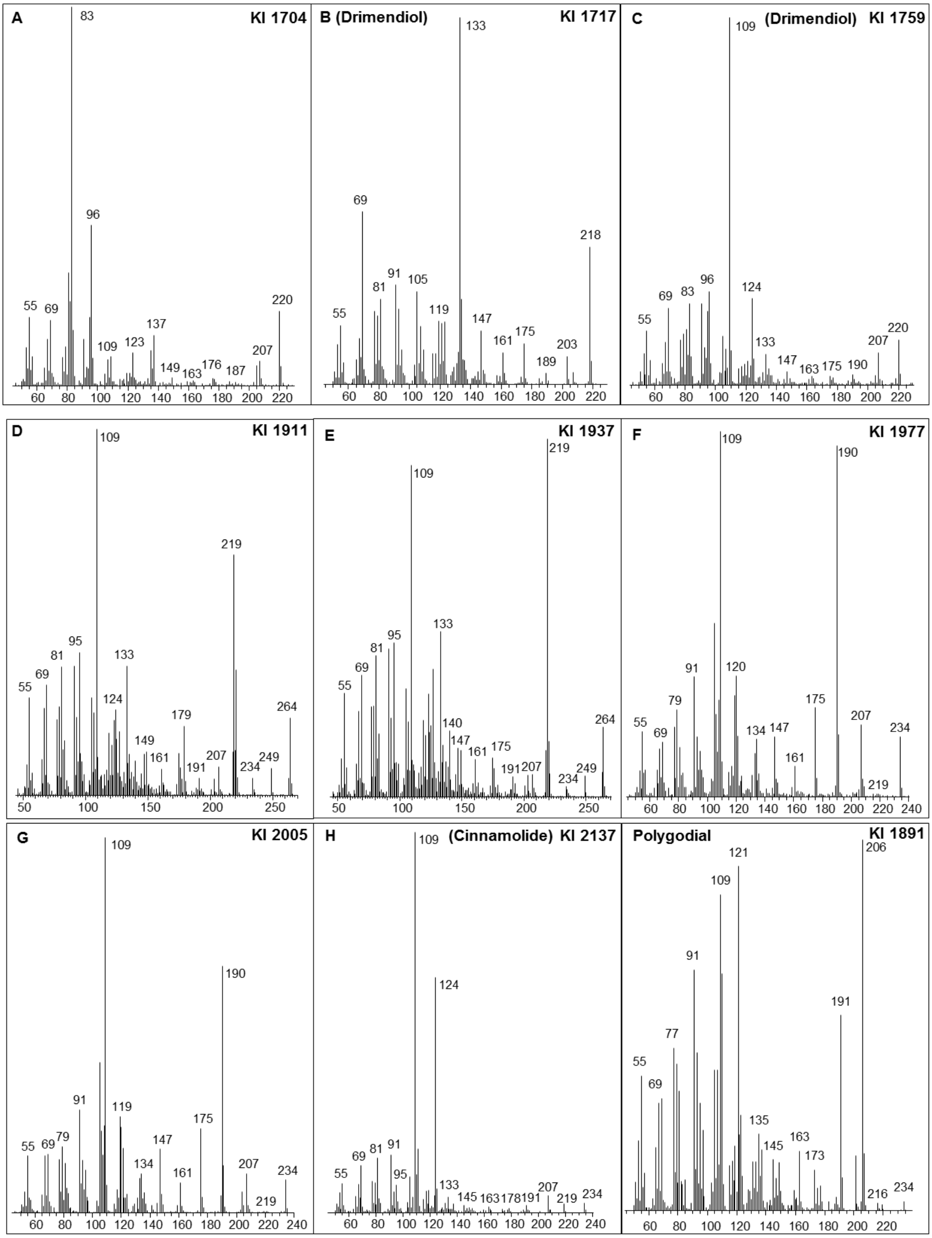
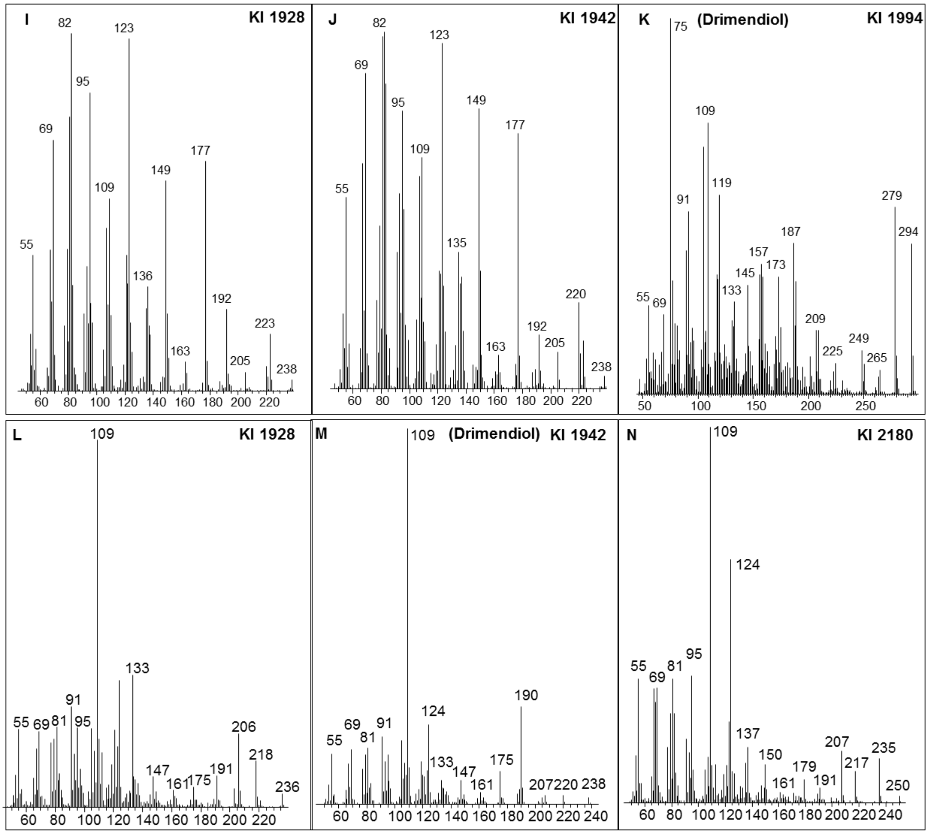
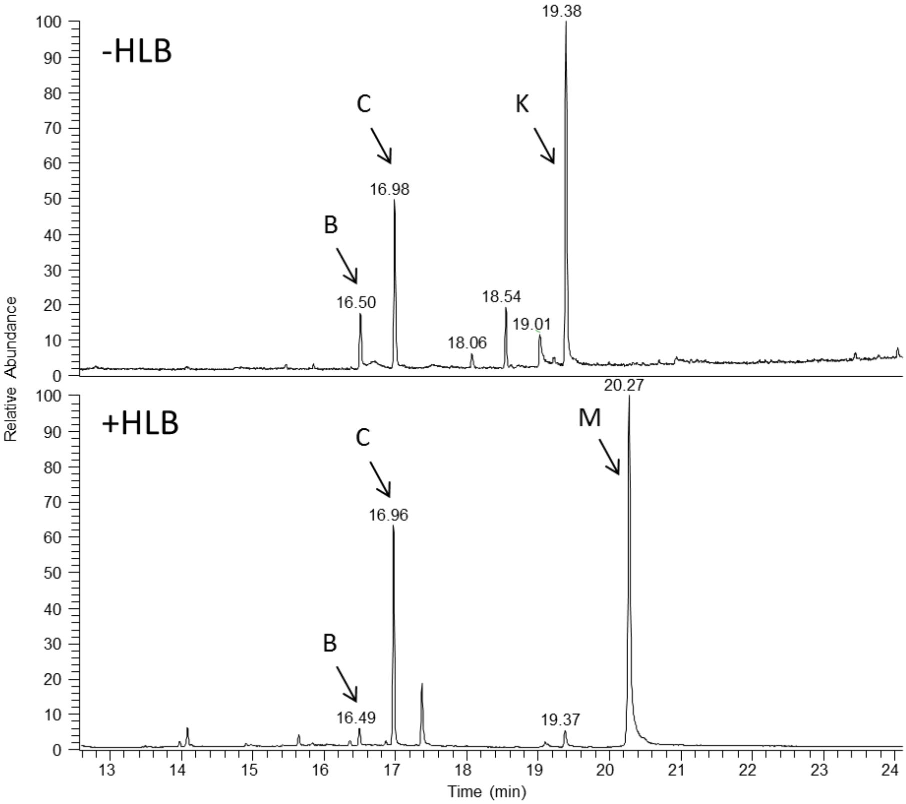
Zevenbergen is acknowledged for making the 454 cDNA library preparation and sequencing.

7. Supplementary material



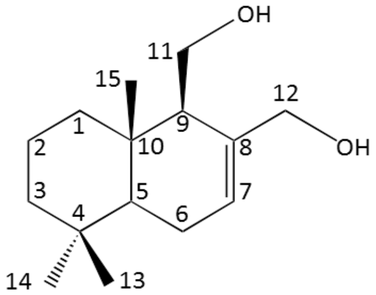


Supplementary Figure S4.1. Mass fragmentation spectra of compounds detected by GCMS on both extracts from yeast co-expressing *PhDS* and *PhDOX1*, and extracts from *PhDS* and *PhDOX1* agro-infiltrated *N. benthamiana* leaves. Mass fragmentation spectrum for the polygodial standard is also shown.



Supplementary Figure S4.2. GC-MS analysis of purified drimendiol before and after HLB-column concentration.

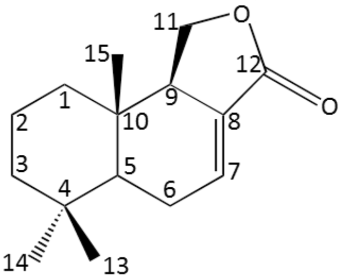
Atom nr.	*C13 (ppm)*	*H1 (ppm)*
1	40.4	2.01 (a); 1.15 (b)
2	43.0	1.44 (a); 1.21 (b)
3	19.5	1.61 (a); 1.48 (b)
4	33.5	n/a*
5	50.8	1.24
6	24.3	2.05 (a); 1.93 (b)
7	126.5	5.78
8	138.5	n/a*
9	55.7	2.05
10	36.5	n/a*
11	61.1	3.84 (a); 3.61 (b)
12	66.7	4.26 (a); 3.96 (b)
13	14.5	0.81
14	33.5	0.88
15	22.0	0.92



Supplementary Figure S4.3. 1H-NMR and 13C-NMR parameters of drimendiol NMR analysis and the structure of drimendiol. Atom numbers are referred to in the table.

*Quarternary carbon atom bearing no hydrogens.

Atom nr.	*C13 (ppm)*	*H1 (ppm)*
1	39.9	1.60 (a); 1.22 (b)
2	19.0	1.62 (a); 1.46 (b)
3	42.9	1.49 (a); 1.27 (b)
4	n.d.	n/a*
5	50.4	1.44
6	25.68	2.42 (a); 2.14 (b)
7	136.70	6.77
8	128.63	n/a*
9	51.39	2.87
10	n.d.	n/a*
11	68.0	4.36 (a); 4.03 (b)
12	171.1	n/a*
13	21.54	0.96
14	33.35	0.92
15	13.57	0.81



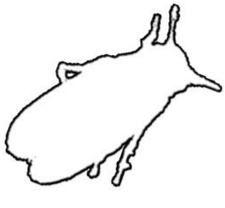
Supplementary Figure S4.4. 1H-NMR and 13C-NMR parameters of cinnamolide NMR analysis and the structure of cinnamolide. Atom numbers are referred to in the table.

*Quarternary carbon atom bearing no hydrogens. n.d. is not detected

Chapter 5

Discovery and characterization of a novel zingiberene synthase and effects on whitefly of its overexpression in *Nicotiana tabacum*

Neli Prota, Katarina Cankar*, Martin Klompmaker, Adele M. M. L. van Houwelingen, Maarten A. Jongsma, Harro J. Bouwmeester, Jules Beekwilder*



*authors contributed equally to this work

1. Abstract

Whitefly is a pest that has systematically spread into colder latitudes for the past two decades and it poses a serious threat to crops, mainly due to the viruses for which it acts as a vector. Zingiberene is a known whitefly repellent, so expressing an enzyme that could produce it directly in the crop plant as a form of pest management is an attractive goal. Here, we describe the isolation and characterization of a zingiberene synthase, CnZIS, from the conifer *Callitropsis nootkatensis*. This sesquiterpene synthase belongs to the TPS-d subfamily of terpene synthases and produces zingiberene as the sole product using (*E,E*)-farnesyl diphosphate (FPP) as substrate. When *CnZIS* was stably expressed in *Nicotiana benthamiana* and targeted to the mitochondria, emission of zingiberene was observed. Insect assays with *N. benthamiana* did not prove successful, as this plant is not a suitable host for whitefly. *N. tabacum*, on the contrary, is a common host for this insect. Insect assays with *N. tabacum* transiently expressing *CnZIS*, however, did not reveal CnZIS-mediated resistance, possibly due to the very low amounts of zingiberene emitted by these plants. When genes encoding rate-limiting steps in the formation of FPP, a truncated form of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) and FPP synthase (FPS), were co-expressed with *CnZIS*, surprisingly, the emission of zingiberene was completely abolished, while strongly increased emission of 5-*epi*-aristolochene (5EA) was observed. Insect choice assays with this material suggested that 5EA is a repellent of whitefly.

2. Introduction

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), though not a naturally occurring species in temperate habitats due to cold sensitivity, has nonetheless spread from the Mediterranean basin to greenhouses at higher latitudes in Central and Northern Europe, claiming habitat there as well (Martin et al., 2000; Bruce et al., 2005). This phloem-feeding insect not only damages plants directly, but, more importantly, acts as a vector for more than a hundred viruses, mainly begomoviruses. Over the past 20 years or so, these viruses have emerged as serious constraints to the cultivation of a variety of vegetable crops (Navas-Castillo et al., 2011). The yield losses that this causes cannot be controlled chemically once plants are infected (Jones, 2003). The most threatened crops belong to the Cucurbitaceae, Leguminosae, Euphorbiaceae, Malvaceae and Solanaceae (Bruce et al., 2005).

Plants are known to display chemical defence mechanisms against pests, directly through production of toxic or deterrent chemical compounds, as well as indirectly through the emission of compounds that will attract natural enemies of the pest (Heil, 2008; Howe and Jander, 2008; Mumm and Dicke, 2010). These compounds are part of the so-called secondary metabolism and can be chemically very diverse. The most abundant and structurally diverse class of plant secondary metabolites involved in signalling and defence are the terpenoids (Cheng et al., 2007). They are usually produced in vegetative tissues, flowers, fruits and occasionally roots.

All terpenoids are derived from the isomeric 5-carbon building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In plants, two separate pathways lead to the synthesis of these compounds: the mevalonate pathway and the MEP pathway (Chen et al., 2011). The rate-controlling enzyme of the mevalonate pathway is 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) and this enzyme has been used in studies that attempt to boost the production of terpenes in plants (van Herpen et al., 2010). The condensation of one molecule of IPP and one of DMAPP will result in geranyl diphosphate (GPP), the general precursor of monoterpenes (C10); the condensation of two molecules of IPP and one of DMAPP will result in the C15 sesquiterpene precursor farnesyl diphosphate (FPP), in either the *trans* or *cis* configuration; the condensation of three molecules of IPP and one of DMAPP will result in geranylgeranyl diphosphate (GGPP), the precursor for diterpenes (C20) (Chen et al., 2011).

Terpene synthases (TPSs) are the enzymes that convert these precursors into the plethora of terpenes and (some of the) terpene alcohols that the plant kingdom produces (Chen et al., 2011). Due to the stochastic nature of bond rearrangements that follow the creation of the unusual carbocation intermediates, a single TPS enzyme using a single substrate often produces multiple products (Degenhardt et al., 2009).

The remarkably similar characteristics of the monoterpene, sesquiterpene, and diterpene synthases are certainly related to the fact that these enzymes carry out similar electrophilic cyclizations involving common steps. This similarity in function is reflected in similarity in primary structure of all of the terpene synthases of plant origin (Bohlmann et al., 1998). According to recent new analysis, the Tps gene family can be divided into seven subfamilies based on the amino acid sequence relatedness of plant terpenoid synthases: Tps-a, b, c, d, e/f, g and h (Chen et al., 2011). This clustering highlights the evolutionary origin of the different TPSs, as the different classes reflect different clades rather than a similarity in the product of the catalysed reaction, thus suggesting that the same function arose independently in several evolutionary events (Chen et al., 2011).

Sesquiterpenes play important roles in interactions of the plant with its ecosystem. Some of them function as phytoalexins involved in the direct defence of plants against herbivores and microbial pathogens: with their proven toxic, antibiotic, antifungal and antifeedant effects, these terpenoids can greatly help the plant to prevent damage caused by insects, nematodes, microorganisms and even vertebrates. Sesquiterpenes can also be volatile and function as attractants for pollinators, repel potential pests, signal to the insect that the plant is undesirable, or can attract predators or parasites of the herbivore attacking the plant (Cheng et al., 2007).

(–)-α-Zingiberene is the major sesquiterpene hydrocarbon constituent of ginger oil and it was first isolated in 1900 (Parthasarathy, 2008). It is a volatile sesquiterpene and a member of the bisabolane family. Olfactometer experiments have shown that at concentrations above 0.5% ginger oil is effective at repelling *Bemisia argentifolii* (= biotype B of *B. tabaci*). The same study reported dose-dependent repellence to whitefly when leaf disks were immersed in ginger oil solutions, as well as reduced settling and oviposition when whole tomato seedlings were dipped in a ginger oil solution (Zhang et al., 2004). Another study assessed the effect of ginger oil on whitefly by means of fumigation, estimating that the LC_{50} was 0.89 $\mu g\ cm^{-2}$; in the same study, the LC_{50} for the residual toxicity of ginger oil against whitefly was 0.03 $\mu g\ cm^{-2}$ (Muigai et al., 2002).

A stereoisomer of α-zingiberene (7-*epi*-zingiberene) has also been shown to occur in glandular trichomes of the wild tomato species, *Solanum habrochaites* accession LA1777 (Carter et al., 1989). In this species it has been demonstrated to help confer resistance to the Colorado potato beetle *Leptinotarsa decemlineata* Say (Carter et al., 1989) and to whitefly (Bleeker et al., 2009). Moreover, this resistance was transferred to cultivated tomato through an interspecific cross with the wild relative bearing the 7-*epi*-zingiberene synthase gene (Bleeker et al., 2012).

Metabolic engineering of terpene biosynthesis has been deployed to manipulate the resistance to insects in plants (Vickers et al., 2014). For instance, transgenic *Arabidopsis* producing either (*E*)-β-farnesene (Beale et al., 2006) or linalool (Aharoni et al., 2003) repels aphids; and transgenic tobacco-producing patchoulol is less attractive to tobacco hornworm than wild-type tobacco (Wu et al., 2006).

The engineering of resistance to whitefly has not been extensively described. In view of the repellent activity of zingiberene on whitefly, a strategy to engineer whitefly resistance in plants could include the expression of a zingiberene synthase. However, the heterologous expression of sesquiterpene synthases (STSs) often yields only minute amounts of the desired sesquiterpene (Wallaart et al., 2001). To circumvent this problem, related to substrate availability, alternative compartmentalization to plastids and mitochondria (Kappers et al., 2005; Wu et al., 2006) and co-expression of genes responsible for the formation of precursors have been shown to significantly improve the accumulation of the desired compound. With regard to the latter, co-expression of FPP synthase (FPS) and a truncated form of HMGR with the STS of interest, either together or individually, was shown to increase the production of the compounds synthesized by the STS (Aquil et al., 2009; van Herpen et al., 2010; Houshyani et al., 2013).

The aim of the current study was to achieve a high production of zingiberene in plants, and to assess the effects of the zingiberene produced in a model plant on whitefly behaviour.

3. Materials and Methods

3.1. Cloning and *in vitro* characterization of zingiberene synthase from *Callitropsis nootkatensis*

3.1.1. Cloning of the zingiberene synthase from C. nootkatensis *cDNA library*

A preparation of an expressed sequence tag (EST) database of a cDNA library containing 34.743 contigs derived from the *C. nootkatensis* heartwood was described previously (Beekwilder et al., 2013). Sesquiterpene synthase contigs were identified by homology approach and RACE PCR (Clontech) strategy was used to amplify the 5'- and the 3'-region of the potential sesquiterpene synthases. The sequence of the RACE fragments was analysed and assembled with contigs of the *C. nootkatensis* EST database using the Seqman II program (DNAstar Inc., Madison, WI).

A putative sesquiterpene synthase was amplified from the *C. nootkatensis* cDNA library using Phusion DNA polymerase (Finnzymes, Finland) and oligonucleotides ATATTACTGCAGCTATTTTAGAGAGATATAGGTTC and ATAATAGGATCCTGCTCAAAAAGCTAATACACAAT. Restriction sites for BamHI and PstI were included in the oligonucleotides and the full ORF was cloned in pACYCDUET-1 (Novagen, Merck Chemicals B.V., Amsterdam, the Netherlands) in frame with the HIS tag. Correct insertion of the ORF was confirmed by restriction analysis and by DETT sequencing.

3.1.2. Heterologous expression in E. coli and enzymatic assay

pACYCDUET-1 construct was transformed to *E. coli* BL21AI (Invitrogen, Bleiswijk, the Netherlands). For expression, 500 µL of the overnight culture was transferred to 50 mL of LB medium supplemented with 50 mg/L chloramphenicol and incubated at 37 °C, 250 rpm. At the OD_{A600} of 0.4–0.6 gene expression was induced by addition of 0.02% arabinose and cultures were incubated overnight at 18 °C and 250 rpm. The next day, cells were harvested by centrifugation, and resuspended in 1 mL Resuspension buffer (50 mM Tris-HCl pH = 8.0, 300 mM NaCl, 1.4 mM 2-mercaptoethanol; 4 °C). Cells were disrupted by sonication (on ice, five times 10 s with 10 s break, MSE Soniprep 150, amplitude 14 µm). After centrifugation (10 min 13 000 g, 4 °C) the soluble protein was further purified using Qiagen Ni-NTA spin columns. 200 µL eluate in imidazole buffer was transferred to a Slide-A-Lyzer Mini Dialysis Unit (10 000 MWCO; Pierce, Rockford, IL) and dialysed for 3 h to 1 L Storage buffer (50 mM Tris-HCl pH = 7.5, 12.5% glycerol, 1.4 mM 2-mercaptoethanol) at 4 °C. After dialysis, enzyme was immediately used for enzyme assays.

Enzyme assays were set up in glass tubes containing 800 µL of MOPSO buffer, (15 mM MOPSO, pH 7.0, 12.5 % (v/v) glycerol, 1 mM Ascorbic acid, 1 mM MgCl2, 2 mM DTT, 0.1% Tween 20), 175 lL of purified enzyme solution and 5 µL of farnesyl diphosphate (10 mM, Sigma FPP, St Louis, MO). This mix was overlaid with 500 µL of pentane and incubated at 30 °C with mild agitation for 2 h. Subsequently, the pentane was collected. The remaining water phase was extracted with 1 mL ethyl acetate. Ethyl acetate and pentane phases were combined, centrifuged at 1200 ***g***, dried over a sodium sulphate column and analysed by GC-MS.

Agilent technology GC-MS instrument was used comprising a 7980A GC system, a 597C inert MSD detector (70 eV), a 7683 autosampler and injector and a Phenomenex Zebron™ ZB-5ms column of 30m length x 0.25 mm internal diameter and 0.25 µm stationary phase with a Guardian precolumn (5 m). In this system 1 µl of the sample was injected using following conditions: injection port at 250 °C, splitless injection, the ZB-5 column maintained at 45 °C for 2 min after which a gradient of 10 °C started, until 300 °C.

3.1.3. *Phylogenetic analysis*

To assess into which of the seven STS gene families the CnZIS belongs, a phylogenetic analysis was performed. The analysis comprised one sesquiterpene synthase from a bacterium as outgroup and 27 from different plant species, selected from a list used by Degenhardt *et al*. (Supplementary table S1) (Degenhardt et al., 2009). Amongst those there were the zingiberene synthases from basil (*Ocimum basilicum*, AAV63788), rice (*Oryza sativa*, ACF05529) and sorghum (*Sorghum bicolor*, C5YHH7) and the 7-*epi*-zingiberene synthase from wild tomato (*Solanum habrochaites*, AFJ67794). In addition to the STS form the Degenhardt list, we included the valencene synthase isolated from the same tissue (Beekwilder et al., 2013) and another previously characterized STS from the Cupressoideae subfamily, namely the β-cadinene synthase from *Chamaecyparis formosensis* (AFJ23663) (Beale et al., 2006).

The sequences were aligned with ClustalW using the standard settings. The phylogeny reconstruction analysis was performed in MEGA 6 using the Minimum Evolution method and 500 bootstrap replicates were used to test the phylogeny. For the substitution model, the Poisson model and the amino acid substitution type were employed. A phylogenetic tree was generated using the neighbour joining clustering method.

3.1.4. *Gene expression analysis of CnZIS*

One microgram of total RNA was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, the Netherlands). Real-time PCRs were carried out in triplicate in a total volume of 20 µL containing 10 µL of 2× iQ SYBR Green Supermix (Bio-Rad), 0.3 µm of forward and reverse primer and 10 ng of cDNA in a MyiQ real-Time PCR machine from Bio-Rad. The following PCR program was used: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers used were CnZis-Fw (GGCTGAATGGTG GGATGAGGCAA) and CnZis-Re (CGTCGCTGCATACTCAGGCTCA), and Actin Fw (TGACAATGGAACCGGAATG) and Actin Re (CACTGCCCTTGGAGCATC). Relative gene expression was calculated as: $2^{-\Delta C_t}$, where $\Delta C_t = C_t$ *CnVS* − C_t actin.

3.2. *In planta* characterization of CnZIS

3.2.1. *Cloning of the zingiberene synthase into pBIN vector*

The *C. nootkatensis* zingiberene synthase was sub-cloned into the BamHI and NotI restriction sites of ImpactVector pIV1A-1.1 (www.impactvector.com) with cytoplasmic targeting. The correct insertion of genes into Impact vectors was confirmed by restriction analysis and DETT sequencing (GE Healthcare). The full expression cassette including the RUBISCO small subunit, RbcS1 promoter and RbcS1 terminator from *Chrysanthemum morifolium* was then transferred to the pBIN+Dest vector by Gateway LR reaction (Gateway LR Clonase Enzyme mix, Invitrogen). Cloning of the truncated 3-hydroxy-3-methylglutaryl-CoA reductase (tHMGR) and the farnesyl diphosphate synthase (FPS2) from *Arabidopsis thaliana* was described previously (van Herpen et al., 2010).

3.2.2. Transient transformation of Nicotiana plants

Agrobacterium tumefaciens strains containing a pBIN+ binary vector with ZIS, AtFPS2, AtHMGR or no gene, were grown at 28 °C at 250 rpm for 24 hours in LB media with kanamycin (50 mg L^{-1}) and rifampicillin (50 mg L^{-1}). Cells were harvested by centrifugation for 20 min at 3500 g and 20 °C and then resuspended in 10 mM MES buffer containing 10 mM $MgCl_2$ and 100 µM acetosyringone (4′-hydroxy-3′,5′-dimethoxyacetophenone, Sigma) to a final OD600 ≈ 0.5, followed by incubation at room temperature and 50 rpm for 180 minutes. Before infiltration, equal volumes of the *Agrobacterium* strains were mixed in the case of co-transformation of the three genes.

Six-weeks-old *Nicotiana benthamiana* or *Nicotiana tabacum* ‘Samsun’ plants, grown in a greenhouse at 25/28 °C with a minimum of 16 h photoperiod, were transiently transformed. Two leaves per plant were infiltrated either with the transformation solution containing *Agrobacterium* harbouring the *CnZIS* or with a mixture of three strains, using a one-millilitre needleless syringe pressed against the abaxial surface of the leaf. As a negative control, leaves were infiltrated with a solution containing *A. tumefaciens* carrying an empty vector. In the case of the *N. tabacum* plants, four individual leaves of each agroinfiltrated type (ZIS, ZIS+AtFPS2+AtHMGR, empty vector) were collected four days post-infiltration (dpi) to firstly trap the emitted volatiles and subsequently perform a dual-choice assay to assess the effect of the volatile blend on the whiteflies’ feeding.

3.2.3. Stable transformation of N. benthamiana plants

The *A. tumefaciens* LBA4404 transformed with mitZIS, cytZIS and empty pBIN vectors were grown in a 10mL LB medium supplemented with (40 µg/mL rifampicin / 50 µg mL^{-1} kanamycin). The starter culture was diluted 1:100 in 25mL fresh LB with antibiotics and grown for 24 h. Cells were harvested by centrifugation and washed with M300 medium (4.4 g L^{-1} MS vitamins, 0.5 g L^{-1} MES, 30 g L^{-1} sucrose, pH 6.0) with acetosyringone (100 µM), spun down again and resuspended in 20 mL of the same medium. The bacterial suspension was 50-fold diluted before being used for transformation of plants.

The leaves of seven-week old *N. benthamiana* plants were cut to leaf discs (5-7 mm) and kept in M300 liquid medium. For the cytZIS, mitZIS and pBin, 125, 125 and 75 explants were prepared, respectively. The M300 medium was then replaced with the agrobacterium suspension and incubated in half-darkness for three days at room temperature. Plant discs were washed with M300 medium supplemented with ticarcillin (500 mg L^{-1}) and placed on solid M300 medium with benzylaminopurine (1 mg L^{-1}), auxin (0.1 mg L^{-1}), ticarcillin (500 mg L^{-1}), kanamycin (50mg L^{-1}) and microagar (6%). After callus-formation, growth medium was changed to shooting medium, without auxin. After shoots were formed, the medium was changed to a medium without hormones to stimulate rooting. After rooting occured, plants were transferred to pots with M400 medium (2.2 g L^{-1} MS vitamins, 15 g L^{-1} sucrose, 6 g L^{-1} agar, pH 6.0).

3.3. Chemical analysis of the transformed plants

3.3.1. Head-space sampling and analysis

3.3.1.1. Trapping conditions

Agroinfiltrated leaves were individually inserted with their petiole into a 5x2x1 cm piece of wet Oasis foam (to preserve turgor) and covered with aluminium foil to limit vapour from escaping from the Oasis foam. Each leaf was placed in a 2L glass jar (Duran). The jar was sealed with a Viton-lined glass lid having an inlet and outlet. Inlet air was filtered by passing through a stainless steel cartridge (Markes, Llantrisant, UK) filled with 200 mg Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, USA). Volatiles were trapped by sucking air out of the jar at a rate of approximately 100 mL min^{-1} through a similar cartridge filled with 200 mg Tenax TA for one hour. Head-space collections were made in a climate chamber at 23 ± 1 °C, 60 ± 5% RH, and 90 ± 5 µmol m^{-2} s^{-1} PAR.

3.3.1.2. Thermal desorption and Gas Chromatography-Mass Spectrometry (GC-MS)

Headspace samples were analysed with a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, USA) connected to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, USA) quadrupole mass spectrometer. Before desorption of the volatiles, the Tenax cartridges were dry-purged with nitrogen at 30 mL min^{-1} for 25 min at ambient temperature to remove moisture. Volatiles were desorbed from the cartridges using a thermal desorption system at 220 °C for 5 min (Model Ultra Markes Llantrisant, UK) with a helium flow of 30 mL min^{-1}. Analytes were focused at 5 °C on an electronically-cooled sorbent trap (Unity, Markes, Llantrisant, UK) and were then transferred in splitless mode to the analytical column (Rtx-5ms, 30 m, 0.25 mm i.d., 1.0 µm film thickness, Restek, Bellefonte, USA) by rapid heating of the cold trap to 250 °C. The GC was held at an initial temperature of 40 °C for 3 min followed by a linear thermal gradient of 10 °C min^{-1} to 280 °C and held for 2 min with a column flow of 1 mL min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 33-280 m/z with a scan rate of 4.22 scans s^{-1}. Compounds were identified comparing the mass spectra with those from the NIST library or, in the case of zingiberene, comparing it with ginger oil. To estimate the amounts produced per g of leaf per hour, the leaves were weighed before trapping the volatiles.

3.3.1.3. SPME-GC-MS

Solid-Phase MicroExtraction (SPME) was used as the preferred method for direct headspace analysis of stably transformed *N. bethamiana* F1 plants. In the setup, 12 mm Ø leaf discs were placed into a 10-mL-glass vial. Samples were analysed directly after sampling. The samples were automatically extracted and injected into the GC-MS via a Combi PAL autosampler (CTC Analytics AG). Headspace volatiles were extracted by exposing a 65-*µ*m polydimethylsiloxane-divinylbenzene SPME fiber (Supelco) to the vial headspace for 20 min and heating at 50°C. The fiber was inserted into a GC 8000 (Fisons Instruments) injection port and volatiles were desorbed for 1 min at 250°C. Chromatography was performed on an HP-5 (50 m×0.32 mm×1.05 *µ*m) column with helium as carrier gas (37 kPa). The GC interface and MS source temperatures were 260 °C and 250 °C, respectively. The GC temperature program began at 45 °C (3 min), was then raised to 280 °C at a rate of 10 °C min^{-1}, and finally held at 280 °C for 3 min. The total run time, including oven cooling, was 29.5 min. Mass spectra in the 35 to 350 *m/z* range were recorded by an MD800 electron impact MS (Fisons Instruments) at a scanning speed of 2.8 scans s^{-1} and an ionization energy of 70 eV.

3.3.2. Analysis of organic extracts by GC-MS

Callitropsis nootkatensis ‘pendula’ was purchased from Plantentuin Esveld in Boskoop (NL). For dichloromethane (DCM) extraction, 0.5 g of plant material was weighed in a precooled glass tube, and 4 mL of dichloromethane was added. The suspension was vigorously shaken for 1 min, sonicated for 5 min in an ultrasonic bath and centrifuged for 5 min at 1500 ***g*** at room temperature. The supernatant was collected, filtered over a column of 1 g sodium sulphate and diluted four-fold in DCM before analysis on a GC-MS. About 2 µL was analysed by GC-MS using a gas chromatograph as described in detail by Cankar *et al*. (Cankar et al., 2011). Curcumene was identified by the comparison of retention time and mass spectrum with the one found in ginger oil.

3.3.3. Data analysis

To quantify the metabolites of interest present in each chromatogram area under their peaks was integrated and compared it to a titration curve obtained with different concentrations of a known sesquiterpene, injected directly into the thermal desorption system.

3.4. Insect dual-choice assays

3.4.1. Petri dish setup

Immediately after the headspace trapping was completed, the collected *N. tabacum* leaves were cut into approximately 3x3 cm squares and placed abaxial-side up in a 9-cm-Petri dish on 45 ml of 8 g L^{-1} water agar substrate. To test the effect of ZIS alone, two of the four leaf squares came from leaves infiltrated with ZIS, and two from leaves transformed with an empty vector. To test the effect of ZIS in combination with the precursor genes (AtHMGR and AtFPS2), two of the four squares were from leaves infiltrated with the mixture (HFZ) and two from leaves transformed with an empty vector. The two treatment- and two control-squares were each placed at two diametrically opposed sides of the Petri dish, to control for potential environmental cues, such as light. The leaves were then covered with a Petri dish bottom through which four holes of 16 mm diameter were drilled, making sure that each leaf square was covered by one hole to allow for insects to feed upon. Insects were put onto the lid of the dishes and then covered with the bottom of the Petri dish containing the leaves. In that way all insects had to fly or walk to the feeding areas without being mistakenly put closer to one disk than to another. The plates were sealed with Parafilm® and kept upside down. On average, 82 and 67 whiteflies were used for the ZIS and HFZ assays, respectively. For each assay, eight biological replicated (separate arenas) were used, two for each leaf transformed with genes of interest (treatment) and four for each leaf transformed with the empty vector. The assays were performed in a climate chamber at 25 °C, 60%

humidity and 16/8 h light/dark photoperiod. The assays were carried on for 24 hours and the feeding insects counted at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 20 h and 24 h. Insects were considered to be feeding when they were immobile on one spot on a leaf disk.

3.4.2. *Whole-plants setup*

A double-choice assay with whole plants placed in a large cage with insects released in the middle of the cage was used to test F2 *N. benthamiana* transformed with mitochondria-targeted *CnZIS*. Four five-weeks old plants were placed at the four corners of a rectangular parallelepiped-shaped cage (30x30x50 cm), which walls were made of a mash that allows for light to penetrate and for air exchange, but with links small enough to not allow whiteflies to escape. Approximately 500 insects (both male and female) were released in the centre using a container that was uncovered once placed inside the cage. Insects were counted at regular intervals before noticing that they were dying within the first few hours.

3.4.3. *Insects*

The insect species used in this study was the silverleaf whitefly *Bemisia tabaci* (genotype B). Whiteflies were reared on tomato (*Solanum lycopersicon*, Moneymaker cultivar) in a greenhouse at 26 °C, at 60% relative humidity with a photoperiod of 16 h light and 8 h dark. To perform the assays, adult flies of both genders were collected from leaves using an aspirator. They were cold-anesthetized at 7 °C in a plastic cylinder (3 cm Ø) covered with Parafilm® for 5 min, before being released into the Petri dish at the start of the dua-choice assay, to ensure they would not fly out before the dishes were sealed.

3.4.4. *Antifeedant index (AI%)*

To express the repellence potency of the emitted volatiles towards whiteflies, the Antifeedant Index (AI%) was calculated according to Kutas and Nádasy, as follows: AI%=[(C-T)/(C+T)]x100, where C indicates the number of insects feeding on the control, and T the number of insects feeding on the treatment (Kutas and Nádasy, 2005). The AI% adopts positive values when the tested compound is an antifeedant and negative values when the compound is a phagostimulant.

4. Results

4.1. Callitropsis nootkatensis contains curcumene

In our search for a zingiberene synthase, we analyzed the terpenoid composition of the Nootka cypress tree *Callitropsis nootkatensis* (also called Alaska yellow cedar). This tree is known to produce several terpenes and significant amounts of nootkatone (Erdtman, 1962; Khasawneh and Karchesy, 2011). Nootka cypress is native to the west coast of North America and is used in temperate regions of Europe as an ornamental conifer in gardens. The tree is known for its highly decay-resistant wood, which makes it suitable as timber, for example in boat decks and Japanese temples (Kelsey et al., 2005). The heartwood oil and several of its components have high fungicidal activity on notorious forest pests, such as *Phytophthora ramorum* and *P. lateralis* (Manter et al., 2006).

Organic extracts of *C. nootkatensis* heartwood and cortex were analysed for their secondary metabolite content by gas chromatography coupled to mass spectrometry (GC-MS) analysis. It was found that the plant stores a variety of mono- and sesquiterpenes in various tissues. No zingiberene could be detected in any of the analysed tissues. However, in all analysed tissues except heartwood, a peak corresponding to curcumene was observed (**Fig. 5.1**), with retention time 13.93 min and a molecular ion of 202 m/z. Curcumene, like zingiberene, is known to occur in ginger oil (Zachariah et al., 2008). Indeed a peak with identical mass spectrum and retention time was found in ginger oil (**Fig. 5.1**). Curcumene is an aromatic compound, which likely derives from zingiberene by dehydrogenation (**Fig. 5.2**) (Chen and Ho, 1988). For this reason, we anticipated the presence of a zingiberene synthase in *C. nootkatensis*.

4.1. Identification of the *Callitropsis nootkatensis* zingiberene synthase

An expressed sequence tag (EST) database of a cDNA library derived from the *C. nootkatensis* heartwood (Beekwilder et al., 2013) was examined for putative terpene synthase candidates. The full-length candidate TPS open reading frames were expressed in *E. coli* and the recombinant proteins were tested for sesquiterpene synthase activity *in vitro* by incubating the enzyme with the universal sesquiterpene precursor farnesyl diphosphate (FPP).

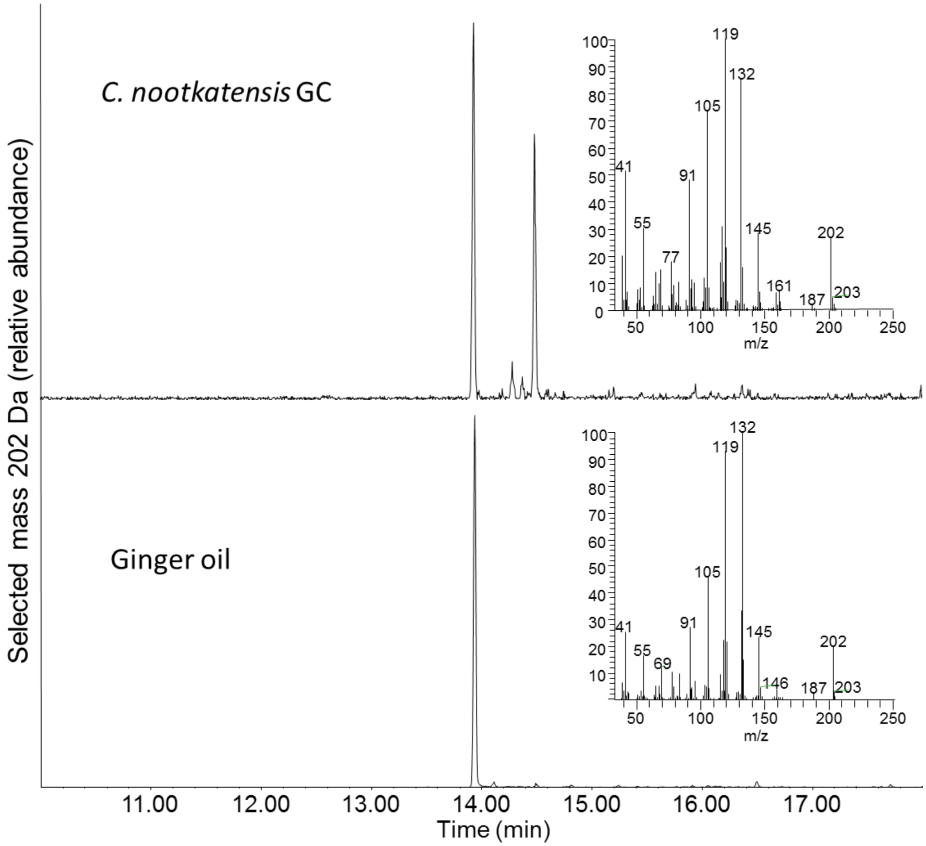


Figure 5.1. Gas chromatogram of a DCM extract of the cortex and epidermis of *Callitropsis nootkatensis* (top, selected ion m/z 202) compared to the one of ginger oil (bottom, selected ion m/z 202), and mass spectra comparison of the compound eluting at 13.93 min, identified as curcumene by its presence in ginger oil and library hits.

In the GC-MS analysis of the products of candidate TPS clone 84-1, one major sesquiterpene was detected and identified as zingiberene by comparison to the NIST library (**Fig. 5.3**). This enzyme was named *C. nootkatensis* zingiberene synthase (CnZIS).

The sequence of *CnZIS* was deposited to the NCBI nucleotide database under the accession number KJ863320. The protein sequence of CnZIS (602 amino acids) contained highly conserved motifs typical of STSs, such as the RRX$_8$W motif at the N-terminus and the DDXXD domain (**Supplermentary Figure S5.1**).

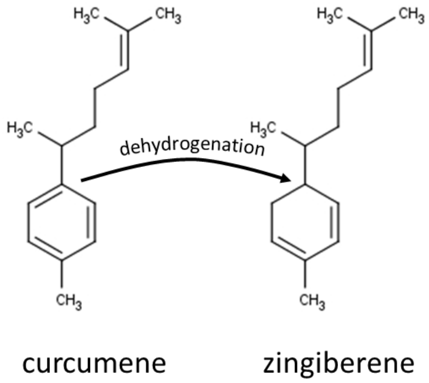


Figure 5.2. Chemical structures of curcumene found in the GC-MS of *C. nootkatensis* and of zingiberene. Curcumene is known to be a dehydrogenation product of zingiberene.

The protein sequence of CnZIS was aligned to that of 26 characterised sesquiterpene synthases, belonging to different TPS families and chosen from a table made by Degenhardt (Degenhardt et al., 2009). A full list of sequences is given in **Supplementary Table S5.1**. CnZIS clustered within the *Tps-d* subfamily of gymnosperm-specific terpene synthases, unlike other zingiberene synthases from other taxa, demonstrating the evolutionary convergence of the function of these enzymes (**Fig 5.4**).

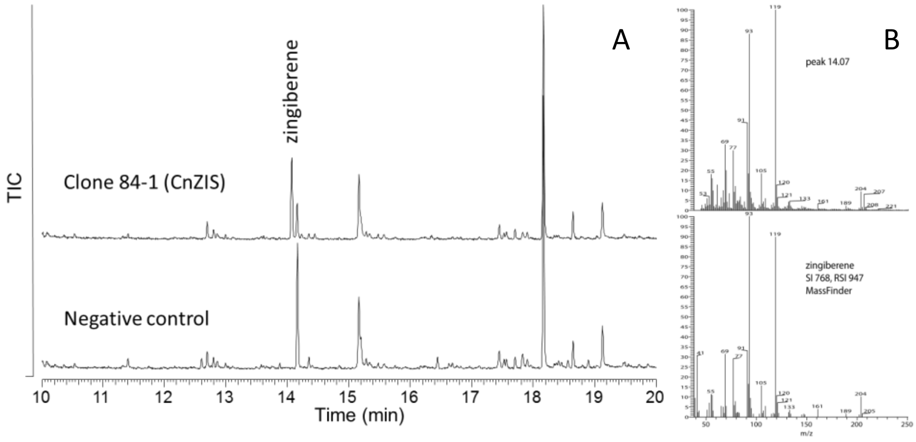


Figure 5.3 A) Gas chromatograms (TIC) of the products of the enzyme assay performed using either the *Callitropsis nootkatensis* zingiberene synthase, CnZIS, heterologously produced in *Escherichia coli* (clone 84-1) or the empty vector control and using (*E*,*E*)-farnesyl diphosphate as substrate. **B)** Comparison of the mass spectrum of the novel peak (14.07 min) with the one of library entry (MassFinder) for zingiberene, which gave the highest similarity index. SI = similarity index, RSI = reverse similarity index

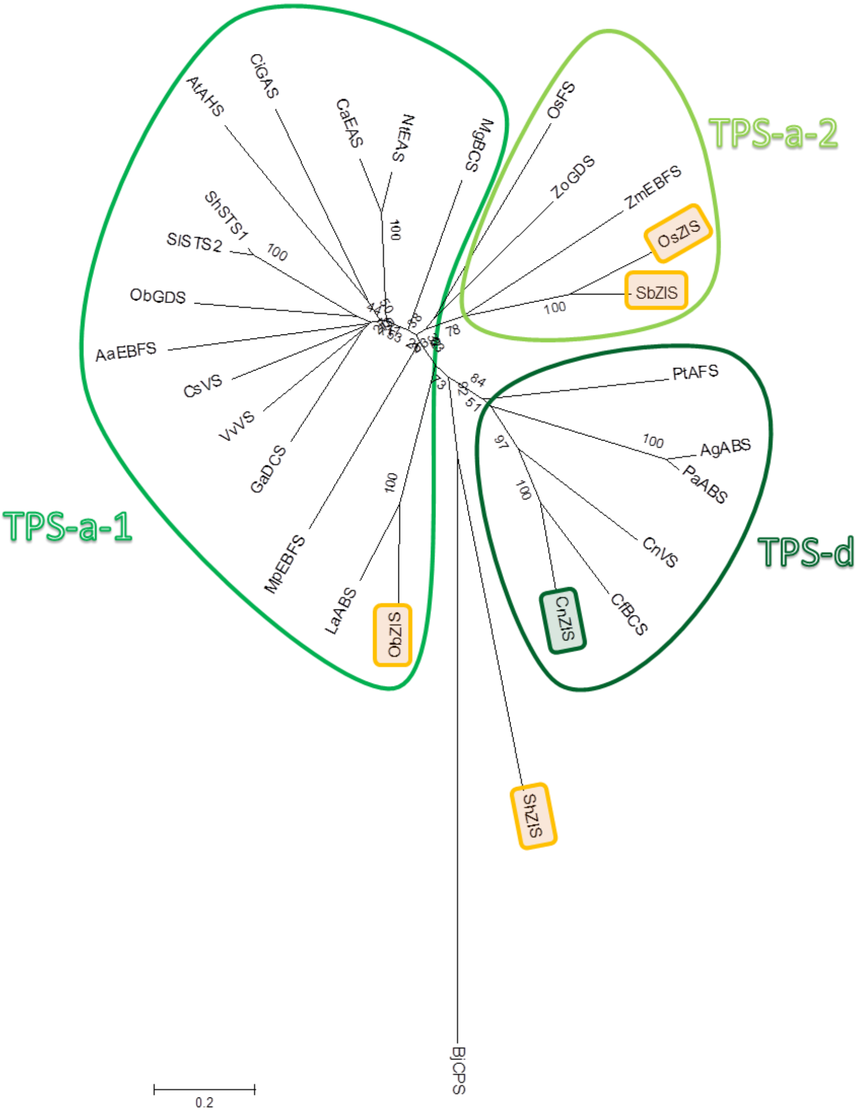


Figure 5.4. Phylogenetic tree representing the clustering of the 28 individual STSs used in the phylogeny analysis. The bacterial copalyl synthase from *Bradyrhizobium japonicum* (BjCPS) is used as the out-group. All the other STSs, except for the ZIS from *Solanum habrochaites* which acts upon a less common substrate – *Z,Z*-FPP and is known to belong to the TPS-e/f family, cluster within one of the three major families. TPS-a-1: dicot STSs; TPS-a-2: monocot STSs; TPS-d: gymnosperm terpene synthases. CnZIS belongs, as predicted, to the TPS-d family (highlighted in dark green). The other zingiberene synthases are highlighted in yellow. The scale bar indicates amino acid substitutions per site; numbers indicate the branch support values in percentage.

4.2. Gene expression analysis of *CnZIS* in *C. nootkatensis* tissues

By performing quantitative RT-PCR, the relative abundance of the *CnZis* mRNA in eight tissues of *C. nootkatensis* was assessed. The highest level of expression was detected in the leaf tissues, followed by the cones, the bottom epidermis and the top cortex/epidermis; the trunk heartwood and cortex had minimal expression levels, while the mRNA was not detected in roots and top heartwood (**Fig. 5.5**). In the same tissues, curcumene was analysed. Though no complete correlation between curcumene accumulation and *CnZIS* expression could be observed, tissues with a high curcumene content such as leaves showed high expression, and tissues with no detectable curcumene such as heartwood showed low *CnZIS* expression.

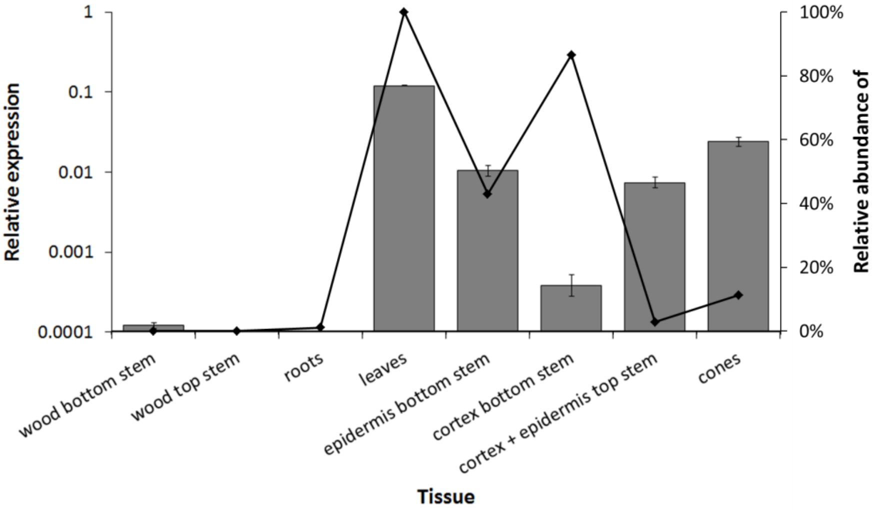


Figure 5.5. The bar plot shows the results of the qRT-PCR representing the expression pattern of zingiberene synthase from *Callitropsis nootkatensis CnZIS* in the different tissues analysed (left Y-axes). The values are relative to the housekeeping gene actin (N=3). The line graph represents curcumene accumulation in the same tissues as measured by GC-MS (N=1), and the relative abundance is given on the right Y-axes in percentage, where the highest accumulation is considered to be 100%.

4.3. Heterologous production of zingiberene in *Nicotiana benthamiana* by transient and stable transformation

To study the performance of CnZIS *in planta*, the gene was transiently expressed in *Nicotiana benthamiana* using *Agrobacterium tumefaciens*-based infiltration of the leaves (van Herpen et al., 2010). *CnZIS* was expressed under the control of a Rubisco Small Subunit promoter from chrysanthemum (Outchkourov et al., 2003) and the resulting protein was targeted to the mitochondria. Upon analysis of the headspace volatiles of *N. benthamiana* leaves by GC-MS, zingiberene was found to be the predominant sesquiterpene in plants expressing *CnZIS* (**Fig. 5.6A**, top 2 chromatograms), followed by 5-*epi*-aristolochene, the most abundant endogenous terpene. Interestingly, also a minor peak, corresponding to curcumene, was observed in GC-MS chromatograms of these plants (**Fig. 5.6A**, peak 3).

After seeing that the transiently transformed plants produced zingiberene, RUBISCO-CnZIS constructs with mitochondrial as well as cytosolic targeting were used for stable transformation of *N. benthamiana*. Twelve transformed plants for each construct were further analysed. To this end, leaf discs of these plants were placed in glass vials and volatiles emitted into the headspace were analysed by Solid-Phase MicroExtraction (SPME) GC-MS. We detected zingiberene production in 8 transgenic lines for the cytosolic construct and 11 transgenic lines for the mitochondrial construct. Although the SPME GC-MS technique is not strictly quantitative, mitochondrial targeting resulted in higher production of zingiberene (**Fig. 5.7**).

4.4. Whitefly choice assays on *N. benthamiana*

Second generation plants, grown from the seeds of lines 1.5_3 and 1.5_4, which derived from the *N. benthamiana* transformants with the highest abundance of zingiberene (both with mitochondrial targeting), were used to assess the effect of the emitted volatiles on whiteflies. Two assays were performed: firstly, whole plants of control and *CnZIS*-expressing lines were placed in a cage, in which adult whiteflies of both sexes were released, and their feeding preference monitored. Secondly, adult whiteflies were released in Petri dishes where leaf discs of control and *CnZIS*-expressing plants were exposed, and again feeding preference was monitored. Surprisingly, in both approaches (whole plants and leaf discs in Petri dish), all whiteflies died within hours of the start of the experiments. This was observed on both the *CnZIS*-expressing and the control plants. Apparently, *N. benthamiana* is not a suitable host for the whitefly used in this experiment.

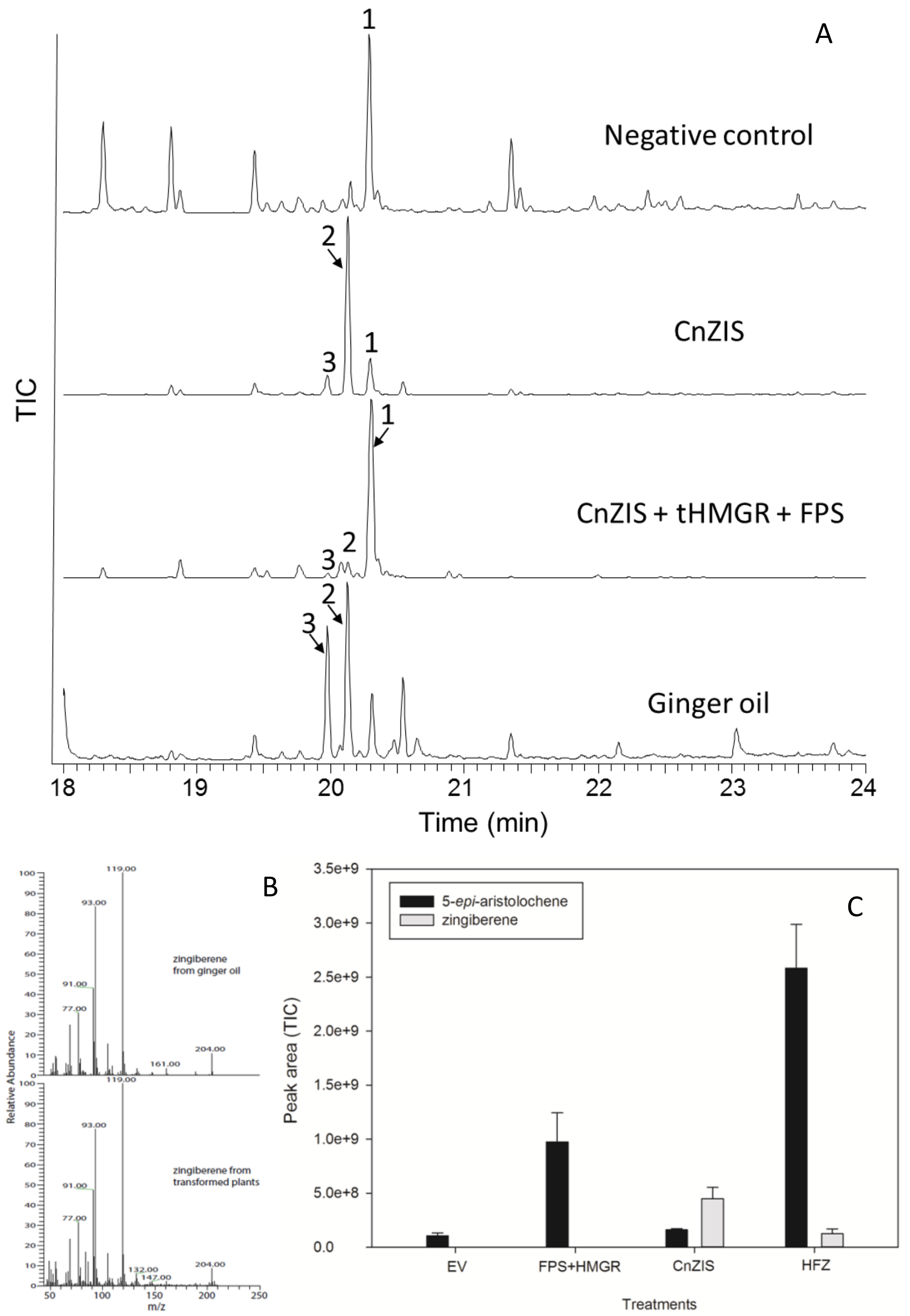


Figure 5.6. see caption on page 122

Figure 5.6. **A)** GC of the volatiles of *N. benthamiana* plants transiently expressing *CnZIS* with mitochondrial targeting. From top to bottom: negative control (plants transformed with empty pBIN+ vector); HFZ (plants co-expressing *AtHMGR*, *AtFPS2* and *CnZIS*); *CnZIS*; ginger oil. Identified peaks: 1 = 5-*epi*-aristolochene, 2 = zingiberene, 3 = curcumene. The relative scales are as follows: ZIS: 100 = 7.85×10^8; HFZ: 100 = 3.09×10^9; EV: 100 = 5.01×10^8; ginger oil: 100 = 2.38×10^9. 1 = 5-*epi*-aristolochene, 2 = zingiberene. **B)** Comparison of the MS of zingiberene from our samples (top) and zingiberene from ginger oil standard (bottom). **C)** Histogram showing the effect of precursor genes on the production of zingiberene and 5-*epi*-aristolochene; the decrease in the amount of zingiberene emitted in the CnZIS and the HFZ plants was statistically significant (P=0.047); error bars are the standard error of the mean (N=3).

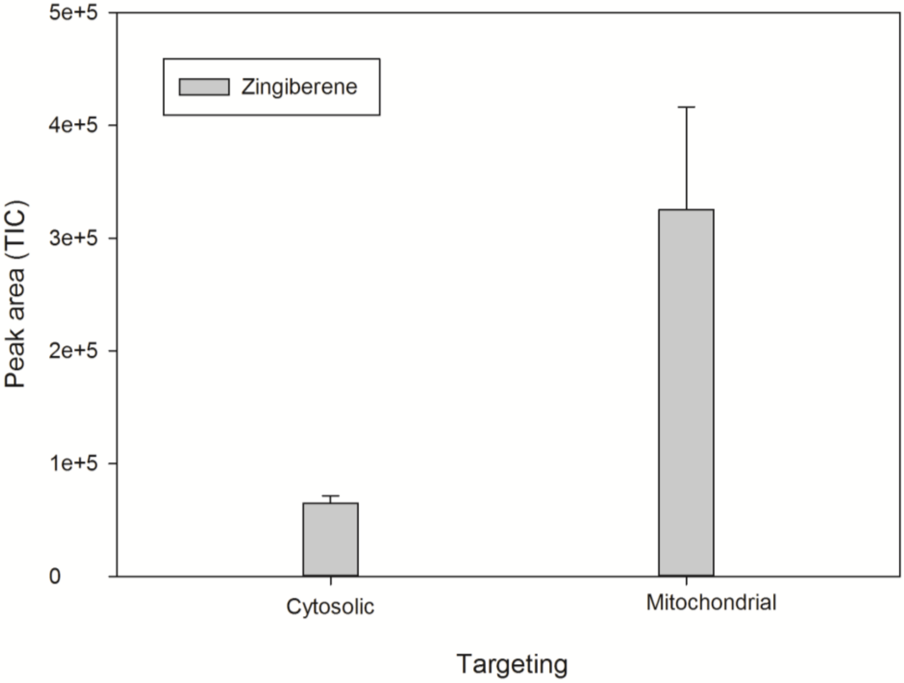


Figure 5.7. Average of the peak areas of zingiberene produced by plants in which *CnZIS* was targeted to the cytosol (left) or to the mitochondria (right) in stably transformed *N. benthamiana* plants, measured by SPME. *n* = 11 for the cytosolic targeting and *n* = 12 for the mitochondrial targeting.

4.5. Whitefly dual-choice assay using *Nicotiana tabacum* transiently transformed with *CnZIS*

In an attempt to overcome the undesired effect of *N. benthamiana* on whitefly, *N. tabacum*, which is known to be a host for whitefly, was used to transiently express *CnZIS* with mitochondrial targeting. The headspace composition of *CnZIS*-expressing plants was analysed by thermal desorption coupled to GC-MS, and showed a low production of zingiberene, which was absent in the plants expressing an empty vector (**Fig. 5.8A**, peak with retention time of 20.02 min). Emission of zingiberene was quantified to be 2.5 ± 0.4 ng h^{-1} gFW^{-1}. The emission was much lower (40-fold) compared with agro-infiltrated *N. benthamiana*.

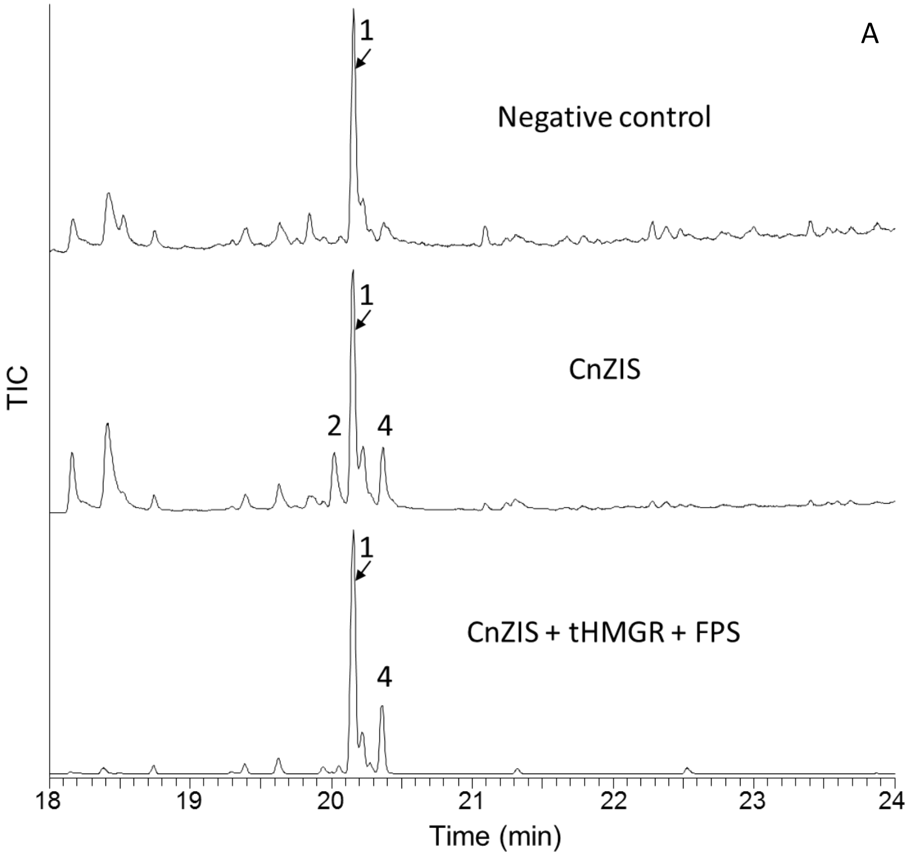


Figure 5.8. see caption page 124

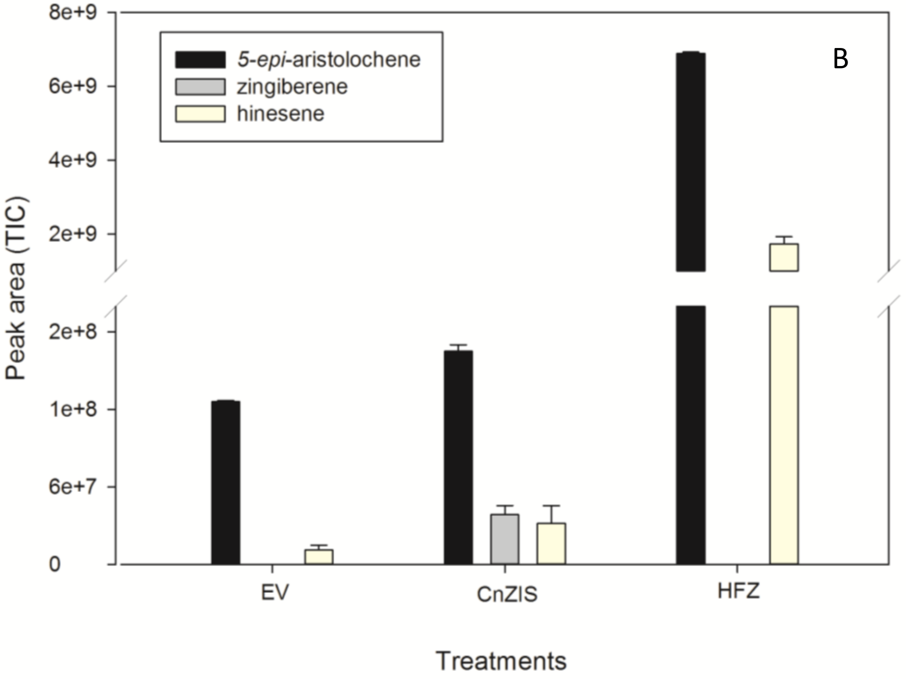


Figure 5.8. **A)** GC of the volatiles of *N. tabacum* plants transiently expressing *CnZIS* with mitochondrial targeting. Top: leaves infiltrated with empty pBIN+ vector (EV); middle: leaves infiltrated with *CnZIS*; bottom: leaves co-infiltrated with *AtHMGR*, *AtFPS2* and *ZIS* (HFZ). Each chromatogram is normalized against the abundance of the compound with retention time 20.16 min, which is 5-*epi*-aristolochene. The relative scales are as follows: ZIS: 100 = 7.99 x 10^7; HFZ: 100 = 2.41 x 10^9; EV: 100 = 4.72 x 10^7. 1 = 5-*epi*-aristolochene, 2 = zingiberene, 4 = hinesene.. **B)** Histogram showing the effect of precursor genes on the production of zingiberene, 5-*epi*-aristolochene and hinesene.

The plants also showed a mild increase (1.3x, $p = 0.04$, $n = 2$) in the amounts of the endogenous sesquiterpene 5-*epi*-aristolochene (**Fig. 5.8B**), similar to *N. benthamiana* transiently expressing *CnZIS* (**Fig. 5.6C**).

Agroinfiltrated leaves were used in the dual-choice assay with whiteflies in the Petri dish setup. The repellent or attractant effect was scored using the Antifeedant Index (AI%). We observed a mild repellent effect against whiteflies of the *CnZIS*-expressing tobacco ($p < 0.05$) only at 2 h after the start of the experiment, while at all other time points there was no effect on whitefly preference in the choice between the leaves transiently expressing *CnZIS* and those expressing an empty vector (**Fig. 5.9**, dashed line).

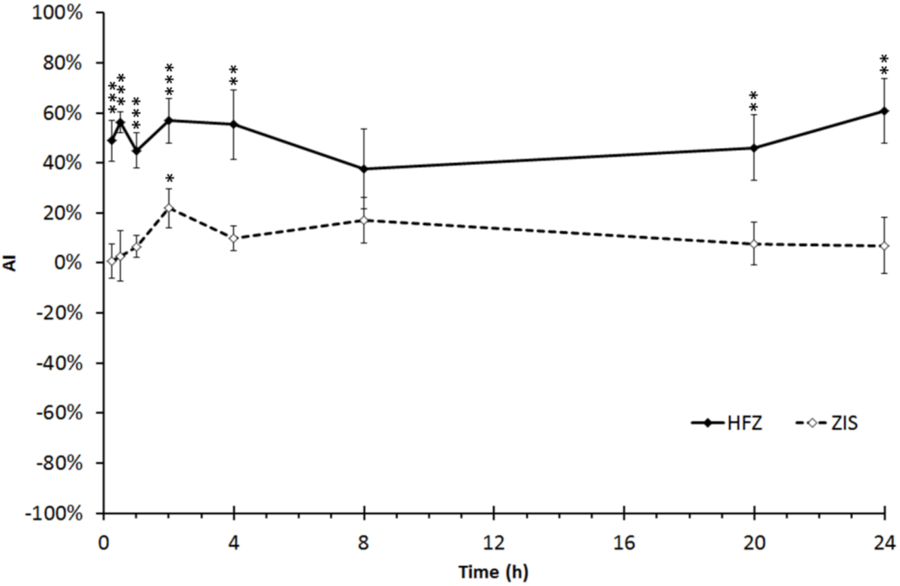


Figure 5.9. Effect on whitefly feeding over time, expressed as Antifeedant Index, of *N. tabacum* transiently expressing either the *C. nootkatensis* zingiberene synthase alone (*CnZIS*) or co-expressing *AtHMGR*, *AtFPS2* and *CnZIS* (HFZ). Positive values indicate an antifeedant effect, negative values a phagostimulant effect. Error bars indicate standard error. (n = 8; 82 and 67 whiteflies on average for the assays with the ZIS and HFZ leaves, respectively). Statistical significance is expressed by the asterisks: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

4.6. Co-infiltration of zingiberene synthase with HMGR and FPS in *N. tabacum* and *N. benthamiana* results in boosting of endogenous but not heterologous sesquiterpenes

In view of the low amounts of zingiberene produced by the *CnZIS*-expressing *N. tabacum* and the resulting negligible effect on whitefly, we resorted to the use of genes that would supply more FPP as a substrate for zingiberene synthase, namely tHMGR and FPS, to try to boost zingiberene production. We therefore transiently expressed *CnZIS* in combination with truncated *AtHMGR* and *AtFPS2* in *N. tabacum* (the treatment is named HFZ for convenience), and subsequently analysed the volatile compounds by GC-MS.

The main differences between the three chromatograms (**Fig. 5.8B**) were seen in the area around 20 min. Surprisingly, zingiberene (peak 1) was absent in the HFZ plants. Furthermore, two compounds were found to be strongly up-regulated in these plants: 5-*epi*-aristolochene (peak 2) and a compound which mass spectrum closely matched the published mass spectrum of the spirocyclic compound hinesene (peak 4). These compounds were increased 54- and 154-fold, respectively, in the HFZ compared to the Empty Vector (EV) sample and 41- and 54-fold, respectively, in the HFZ compared to the ZIS samples (**Fig 5.8C**).

After observing the interesting fact of having the endogenous sesquiterpenes of *N. tabacum* boosted by transient co-expression of *tHMGR* and *FPS*, but no detectable levels of zingiberene, we decided to test the effect of *tHMGR* and *FPS* in *N. benthamiana*, for comparison. In this species, we also observed a strong increase in production of 5-*epi*-aristolochene upon co-expression of *CnZIS* with *tHMGR* and *FPS*, making it the main product in the headspace of these plants. Nevertheless, there was a detectable production of zingiberene as well (**Fig. 5.6A**), contrary to what was observed in *N. tabacum*.

4.7. Whitefly bioassay using co-infiltrated *N. tabacum* leaves

Dual-choice whitefly assays were performed using the *N. tabacum* HFZ leaves, in the same fashion as for the leaves transiently expressing *CnZIS*. Curiously, in the case of the HFZ plants, which did not produce any detectable zingiberene, the antifeedant effect was much stronger than with CnZIS alone. The average AI% of the products of those leaves was around 50, meaning that three times as many whiteflies were feeding on the control leaves than on the HFZ ones. The repellent effect was statistically significant ($p < 0.01$) for all time-points except 8 h (**Fig. 5.9**, solid line). Those leaves also displayed some wilting and mild damage compared with the ones transiently expressing only *CnZIS*.

5. Discussion

5.1. *C. nootkatensis*' CnZIS produces α-zingiberene *in vitro* and when overexpressed in *Nicotiana* spp.

Within a pool of terpene synthases, selected from the EST library of *C. nootkatensis* based on sequence analysis, candidate TPS clone 84-1 was identified as producing the bisabolane sesquiterpene zingiberene *in vitro* (Fig 3). The translated amino acid sequence was compared to that of several other sesquiterpene synthases and phylogenetic analysis confirmed it belongs to the TPS-d subfamily of terpene synthases, which encompasses all gymnosperm mono- and sesquiterpene synthases (**Fig. 5.4**). The role of CnZIS in *C. nootkatensis* is still unclear. Possibly, it is involved in the biosynthesis of curcumene, based on the relationship between the structures of curcumene and zingiberene (**Fig. 5.2**). Conversion of zingiberene to curcumene would require a dehydrogenation step. In the past, the incidence of curcumene in ginger oil has been ascribed to thermal conversion of zingiberene, rather than to enzymatic dehydrogenation (Cornell and Jordan, 1971). Support for a relationship between zingiberene synthase and curcumene formation was obtained by the finding of curcumene in the headspace of CnZIS expressing *N. benthamiana*. Apparently, this plant contains also a dehydrogenation activity that can convert zingiberene to curcumene. Also, we assessed the potential activity as curcumene synthase of CnZIS in *C. nootkatensis* by looking for a correlation between the accumulation of curcumene and the expression of *CnZIS* in various tissues. **Figure 5.5** shows that tissues that do not express the gene, such as the heartwood and the roots, also do not accumulate any curcumene. However, in the tissues that do contain curcumene there is no close correlation between the abundance of curcumene and the expression of *CnZIS*. Possibly, its formation is therefore a process performed by endogenous enzymes or, in some of those tissues, the compound is further converted or conjugated and therefore not detected in our analysis.

5.2. Zingiberene does not have an effect on whitefly in *N. tabacum* transiently expressing *CnZIS*

It has been shown that both α-zingiberene and its enantiomer 7-*epi*-zingiberene have a repellent effect on whitefly. The first one was proven effective in olfactometer studies as well as in choice assays using leaf disks that were immersed in a solution containing ginger oil (Zhang et al., 2004). The effect of the latter was studied using hybrids of cultivated (*S. lycopersicon*) and wild tomato (*S. habrochaites*) that were emitting 7-*epi*-zingiberene (Bleeker et al., 2012). In a previous study by the same group, the repellent effect of zingiberene, as well as curcumene, was shown to depend on the stereochemistry at C-7; although both isomers could be detected by the whitefly's antennae, only 7-*epi*-zingiberene was effective at repelling whiteflies from a distance triggering an innate response in the insects (Bleeker et al., 2011). In our study, however, when tested in the dual-choice Petri dish setup, the *N. tabacum* leaves producing zingiberene did not have any effect on whitefly feeding preference. One might speculate that such setup, which proved effective when studying an applied non-volatile antifeedant compound (polygodial) to tomato leaves (Prota et al., 2013), does not provide the optimal way to study the effects of a volatile compound that potentially easily saturates the small volume of air in the Petri dish. However, in the assays with *N. tabacum* leaves producing high amounts of 5EA the set-up worked well. Therefore, the most plausible conclusion is that the amount of zingiberene emitted by *N. tabacum* is too low to have a significant effect.

Higher levels of zingiberene were produced by *N. benthamiana* transiently expressing *CnZIS*. It appeared impossible, nonetheless, to study the effect of zingiberene in this species, as virtually all whiteflies were found to be dead hours after being introduced into a cage or a Petri dish containing WT and *CnZIS*-expressing *N. benthamiana* plants. This phenomenon can be most likely explained by the fact that whiteflies were trapped by the glandular trichomes and/or poisoned by their contents (such as $CaCl_2$), as is the case for aphids (Neal et al., 1990; Harada et al., 1996).

5.3. Overexpression of precursor genes of the sesquiterpene pathway together with zingiberene synthase results in boosting of the endogenous 5-*epi*-aristolochene but not of the heterologous zingiberene

As reported by van Herpen *et al.* (2010) and Wu *et al*. (2006), over-expression of a truncated version of HMGR (targeted to the cytosol) together with an FPS targeted to the same subcellular compartment as the sesquiterpene synthase of interest, boosts the production of the desired sesquiterpene. In our work, we observed rather low amounts of zingiberene produced upon overexpression of only the *CnZIS* in *N. tabacum*. We therefore proceeded to boost the production by overexpressing the two precursor genes (*tHMGR* and *FPS2* from *Arabidopsis thaliana*). To our surprise, however, the plants co-expressing the three genes (HFZ) produced no zingiberene anymore (**Fig. 5.8B**). Furthermore, the levels of the endogenous sesquiterpenes 5-*epi*-aristolochene (5EA) and hinesene increased dramatically in those plants (up to 60- and 154-fold, respectively, when compared with the empty vector control). A similar phenomenon was observed for *N. benthamiana* (although hinesene is absent in this species) as well (**Fig. 5.6C**), where the introduction of the three genes of the pathway resulted in a drastic increase in 5EA production, while reducing the amount of zingiberene when compared with plants only transiently expressing *CnZIS* ($p = 0.047$, $n = 3$). The reasons for this phenomenon could be several. For instance, the co-infiltration of *CnZIS* with *tHMGR* and *FPS* lowers the gene dosage of *CnZIS*, compared with the single-construct experiment. As observed before, gene dosage may strongly affect the outcome of agro-infiltration (Ting et al., 2013). An alternative explanation may be a lower affinity or reaction velocity of CnZIS in comparison with the endogenous enzymes such as 5EA synthase (EAS). In that case, the induction of EAS upon up-regulation of the FPP pool may lead to stronger sink-formation in 5EA, compared with zingiberene. Finally, another possibility may be that metabolon formation occurs preferentially between the heterologous FPS (from *Arabidopsis*) and the endogenous EAS. Metabolon formation has been reported to occur in other secondary metabolic pathways in plants (Jørgensen et al., 2005), and would allow for substrate channelling and thereby a more efficient formation of 5EA compared with zingiberene.

Unlike *N. tabacum*, *N. benthamiana* plants still produced some zingiberene when co-infiltrated with all three genes. In fact, the level of zingiberene was generally notably lower in *N. tabacum* than in the analogously agro-infiltrated *N. benthamiana*, where zingiberene was, for instance, the main compound produced after the overexpression of ZIS alone (**Fig. 5.6**). This difference between the emissions observed in the two species might be due to a species-specific reaction to the overexpression of the aforementioned genes or innate differences in their terpene transport capacity.

5.4. Plants transiently expressing *HMGR*, *FPS* and *CnZIS* are repellent against whitefly, despite the undetectable levels of zingiberene in their head-space

When choice assays were carried out to assess the effect of transient expression of *CnZIS* alone or in combination with *tHMGR* and *FPS* (HFZ), the *CnZIS*-expressing leaves had negligible effect, while on the contrary, the HFZ leaves proved to be highly unappealing to the insects when compared with an empty vector control. The HFZ leaves appeared slightly more damaged at four days post-infiltration compared with those infiltrated with ZIS alone, which might have contributed to the whiteflies' preference for the "control" leaves. However, as seen in the GC of the headspace (Fig. 8), no major differences in the volatile composition occurred between the HFZ leaves and the ones transformed with an empty vector, except for hexenol, 5EA and hinesene. This argues in favour of a direct effect of the increase in one of these compounds on the feeding preference. Particularly, it is known that 5EA synthase (EAS) is induced upon wounding (Korth and Dixon, 1997) as well as being a JA-associated gene and induced by the SA-analogue benzothiadiazole (Heidel and Baldwin, 2004). 5EA is a precursor of the antifungal sesquiterpenoid capsidiol, and, accordingly, EAS was induced when tobacco was treated with an elicitor (cellulase) (Facchini and Chappell, 1992). Clearly, the use of empty-vector-infiltrated leaves as controls would exclude an effect of Agrobacterium or the agro-infiltration procedure on up-regulation of these compounds, or on the feeding behaviour of whitefly. These observations lead us to postulate that 5EA has an, until now undemonstrated, effect on whitefly as well. Alternatively, the concerted effect of HMGR and FPS might work to boost other terpenes (such as triterpenes, not detectable by our methods), which are known to act as antifeedants, and could therefore discourage whiteflies from settling. Assessment of the effect of isolated 5EA on whitefly in an olfactometer setup or overexpression/silencing of the enzyme EAS would be an interesting next step (Silencing has been successfully obtained for this gene in an attempt to boost production of heterologous sesquiterpenes (Cankar et al., 2014)).

Our research is not yet in a stage that conclusions can be drawn on the effect of CnZIS mediated zingiberene production on whitefly feeding behaviour. One of the reasons for this is that *N. benthamiana* is not suitable as a model for whitefly resistance. Future experiments should be directed at improved methods for overproduction of zingiberene, and stable transformation of *N. tabacum*, *S. lycopersicon* or other species that are hosts for whitefly.

6. Acknowledgements

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7. Supplementary material

Supplementary Table S5.1. Selection of previously characterized sesquiterpene synthases (Degenhardt et al., 2009; Chen et al., 2011; Bleeker et al., 2012; Beekwilder et al., 2013) to which CnZIS was aligned to build a phylogenetic tree.

ID	Major product	Name[1]	Species	Characterized by
AAC24192	(*E*)-α-Bisabolene	AgABS	*Abies grandis*	Bohlmann et al. (1998)
AAO85539	(-)-(*E*)-β-Caryophyllene/ α-humulene	AtAHS	*Arabidopsis thaliana*	Chen et al. (2003)
AAX39387	(*E*)-β-Farnesene	AaEBFS	*Artemisia annua*	Picaud et al. (2005)
BAC47414	Copalyl diphosphate	BjCPS	*Bradyrhizobium japonicum*	Kaneko et al. (2002)
AAC61260	5-*epi*-Aristolochene	CaEAS	*Capsicum annuum*	Back et al. (1998)
AFJ23663	β-Cadinene	CfBCS	*Chamaecyparis formosensis*	Kuo et al. (2012)
AAM21659	(+)-Germacrene A	CiGAS	*Cichorium intybus*	Bouwmeester et al. (2002)
AAQ04608	Valencene	CsVS	*Citrus sinensis*	Sharon-Asa et al. (2003)
AAA93064	(+)-δ-Cadinene	GaDCS	*Gossypium arboreum*	Chen et al. (1995)
ABB73046	(*E*)-α-Bergamotene	LaABS	*Lavandula angustifolia*	Landmann et al. (2007)
ACC66281	β-Cubebene	MgBCS	*Magnolia grandiflora*	Lee and Chappell (2008)
AAB95209	(*E*)-β-Farnesene	MpEBFS	*Mentha x piperita*	Crock et al. (1997)
AFJ04408	5-*epi*-Aristolochene	NtEAS	*Nicotiana tabacum*	Zhang (2012)
AAV63788	α-Zingiberene	ObZIS	*Ocimum basilicum*	Iijima et al. (2004)
AAV63786	Germacrene D	ObGDS	*Ocimum basilicum*	Iijima et al. (2004)
ACF05529	Zingiberene	OsZIS	*Oryza sativa*	Yuan et al. (2008)
ABJ16554	(*E*,*E*)-Farnesol	OsFS	*Oryza sativa*	Cheng et al. (2007)
AAS47689	(*E*)-α-Bisabolene	PaABS	*Picea abies*	Martin et al. (2004)
AAO61226	(*E*,*E*)-α-Farnesene	PtAFS	*Pinus taeda*	Phillips et al. (2003)
AAG41890	δ-Elemene	SlDES	*Solanum lycopersicum*	van der Hoeven et al. (2000)
AFJ67794	7-*epi*-zingiberene	ShZIS	*Solanum habrochaites*	Bleeker et al. (2012)
AAG41891	Germacrene B	SlGBS	*Solanum hirsutum*	van der Hoeven et al. (2000)
C5YHH7	α-Zingiberene	SbZIS	*Sorghum bicolor*	Zhuang et al. (2012)
AAS66358	(+)-Valencene	VvVS	*Vitis vinifera*	Lucker et al. (2004)
ABY79212	(*E*)-β-Caryophyllene	ZmEBCS	*Zea mays mexicana*	Köllner et al. (2008)
AAX40665	(+)-Germacrene D	ZoGDS	*Zingiber officinale*	Picaud et al. (2006)
JX040471	Valencene	CnVS	*Callitropsis nootkatensis*	Beekwilder et al. (2013)
KJ863320	α-Zingiberene	CnZIS	*Callitropsis nootkatensis*	Present study

[1] these names refer to those used in **Fig. 5.4**.

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              5         15         25         35         45         55         65         75         85
CnZIS     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
ObZIS     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SbZIS     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
OsZIS     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
ShZIS     MIVGYRSTII TLSHPKLGNG KTISSNAIFR RSCRVRCSHS TPSSMNGFED ARDRIRESFG KVELSPSSYD TAWVAMVPSK HSLNEPCFPQ
CnVS      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
             95        105        115        125        135        145        155        165        175
CnZIS     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---MAQKANT
ObZIS     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SbZIS     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
OsZIS     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
ShZIS     CLDWIIENQR EDGSWGLNPS HPLLLKDSLS STLACLLALT KWRVGDEQIK RGLGFIETQS WAIDNKDQIS PLGFEIIFPS MIKSAEKLNL
CnVS      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---MAEMFN-
            185        195        205        215        225        235        245        255        265
CnZIS     QFDAIYFNGE EKTEEKGVIR RMGEYHANVW ---------- ------DRDF LLSLSSPYGA PSYMERLETL VNEIKMDVFI NSLLVGDEEI
ObZIS     ---------- ------MESR RSANYQASIW ---------- ------DDNF IQSLASPYAG EKYAEKAEKL KTEVKTMIDQ TR--------
SbZIS     ------MAAL QYNCDAGLAK APT-FHPSLW ---------- ------GDFF LTYQPPTAPQ HVYMKERAEL LKEEVREIVK GT--------
OsZIS     ---MSSTPAA NFSNEDDERK APTGFHPSLW ---------- ------GDFF ISYQPPTAPK HAYMKERAEV LKEEVRKVVK GS--------
ShZIS     NLAINKRDST IKRALQNEFT RNIEYMSEGF GELCDWKEII KLHQRQNGSL FDSPATTAAA LIYHQHDKKC YEYLNSILQQ HKNWVPTMYP
CnVS      --GNSSNDGS SCMPVKDAIR RTGNHHPNLW ---------- ------TDDF IQSLNSPYSD SSYHKHREIL IDEIR-DMFS NG--EGDE--
            275        285        295        305        315        325        335        345        355
CnZIS     NPSSYDLIER FSIVDVVQRL GIDRHFEKEI KAVLDYTYRY WSENGISWRR ENSIIDLYTT ALGFRILRLN GYSVSPDVFH NFKDGDGQFV
ObZIS     -----DELKQ LELIDNLQRL GICHHFQDLT KKILQKIYGE ERNGDHQHYK E---KGLHFT ALRFRILRQD GYHVPQDVFS SFMNKAGDFE
SbZIS     ----NELPKL LDLIITLQRL GLDNHYEIEI DEHLHFIYNS SCDV------ ----KDLNLV SLRFYLLRKN GYDVSSDVFL NFKDKDGNFA
OsZIS     ----NEVPEI LDLVITLQRL GLDSYYKAEI DELLCTVYNT DYND------ ----KDLHLV SLRFYLLRKN GYDVSSDIFQ HFKDKEGSFV
ShZIS     ----TKIHSL LCLVDTLQNL GVHRHFKSEI KKALDEIYRL WQQKNEEIFS -----NVTHC AMAFRLLRIS YYDVSSDELA EFVDEEHFFA
CnVS      ----FGVLEN IWFVDVVQRL GIDRHFQEEI KTALDYIYKF WNHDSIFG-- -----DLNMV ALGFRILRLN RYVASSDVFK KFKGEEGQFS
            365        375        385        395        405        415        425        435        445
CnZIS     -FPTKQSDNQ IRIMLSLYQA SEISFPGE-- YIMREAKEFS YKYLEEALGK WKGLN-EKTQ LIEEVEYIMK YPWRCRVPRW ETWNSIKILR
ObZIS     ES----LSKD TKGLVSLYEA SYLSMEGE-- TILDMAKDFS SHHLHKMVED AT-----DKR VANQIIHSLE MPLHRRVQKL EAIWFIQFYE
SbZIS     -------SDD IRSLLSLYNA AYLRTHGE-- EVLDEAIIFT RRHLEAALTS L------ESK LADEVSLSLQ TPLFRRVRIL ETRNYIPIYE
OsZIS     -------ADD TRSLLSLYNA AYMRTHGE-- KVLDEAVVFT TNRLRSELKH L------KSP VADEVSLALD TPLFRRVRII ETQNYIPIYE
ShZIS     TSG---KYTS HVEILELHKA SQLAIDHEKD DILDKINNWT RTFMEQKLLN NG---FIDRM SKKEVELALR N-FYIISDLA ENRRYIKSYE
CnVS      GFESSDQDAK LEMMLNLYKA SELDFPDE-- DILKEARAFA SMYLKHVIKE YGDIQESKNP LLMEIEYTFK YPWRCRLPRL EAWNFIHIMR
|           455        465        475        485        495        505        515        525        535
CnZIS     QDMGAWMLME GIYKVQNEWS EKILEVAILD FNILQSHHHN ELKHLAEWWD EA-IVKQLSF FRHRHVEYYF WYACGLYEPE YAATRLCYAK
ObZIS     CG-------- ------SDAN PTLVELAKLD FNMVQATYQE ELKRLSRWYE ETGLQEKLSF ARHRLAEAFL WSMGIIPEGH FGYGRMHLMK
SbZIS     ME-------- ------PSRN EAMLEFAKLN FNLLQILYCE ELKTVTAWWK QLNIETDLSF IRDRIVEMHF WMAGACSEPK YSLSRVILTK
OsZIS     SA-------- ------TTRN EAILEFAKLN VNLLQLIYCE ELKTITRWWK ELNVESNLSF IRDRIVEMHF WMTGACSEPH YSLLRIILTK
ShZIS     EN--NFKILK AAYRSPNINN KDLFIFSIRD FELCQAQHQE ELQQLKRWFE DC-RLDQLGL SEQFISASYL CAIPIVPGPE LSDARLVYAK
CnVS      QQDCNISLAN NLYKIPKIYM KKILELAILD FNILQSQHQH EMKLISTWWK NS-SAIQLDF FRHRHIESYF WWASPLFEPE FSTCRINCTK
            545        555        565        575        585        595        605        615        625
CnZIS     LGTLITVIDD IFDTYGTIDE LIPFREALIN WDMSIVDQLP -NYMQISLQF AHKTYMEIAT EAEKIHGPHV QKWMEDYWKT LIWAEFQD-A
ObZIS     IGAYITLLDD IYDVYGTLEE LQVLTEIIER WDINLLDQLP -EYMQIFFLY MFNSTNELAY EILRDQGINV ISNLKGLWVE LSQCYFKE-A
SbZIS     MTAFITILDD IIDTHSTTEE GKLLAKAIDR CSQDANEVLP -DYMKHFYMF LLKTFDSCED ELGPNK-RYR -------LKI LVRGYSQE-I
OsZIS     MTAFITILDD IFDTYATTEE SMMLAKAIYM CNESATVLLP -KYMKDFYLY YLKTFDSFEE ALGPNK-SYR VLYFKELFKI LIKGYSEE-I
ShZIS     YVMLLTIVDD HFESFASTDE CLNIIELVER WDDYASVGYK SERVKVLFSM FYKSIEEIAT IAEIKQGRSV KNHLINLWLK VMKLMLMERV
CnVS      LSTKMFLIDD IYDTYGTVEE LKPFTTTLTR WDVSTVDNHP -DYMKIAFNF SYEIYKEIAS EAERKHGPFV YKYLQSCWKS YIEAYMQE-A
            635        645        655        665        675        685        695        705        715
CnZIS     EWIANNYHPT -LIQYLKNSL ISSTVPVVTL FPMILINTNL PNDILNKVNK FESK----VA WGCRLIDDSK DFQHEQEHGE TASWIECYVR
ObZIS     TWFHNGYTPT -TEEYLNVAC ISASGPVILF SGYFTTTNPI NKHELQSLER HAHS----LS MILRLADDLG TSSDEMKRGD VPKAIQCFMN
SbZIS     EWRDEHYVPE TIDKHLEISR VTVGAFQLAC SSFVGMGDII TKEVLDWLLT YPELLKC-FT TFVRLSNDIT ST-------- ----------
OsZIS     KWRDDHYIPK TIEEHLELSR MTVGAFQLAC ASLVGMGDFI TEDTLDYLLT YPKLIKS-YT TCVRLSNDIA STKREQAGDH YASTIQCYML
ShZIS     EWCSGKTIPR -IEEYLYVSS ITFGSRLIPL TTQYFIGIKI SKDLLESDEI YGLCNFT--G IVLRLLNDLQ DSKREQKEG- ---SINLVTL
CnVS      EWIASNHIPG -FDEYLMNGV KSSGMRILMI HALILMDTPL SDEILEQLDI PSSKSQALLS LITRLVDDVK DFEDEQAHGE MASSIECYMK
            725        735        745        755        765        775        785        795        805
CnZIS     DNPGTTREQA LDHVNMLIEL NIEELNKERL FNEH-DIPTC CKRLYFDSMY RSVSFIWRDI DGFTISHQG- TKDDIMKILI EPISL--
ObZIS     -DTGCCEEEA RQHVKRLIDA EWKKMNKDIL MEKP-FKNFC PTAMNLGRIS ---MSFYEHG DGYGGPHSD- TKKKMVSLFV QPMNITI
SbZIS     -EHSTTMHDA CEKIKGLIED SWKDMMQLYL TPT--EQSKV VAQTVVDFAR -TGDYMYKKT DAFTFSHTI- -KDMIALLYV EPILF--
OsZIS     -QHGTTIHEA CIGIKELIED SWKDMMKEYL APTN-LQPKI VARTVIDFAR -TGDYIYKQA DSFTFSHTI- -KDMIASLYV EPYSI--
ShZIS     LMKSISEEEA IMKMKEILEM KRRELFKMVL VQKKGSQLPQ LCKEIFWRTC KWAHFTYSQT DRYRFPEEM- -ENHIDEVFY KPLNH--
CnVS      DNHGSTREDA LNYLKIRIES CVQELNKE-L LEPS-NMHGS FRNLYLNVGM RVIFFMLNDG DLFTHSNRKE IQDAITKFFV EPIIP--
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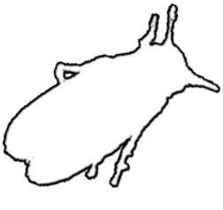
Supplementary Figure S5.1. sequence alignment of the amino acid sequence of CnZIS and four other zingiberene synthases from different species and the valencene synthase from *C. nootkatensis*. The alignment was performed using ClustalW. The boxed residues are highly conserved sequences in sesquiterpene synthases.

Chapter 6

General discussion

"Give a farmer a bag of hybrid seeds, some synthetic pesticides and fertilizers, and you will feed him for a season. Give a farmer the knowledge about how the members of the agroecosystem interact with each other, and you will feed him for a lifetime."

(Neli Prota, adapted from the quote by Anne Isabella Thackeray Ritchie)



1. Whitefly infestation and potential approaches at combating it

"Death is now caused by the Whitefly infection actually starving the life out of your landscaping. Without proper treatment, no infected plant will survive." - http://www.whitefly-treatment.com/

In the last two decades several species of whitefly, especially from the genus *Bemisia*, have acquired the status of global pests (Dalton, 2006), spreading to climates different from those where they originated, as a result of the intensification of agriculture, monocropping, globalization and the development of resistance to most common insecticides. Approaches at combating this growing agricultural nuisance have involved use of novel pesticides (neonicotinoids) for which resistance had not yet emerged (Dennehy et al., 2005; Caballero et al., 2013), classical as well as molecular breeding for resistant crop varieties (Bleeker et al., 2012; Firdaus et al., 2012; Firdaus et al., 2013), the use of new insect-growth regulators (Dennehy et al., 2005; Dalton, 2006), next to attempts at controlling the infestation with biological control methods, such as employing predators, parasitoids (Naranjo, 2001) and fungi (Faria and Wraight, 2001). In my thesis, I focussed on the potential of natural compounds, polygodial and zingiberene as sprayable deterrents, and at employing the genes responsible for their biosynthesis to create resistant transgenic plants. Although the original aim was to use tomato, a well-known host of *Bemisia spp.*, as target organism for the expression of the genes involved in the synthesis of the two aforementioned compounds, eventually the proof of principle was achieved using *Nicotiana benthamiana*, a model plant more amenable to transformation.

2. The role of natural compounds in pest control

"Plants are nature's alchemists, expert at transforming water, soil and sunlight into an array of precious substances, many of them beyond the ability of human beings to conceive, much less manufacture." – Michael Pollan

Natural plant-derived compounds have been exploited by man for agricultural purposes since times immemorial (Dayan et al., 2009). In plants, these so-called secondary metabolites have diverse functions. Many of them will, however, serve as communication tools between the plant and its environment (Reinecke and Hilker, 2014). For example, some of these compounds will warn neighbouring plants that the one emitting it is being attacked and that they should quickly deploy their own defences (Karban and Maron, 2002; Dicke et al., 2003; Baldwin et al., 2006); others will notify the hungry bee that juicy nectar is awaiting in return for spreading the pollen to other flowers (Pichersky and Gershenzon, 2002); some will inform the moth that the leaves emitting them are not suitable for its offspring (De Moraes et al., 2001); yet others will attract the wasp that looks to parasitise the pest that is attacking the plant sending the signal (Mumm and Dicke, 2010; Rodriguez-Saona et al., 2012). Other compounds, if they even can be said to convey a message, will do so only after they are ingested or digested, at which point they may taste bitter or hot (antifeedants) or exert their potent toxicity (toxins) against the unfortunate herbivore (Isman, 2002; Koul, 2008).

As the use of manmade pesticides has proven not to be free of (unforeseen) side effects, both in terms of resistance acquisition by the target insect as well as potential damage to other players of the ecosystem – including man – alternatives have been sought for some decades (Isman, 2006). Though microencapsulation of pesticides is still a promise to aid in minimizing toxicity and solving other issues (Ghormade et al., 2011), and there has been even some advance in the field of nanotechnology applied to crop protection (Khot et al., 2012), the use of natural compounds offers an attractive solution due to the usually extensive knowledge about them and their generally low environmental impact (Dayan et al., 2009). However, there are also drawbacks to the use of natural compounds, and essential oil formulations, such as their lack of persistence, limited efficacy against larger chewing insects (e.g. lepidopterans and coleopterans), and potential phytotoxicity (Isman et al., 2011).

The array of potential deterrents or antifeedants amongst plant secondary metabolites is vast (Koul, 2005; Koul, 2008; Dayan et al., 2009; Shrivastava et al., 2010). The many classes of natural compounds with known insect-repellent activity are dealt with in detail in the General Introduction (**Chapter 1**). In my work, I focussed on two families of sesquiterpenoids and on their effect on whitefly feeding behaviour. In **Chapter 2** of this thesis, I described the thus far unreported antifeedant effect on whiteflies of polygodial, a sesquiterpene dialdehyde belonging to the drimane family, comparing the efficacy to natural pyrethrins. Another drimane sesquiterpenoid, cinnamolide, was one of the products resulting from the overexpression of genes of the polygodial biosynthetic pathway in yeast and *Nicotiana benthamiana* and also proved to be a whitefly antifeedant (**Chapter 4**). The effects on whiteflies of zingiberene, a bisabolane sesquiterpene, produced in *Nicotiana tabacum* by transient expression of a zingiberene synthase are reported in **Chapter 5**.

3. Polygodial and the drimane biosynthetic pathway

"Polygodial, a compound in Polygonum hydropiper (water smartweed) is among the most potent deterrents to insect feeding known." - http://www.asknature.org

Polygodial, a sesquiterpene dialdehyde member of the drimane family, possesses several biological activities, described in detail in **Chapter 2**. This compound attracted interest as an insect antifeedant already in the 1980s and 1990s (Kubo and Ganjian, 1981; Caprioli et al., 1987; Asakawa et al., 1988; Powell et al., 1995; Gols et al., 1996; Messchendorp et al., 1998). It became a target of research again in recent years as the need for alternatives to synthetic pesticides grew, both as result of the Food Quality Protection Act (1996) by the US-EPA and of the growth of the organic agriculture sector (ca. 5% between 2006 and 2007 (Yussefi, 2008)).

Polygodial is produced by a handful of plant genera (Castelli et al., 2005; Munoz-Concha et al., 2007), including some ferns and liverworts (Asakawa et al., 2001), as well as by some marine organisms (Avila et al., 1991; Paul et al., 1997) (see also **Chapter 2**). In temperate climates, the most important source of polygodial is *Persicaria hydropiper* (water pepper) and the accumulation patterns in its tissues and two of its congeners are described in **Chapter 3**. This metabolite has the peculiarity of being perceived as hot-tasting by humans (Kubo and Ganjian, 1981). This property is conveyed by the dialdehyde moiety (Caprioli et al., 1987) and exerted through the activation of the TRPA1 ion channel (Escalera et al., 2008) that is also activated by natural compounds like ally lisothiocyanate from garlic and horseradish, which can be used as fumigant against insect pest on stored grains. The activation of this ion channel by drimane compounds leads to effects on neurotransmission (Escalera et al., 2008), which mammals experience as a pungent taste (Kubo and Ganjian, 1981; Jansen and de Groot, 2004) and dipteran insects as heat (Rosenzweig et al., 2005; Al-Anzi et al., 2006; Wang et al., 2009). We reported for the first time that polygodial may be a suitable natural compound to use in fighting whitefly infestation (**Chapter 2**) due to the antifeedant activity recorded in dual-choice assays.

The interest in the insect antifeedant properties of polygodial, prompted us to try to elucidate the biosynthetic pathway that leads to its accumulation in water pepper (**Chapter 4**), in the hope to be able to achieve heterologous production of this compound in crops under high whitefly pressure, such as tomato (*Solanum lycopersicum*). A tentative pathway for the biosynthesis of polygodial was proposed by Pickett (Pickett, 1985); in the suggested pathway the sesquiterpene alcohol drimenol is a precursor for polygodial. Drimenol was suggested to be synthesized directly from farnesyl diphosphate (FPP) by a sesquiterpene synthase. The conversion to polygodial was considered to likely occur through one or more oxidases belonging to the cytochrome P450 family, but neither the drimenol synthase nor the oxidases had been identified before. We succeeded in identifying a drimenol synthase (*PhDS*) and drimenol oxidase (*PhDOX1*) in a young flower EST library of *P. hydropiper*. To isolate these genes, the transcriptome of *P. hydropiper* was sequenced and compared with that of the closely related *P. maculosa*, which does not contain polygodial. *PhDS* was identified by characterizing the most abundant sesquiterpene synthase found in the EST library of *P. hydropiper*. Of the several CytP450 sequences with homology to other terpene hydroxylases, the one with the highest number of sequence reads was cloned and expressed in yeast. When co-expressed – in yeast or *N. benthamiana* - with *PhDS*, the enzyme was able to convert the drimenol synthesized by PhDS into further oxidised sesquiterpenoids (**Fig. 4.3** and **4.4.**). Of the array of compounds that was produced in *N. benthamiana* and yeast, only two could be identified with certainty – the sesquiterpene lactone cinnamolide and the sesquiterpenediol, drimendiol. In contrast to our prediction, no polygodial was produced (**Chapter 4**). It is known that polygodial is phytotoxic (Pickett, 1985; Asakawa et al., 1988) and we have shown this effect on tomato leaves as well (**Chapter 2**). This property of polygodial might be the reason we do not detect it in our experiments. In fact, heterologous plant hosts might have a strategy for sequestering it by creating conjugates to limit toxicity. Polygodial is also a very reactive compound in the presence of proteins. Previous studies reported that polygodial forms a pyrrole derivative with compounds possessing a primary amine group (Caprioli et al., 1987; Kubo et al., 2005). Polygodial, if formed by the combined activity of PhDS and PhDOX1, may therefore immediately react inside the cells with any compound possessing an amine group, rendering it undetectable with our analytical techniques. This phenomenon makes a heterologous expression system like yeast or tobacco a suboptimal platform for the production of polygodial. In *P. hydropiper*, polygodial accumulates in cavities devoid of other cell content (Hagendoorn et al., 1994; Derita et al., 2008), which suggests that the storage and production of the dialdehyde *in planta* may require specialised secretory structures (cavities or valvate glands and the cells surrounding them, respectively, see **Fig. 3.1**) to prevent the aldehyde groups to react with amino-groups of proteins or to be converted to the lactone form. It would be interesting to study the gene expression of *PhDS* and *PhDOX1* in the cells surrounding the cavities in *P. hydropiper* by dissecting them from the adjacent tissue and performing RT-qPCR analysis; a similar *in situ* experiment could be performed to assess the accumulation of drimanes in these cells and/or the cavity, through LASER ablation electrospray ionization (LAESI)(Shrestha et al., 2011).

Other drimane compounds, such as warburganal and muzigadial (Kubo et al., 1977; Fukuyama et al., 1982), as well as some sesquiterpene lactones (Rodriguez et al., 1976), have been shown to exhibit antifeedant properties. We therefore decided to test the effect on whiteflies and aphids of cinnamolide, which we obtained from the combined expression of *PhDS* and *PhDOX1*. Cinnamolide was tested in the same fashion as polygodial. In dual-choice assays it showed a clear insect antifeedant activity, though milder than polygodial: the ED_{50} (effective deterrent dose for 50% feeding deterrence) values of cinnamolide against whiteflies and aphids were 195 and 423 µg gFW^{-1} , respectively, while in the case of polygodial the ED_{50} values were 25 and 54 µg gFW^{-1} (**Chapter 2** and **Chapter 4**).

An attractive possibility to assess the ecological significance of polygodial arises now that the two genes of this drimane pathway have been characterized. If a suitable transformation protocol is developed, transgenic *P. hydropiper* lines with reduced drimane production could be created and evaluated for the ecological consequences. Testing the effects on larger herbivores (mammals or birds) and on chewing insects (for instance lepidopterans), besides testing the piercing/sucking insects, could give a more complete picture of the natural role of these compounds.

4. Chemical ecology of the *Persicaria* genus

"Several species can be eaten cooked, for example during famines." - http://en.wikipedia.org/wiki/Polygonum

The *Persicaria* genus, sometimes referred to as *Polygonum* (for the taxonomic debate as to the proper classification of the many species, see the work of Ekman & Knutsson and of Sanchez & co-workers (Ekman and Knutsson, 1994; Sanchez et al., 2011)), comprises 68 species, commonly called smartweeds. A number of these cosmopolitan species produce drimane sesquiterpenoids that display various biological activities, one of which being a disruptive effect on the feeding pattern of some insects. Among these species are *P. hydropiper* and *P. minor* (**Chapter 3**). As for *P. maculosa*, previous studies determining the presence of polygodial and other drimanes in this species were somewhat inconsistent. In fact, *P. maculosa* was found not to produce any polygodial in a study of Dutch specimens by Hagendoorn *et al*. (1994), while a study on plants grown in Argentina showed that small amounts of the compound were present in that species (Derita et al., 2008). The methods used in those two studies might be the source of these differences: the Argentinian group used GC-MS on dry leaf material, while the Dutch investigators used microscopy – demonstrating the absence of cavities – and Fourier transform infra-red microspectroscopy (FTIR) on the tepals of *P. maculosa*. In our study, we did not, in contrast to Derita *et al.*, observe in *P. maculosa* any valvate glands (cavities); they were on the other hand abundant in the tepals of *P. hydropiper* (**Fig. 3.1**). However, just as Derita *et al.*, we did also detect trace amounts of polygodial, but not of other drimanes, in *P. maculosa*.

Comparing the different levels of polygodial between leaves and flowers of the three species I studied, this metabolite was 10x more abundant in flowers than in leaves of *P. hydropiper*, equally abundant in *P. minor* leaves and flowers and 10x less abundant in flowers than in leaves of *P. maculosa.* The high abundance in *P. hydropiper* flowers of defence-related compounds is in line with the Optimal Defence Theory (ODT), according to which most plants will accumulate a higher proportion of defence compounds in their reproductive organs and valuable young tissues (Zangerl and Bazzaz, 1992; Van Dam et al., 1996; Kaplan et al., 2008). That does not hold true for the other two species, which accumulate just as much or less defence-related compounds in their flowers compared with the leaves. This fact suggests that different species invest either a lot or not at all in terpenoid-based defence strategies. The benefits of investing in a specific defence strategy must outweigh the costs. If the outcomes of chemical defence are moderate, the selection pressure might favour plants that have evolved other mechanisms to limit herbivory (thorns, trichomes, thick cuticles...).

As plants are known to emit volatile compounds to discourage some herbivores, attract natural enemies or warn conspecifics of a potential threat (Baldwin et al., 2006), I also analysed the headspace of *P. hydropiper* inflorescences and leaves. No drimanes were detected in this analysis, possibly due to their low volatility, but the headspace of both flowers and leaves was dominated by a combination of (*E*)-β-farnesene and β-caryophyllene. These compounds are known to play a role in plant-insect communication: (*E*)-β-farnesene – the aphid alarm pheromone – was shown to repel aphids and attract parasitic wasps when emitted by a wild potato species and in engineered Arabidopsis (Gibson and Pickett, 1983; Beale et al., 2006). However, (*E*)-β-farnesene may be masked by other plant volatiles, such as β-caryophyllene – known to act as an alarm pheromone antagonist, which is also dominant in *P. hydropiper* (Dawson et al., 1984; Mostafavi et al., 1996; Bruce et al., 2005). Another role for these compounds is pollination. (*E*)-β-farnesene, β-caryophyllene and limonene are amongst the many terpenoids produced by plants that attract pollinators (Beale et al., 2006). For example, the flower scent of caraway, *Carum carvi* (Apiaceae), is dominated by these three terpenoids and this plant is mostly visited by flies (Borg-Karlson et al., 1993). Although *P. hydropiper* has predominantly cleistogamous flowers, and, therefore, relies mainly on self-pollination, we cannot exclude that some insects use the volatile cues of the flowers for the localization of a potential source of nectar or pollen.

The production of polygodial – its biosynthesis as well as the need to store it in specialized organs to avoid damage to the plant itself – is a costly process. Compared with *P. hydropiper*, which, as the name implies, thrives in damp environments, *P. maculosa* grows in drier habitats, and this necessitates a thicker cuticle on its leaves and flowers to prevent them from dehydration; this species may hence rely, for example, on such a physical feature to discourage foraging by various herbivores, rather than on toxic secondary metabolites. *P. minor* might have evolved yet another defence strategy. Furthermore, despite the very high abundance of polygodial in our *P. hydropiper* specimens (**Tables 3.1** and **3.2**), we nevertheless observed colonies of the aphid *Aulacorthum circumflexum* (Buckton) on the spikes of many plants grown in the greenhouse (**Fig. 6.1**). Possibly, *A. circumflexum* is insensitive to polygodial, or alternatively, polygodial stored inside cavities may not get into contact with the aphids' sensilla. Likely, the phloem feeding habit of aphids also avoids any contact through their diet. Moreover, when observed in their natural environment, plants appeared to host some small lepidopteran larvae, as well as a few dipteran and hemipteran species. These insects were present on distinct preferred plant parts, which could imply that they are either insensitive to the concentrations of polygodial in those tissues or that their feeding habits avoid contact with the polygodial-containing cavities. Alternatively, these insect species may rely on a detoxification mechanism allowing them to specialize in foraging on *P. hydropiper*.

Figure 6.1. The aphid *Aulacorthum circumflexum* (apterous adult on the left, nymphs on the right) on the inflorescence of *P. hydropiper*

Because of the polygodial-mediated activation of the TRPA1 ion channel, present in mammals as well as insects, *P. hydropiper* may also benefit from the presence of polygodial by discouraging larger herbivores (vertebrates like mice or birds) from feeding, especially on the fruits. Also, the tepals containing polygodial still surround the fruits after they are shed; the antifeedant and antimicrobial properties of the chemical could then be exerted against various soil organisms, including microorganisms against which polygodial has been proven to be active under laboratory conditions (Jansen and de Groot, 2004).

5. α-zingiberene and 7-*epi*-zingiberene

"He's of the colour of the nutmeg. And of the heat of the ginger... he is pure air and fire." - William Shakespeare

First isolated in 1900, (–)-α-zingiberene is the major sesquiterpene in ginger oil (Parthasarathy, 2008). It is a volatile hydrocarbon, member of the bisabolane family. Because of its volatility and the proven toxic effects on *Bemisia argentifolii* (= biotype B of *B. tabaci*), in which it effectively reduced settling and oviposition (Vijverberg et al., 1982; Zhang et al., 2004), we considered it interesting as a first line of defence against whiteflies in our attempt to employ an integrated strategy to battle this growing pest problem. A crop producing both zingiberene and polygodial, would then first discourage whitefly landing due to the olfactory cue (zingiberene) and, if landing occurred, would be unpalatable to the insect due to the presence of the antifeedant (polygodial).

A recent study by Bleeker and co-workers shows that the repellent effect of zingiberene (and of the related compound curcumene) depends on the stereochemistry at C-7, and that although both isomers are detected by the whitefly's antennae, only 7-*epi*-zingiberene is effective at repelling whiteflies from a distance triggering an innate response in the insects (Bleeker et al., 2011). 7-*epi*-zingiberne is produced from (*Z,Z*)-FPP, and it has been demonstrated to help confer resistance to whitefly (Bleeker et al., 2009) and to the Colorado potato beetle *Leptinotarsa decemlineata* Say (Stenersen, 2004). This compound occurs in glandular trichomes of a wild tomato species, *Solanum habrochaites* accession LA1777 (Stenersen, 2004). Through an interspecific cross between this wild species, bearing the 7-*epi*-zingiberene synthase gene, and cultivated tomato *Solanum lycopersicon*, resistance against whitefly was transferred to the latter (Bleeker et al., 2012).

We found that *Callitropsis nootkatensis* (D.Don) 'Pendula' produces curcumene, a bisabolane sesquiterpene closely linked to zingiberene (**Fig. 5.2**). In fact, this molecule is an oxidation product of zingiberene and has been suggested to be produced in plants through non-enzymatic conversion (Cornell and Jordan, 1971). As we sought for potential sesquiterpene synthases expressed in *C. nootkatensis*, we found among the ESTs one that effectively converted FPP into zingiberene when tested *in vitro*. We named it *CnZIS*. We only used (*E,E*)-FPP as substrate for the enzyme, so we assume we are dealing with α-zingiberene. However, the type of column used in our GC-MS analysis does not discriminate between the two stereoisomers of zingiberene and therefore we cannot confirm if it is indeed α-zingiberene.

When *CnZIS* was expressed in *Nicotiana benthamiana*, we observed zingiberene as well as curcumene being produced. Stably transformed plants even produced more curcumene than zingiberene. This was not the case in *N. tabacum*, in which no curcumene was detected. In this species, the amount of zingiberene was also notably lower (about 40-fold). When *N. benthamiana* was used to assess the effect of zingiberene on whitefly, an unexpected phenomenon was observed: both on whole plants and on leaf disks, most insects died within hours. However, the same happened when control plants (transformed with an empty vector) were used. In **Chapter 5** we discuss in more detail the possible causes of this. Because *N. benthamiana* hence appeared to be an unsuitable host for *Bemisia*, we proceeded with transient expression in *N. tabacum*, which is a suitable host for whitefly. The setup worked as desired; however, no difference in the feeding preference of whitefly was observed, except for one time point (**Fig. 5.9**), where a mild repellence was observed (AI% = 22%, p = 0.025). Therefore, we transiently co-expressed *CnZIS* with *tHMGR* and *FPS*. The latter two code for key enzymes upstream in the biosynthetic pathway, and were previously shown to help boost the production of sesquiterpenes (van Herpen et al., 2010) by increasing the pool of the precursor(s). In our experiments, we observed once again an unexpected outcome of this co-expression. In both *N. tabacum* and *N. benthamiana*, the level of zingiberene emitted was reduced compared with plants expressing *CnZIS* alone. However, the emission of 5-*epi*-aristolochene, a sesquiterpene endogenous to these *Nicotiana* spp., increased dramatically (**Fig. 5.6C** and **5.8B**). When tobacco plants – producing enhanced levels of 5-*epi*-aristolochene – were used in choice assays with whiteflies, they strongly repelled the insects on most time points (**Fig. 5.9**).

In short, we cannot conclude that zingiberene emitted at the level we observed in agroinfiltrated *N. tabacum* is effective at repelling whiteflies. It is likely that the effect we do observe in plants transiently co-expressing *CnZIS*, *tHMGR* and *FPS* is attributable at least partially to 5EA. Purifying or synthesising 5EA and applying it onto leaves of plants that do not emit 5EA, such as tomato, would be a way to prove this hypothesis. Another would be to overexpress or knock down the 5EA synthase in *N. tabacum* as it has been done by Cankar and colleagues in an attempt to reduce the competition for the precursors when overexpressing a transgenic sesquiterpene synthase (Cankar et al., 2014).

6. Genetic engineering: promises and limitations

"Every new step in the direction of simplification – toward monoculture, say, or genetically identical plants – leads to unimaginable new complexities." – Michael Pollan

In the attempt to produce plants that emit enough zingiberene to have a measurable effect, when overexpressing *CnZIS*, we investigated the effect of subcellular targeting by using constructs that would target CnZIS to the mitochondria (Hurt et al., 1985) or retain it in the cytosol. This approach of alternative targeting was proven successful before (Kappers et al., 2005; Wu et al., 2006). In *N. benthamiana* stably transformed with these two constructs, we observed that mitochondrial targeting resulted in a much higher emission of zingiberene compared with plants retaining CnZIS in the cytosol (**Fig. 5.7**). However, there was a very high variation between different primary transformants and some had virtually no emission of zingiberene. Gene copy number and position of the incorporation of the transgene in the host genome are likely causes for this phenomenon. In the transiently expressing *N. tabacum* and *N. benthamiana*, we subsequently only targeted the enzyme to the mitochondria. The levels of zingiberene emitted were much lower in *N. tabacum* than in *N. benthamiana*, showing that results cannot always be extrapolated from one species to another. It also points at potential pitfalls of a technique such as transient, *Agrobacterium tumefaciens*-mediated, expression. The unpredictability was not limited to the outcome of the same experiment in different species and at different times: what was equally unexpected was that introducing genes upstream in the biosynthetic pathway would boost the emission of an endogenous terpene (5EA) rather than that of the product of the transienstly expressed heterologous sesquiterpene synthase.

For my thesis work, I have also attempted to create transgenic plants with tissue-specific expression of terpene synthases, with the objective to optimize the production and minimize potential side effects of constitutive expression. These side effects can be: phytotoxicity (in the case of polygodial for instance, see **Fig. 2.5**); unwanted expression in edible tissues of the crop of interest; and a potential growth retardation and yield decrease due to resource reallocation for the purpose of making the secondary metabolite(s) (Herms and Mattson, 1992; Kooke and Keurentjes, 2012). My attempts, by using reporter genes and terpene synthases other than those used in the rest of the study, showed remarkable difficulty at obtaining reproducible and consistent results.

In my work, I also tried to implement a self-splicing polyprotein (de Felipe et al., 2006) possibly containing three or four genes of a biosynthetic pathway, which would have avoided the need for multiple selection media in case of transformants co-expressing more than one transgene at the time. This operon-like construct would have also solved issues like silencing due to the repetition of the same promoter sequence many times. Earlier, I tried to overcome the same and other technical obstacles by adopting recombination-based cloning technology (Gateway®) (Karimi et al., 2002; Busso et al., 2005). Those attempts also proved vain, and their outcomes do not feature in this thesis.

These hurdles clearly show that even 30 years after the introduction of genetic engineering for plant improvement (Fraley et al., 1986), the practice of producing genetically modified plants that display the desired phenotype and that have minimal side-effects is still not straightforward. The somewhat outdated review by Hilder and Boulter sketches the potentials and the drawbacks of genetic engineering for crop protection against insects (Hilder and Boulter, 1999).

Genetic engineering is just one in a series of responses to the unforeseen consequences of the ever-increasing intensification of agriculture, which has its roots in the Green Revolution. After the introduction, in the mid-1940s, of synthetic pesticides and herbicides, in combination with improved varieties and the use of fertilisers and improved irrigation techniques, crop losses dramatically decreased and yields increased allowing world food production to double between 1965 and 2000 (Tilman, 1999). However, the concomitant increase in the use of fertilisers, pesticides and herbicides had serious consequences for the ecosystem as well as human health. According to estimates, close to 70 million birds are killed annually in the USA as a direct effect of pesticide use, and 10-20 thousands of cases of pesticide poisoning occur in agricultural workers each year in the USA; pesticide toxicity issues are likely much worse in developing countries. Studies have shown that the use of genetically modified (GM) crops allows for a reduction of sprayed pesticides or herbicides that ranges between 10 and 60% in countries where herbicide tolerant or insect resistant varieties are being cultivated. It has been estimated that introducing GM crops in the EU and growing 50% of maize, oils seed rape, sugar beet and cotton varieties with herbicide-tolerance or insect-protection traits, would reduce the amount of annually applied chemicals by 14.5 million kg, and the amount of tractor fuel from less spraying by 20.5 million litres of diesel (Phipps and Park, 2002). Furthermore, there are reports that claim that GM crops can be beneficial for preserving the natural biodiversity through e.g. no-tillage practices (improved soil ecology) and reduced pesticide sprays (survival of non-target organisms) (Ammann, 2005).

This last point also has another side to it, in the sense that the biodiversity of the cultivated crops is – at least at the present time – limited to no more than a couple of varieties per species. Moreover, GM crops have been a widely debated topic in recent decades due to concerns about their potential negative effects on human health, on the environment as well as their impact on the socio-economic conditions of farmers, especially in developing economies. According to a recent review, potential health hazards of GM food for animals and populations exposed to a diet containing GM products are claimed to include: possible pleiotropic and insertional effects (production in GM plants of unforeseen compounds with unknown effect on health); effects on animal and human health resulting from the increase of anti-nutrients (such as phytoestrogens, glucinins, and phytic acid); possible effects of GM foods on allergic responses, especially in immunocompromised individuals (Dona and Arvanitoyannis, 2008). The primary potential environmental threats are: invasion of GM crops into natural habitats, hybridization of GM crops with wild relatives, horizontal gene transfer, development of resistance in target organisms and detrimental effects on non-target organisms (Houshyani, 2012). Moreover, it has been documented that the use of, for instance, *Bt* cotton, can also cause a rise in secondary pests, previously controlled by the use of broad-spectrum insecticides, which points to unforeseen ecological changes (Azadi and Ho, 2010). The potential health concerns of GM foods might be negligible and the proposed environmental threats unproven as; however, the impact that genetic engineering has on the socio-economic and political dynamics is nevertheless a complex problem that is only marginally studied (Qaim and Traxler, 2005). On the one hand, there are reports of improved social and economic conditions, for both large- and small-scale farmers and their families. A study of South African cotton farmers showed an overall improvement in terms of higher yields, lower pesticide use, less labour for pesticide application and substantially higher gross margins per hectare, as well as less poisoning due to pesticide spraying, when comparing GM and regular cotton cropping (Bennett et al., 2006). Likewise, increased family incomes in Indian and Pakistani cotton farmers who had adopted insect resistant GM varieties are an important component in reducing rural poverty and in the concept of food security (Ali and Abdulai, 2010; Qaim and Kouser, 2013). On the other hand, agribusiness domination of the global seed market and aggressive campaigns to promote broad patenting rights for biotech seeds and plants, whether transgenic or not, challenge traditional farming practices such as seed saving and seed sharing (Stein, 2004). What is more, the agricultural biodiversity and the wide range of genetic resources for food and agriculture, are also under threat from their privatisation. Plant variety protection through patents imposes a system that weakens informal sector knowledge (that of farmers), facilitating concentration of knowledge and power in the hands of few (Mulvany, 2005). In the context of sustainable development, defined as ‘development that meets the needs of the present without compromising the ability of future generations to meet their own needs’, GMOs could either promote such development or jeopardise it. To assure the first one happens, carefully derived and fully implemented biosafety principles are needed (Karlsson, 2003). There are currently three principles used to assess whether the commercialization of GMOs will foster sustainable development: the precautionary principle, the polluter pays principle and the principle of public participation. They are described in great detail by Karlsson (2003), but in conclusion, it is safe to state that each GMO is different, and has a different “mode of action” and careful evaluation will be necessary on individual basis. Therefore, it is neither possible to draw general conclusions on potential adverse effects nor to infer them based on what is known about other pre-existing varieties.

As it appears, both at the practical, laboratory level, and at the implementation level, genetic engineering presents numerous hurdles to be overcome. Based on the precautionary principle, which advocates the risk assessment to consider alternative ways of fulfilling the underlying and social purposes of introducing a specific GMO (Karlsson, 2003), I here want to propose one potential alternative approach.

7. A new paradigm in agricultural practices: from intensive agriculture to mid-scale sustainable agriculture – an approach in concert with nature

“For great many species today, “fitness” means the ability to get along in a world in which humankind has become the most powerful evolutionary force.” – Michael Pollan

Since the onset of the Green Revolution in the middle of the 20th century, agricultural practices have witnessed an ever-increasing intensification, in the attempt to meet the feeding needs accompanying the demographic explosion of our times. The desired outcomes of these efforts and developments have been reducing the price of food and allegedly saving one billion from starvation. These initiatives included the development of high-yielding varieties of cereal grains, expansion of irrigation infrastructure, modernization of management techniques, distribution of hybrid seeds, synthetic fertilizers and pesticides to farmers (Glaeser, 2010). As described above, however, the unforeseen drawbacks of the use of pesticides and herbicides, in combination with an ever-increasing demand for food and feed, has led scientist to pursue solutions in the form of genetically enhanced varieties that can withstand pest pressure better, be tolerant to more environmentally friendly herbicides (glyphosate) or have higher yields, even under adverse conditions such as drought (Karlsson, 2003; Ammann, 2005; Lemaux, 2009). There are models that describe the potential of the use of GM crops in combination with other, non-transgenic species, which could function as refuges for the deterred insects by allowing the fixation of choice/preference traits, thereby limiting the development of resistance in the insects as well as increasing biodiversity (Jongsma et al., 2010). Nevertheless, GM crops still require a very intensive land use and do not tackle other pitfalls of the so-called industrial agriculture; rather they are an extension of it.

The use of mineral fertilizers, monocropping and the application of modern technology for cultivation of the land, have a variety of effects on the environment, which are not limited to agroecosystems, but also affect the landscape and its structural and species diversity. Fertilizers, primarily nitrate and phosphate, pollute waters and soil, causing eutrophication of rivers, lakes and coastal oceans (Bennett et al., 2001). Pesticides, aside from their direct effects discussed above, can enter the food chain through the underground waters (Martin and Sauerborn, 2013). The use of machinery in agriculture necessitates large and, preferably, homogeneous fields. This homogenization, or the use of monocultures, can cause increased pest pressure and selection, as well as soil depletion (Altieri, 1999). Another aspect of annual cropping systems is the nature and frequency of soil disturbance regimes. Periodic tillage and planting continually, results in soil biodiversity reduction, which is negative because, for the recycling of nutrients and proper balance of the organic matter, soil organisms diversity and plant diversity are necessary components of a productive and ecologically balanced soil environment (Altieri, 1999). As a consequence of mechanization, habitats such as hedgerows, ridges, and wetlands are increasingly lost from the landscape, and heavy machinery also leads to compaction of the soil (Martin and Sauerborn, 2013).

Sustainable agriculture is a generic concept that is founded on the principle that also agricultural production must meet our current needs without jeopardising the ability of future generations to meet theirs. The main objectives of sustainable agriculture, rooted in the same precepts of sustainable development in general, are: environmental soundness, economic profitability, and social equity. These objectives can be met by either: a) organic farming, which attempts at creating nutrient cycles within the farm that are as closed as possible, thereby entirely avoiding the application of synthetic pesticides and fertilizers; or b) integrated agriculture – a method that uses the techniques of conventional agriculture, but tries to minimize its negative effects by combining biological, technical, and chemical measures that enable production of a high-quality harvest while conserving natural resources (Martin and Sauerborn, 2013).

The science that studies these alternative and more sustainable approaches to agricultural practices is called agroecology. Based on the latest literature in that field, an optimistic view arises as to the potential of implementing agricultural strategies in concert with nature.

Of major importance is, for instance, the conservation and promotion of soil fertility. This is achieved by means of site-adapted crop rotation in which the use of legumes for fixation of nitrogen plays a crucial role, as does the utilization of organic fertilizers (compost, manure) (Martin and Sauerborn, 2013). In the last decade, there has been an increasing focus on the so-called ‘unseen majority’, that is the microbial community living in the rhizosphere and interacting with the plant to promote plant performance. The roots of a plant are comparable to the human gut, inasmuch that they are both responsible for the absorption of nutrients. The parallel between plant roots and the mammalian intestine holds true also when it comes to the close interaction that the two organs have with the microbes populating them, and the effect on health that derives from that interaction (Berendsen et al., 2012). It is also known that plant roots release a broad variety of chemical compounds to attract and select microorganisms in the rhizosphere. In turn, these plant-associated microorganisms, via different mechanisms, influence plant health and growth (Huang et al., 2014). In their role as regulators of soil ecosystem processes, soil organisms perform a number of vital functions: decomposing litter and cycling nutrients; converting atmospheric nitrogen into organic forms, and reconverting organic nitrogen to gaseous nitrogen; suppressing soil-borne pathogens through antagonism; synthesizing enzymes, vitamins, hormones, vital chelators and allelochemicals that regulate populations and processes; altering soil structure; directly interacting with plants through mutualism, commensalisms, competition, and pathogenesis (Altieri, 1999). So, by taking care of the soil, through ‘feeding’ it the most complete and natural type of ‘food’, i.e. compost and manure, and through the avoidance of methods that physically alter the top layer of the soil, the delicate balance of the rhizosphere micro-flora is preserved, as are the other vital players of the belowground ecosystem. This not only aids in reducing the use of artificial fertilizers and its side effects, but also promotes better defence of plants against insects and pathogens by enhancing the natural immunity (Van Oosten et al., 2008; Berendsen et al., 2012). Both phytophagous insects and their carnivorous enemies of the third trophic level are affected by the interaction between the host plant and the rhizosphere microbiota (Pineda et al., 2013). In some cases, the effect on the herbivores is even opposite to what would be hypothesised based on the knowledge of some of the underlying signalling mechanisms (Pineda et al., 2012). It is interesting to note, in this context, that both aphid (Lee et al., 2012) and whitefly (Yang et al., 2011) infestation of bell pepper produced changes in the soil micro-flora, thereby priming plant immunity against bacterial pathogens. All these facts, taken together, point at how little we understand about the vastness of the ecological interactions between plants and the below- and above-ground communities. Equally vast is, therefore, the potential that the understanding of the mechanisms that facilitate such interactions can have on the development and optimization of agricultural practices that minimize human input and take advantage of the available ecological resources. In parallel to the acquisition of new knowledge, participatory extension programs are necessary, to educate the farmers and integrate the knowledge within their specific biogeographic and social setting.

Effective soil management is crucial, as we have seen, in the process of developing sustainable agricultural approaches. Equally important is, however, the creation or the maintenance of wide biological and genetic diversity within the cultivated fields. Agroecosystems, in contrast with natural ecosystems, are often stripped of the vast majority of the organisms that used to populate that land. It is estimated that no more than 70 different crop species are currently cultivated in modern agricultures, with just a handful of varieties for each major crop. The economic and environmental costs of such biological simplification can be quite significant. Economically, there is a need to supply crops with costly external inputs, because agroecosystems lack the capacity to sponsor their own soil fertility and pest regulation. In addition, the need of constant human intervention and the contamination of soil, water and food, create economic problems as well as health and environmental ones. From a pest pressure perspective, the expansion of crop monocultures and therefore the decreasing local habitat diversity, result in heavy pest damage that is directly proportional to the intensity with which such communities are modified. Therefore, enhancing functional biodiversity in agroecosystems is a key ecological strategy to bring sustainability to production and to reduce the vulnerability associated with genetic uniformity. In contrast to the modern Western systems, biodiversity preservation is very common to traditional farmers in the Third World. Polyculture (intercropping), agroforestry and shifting cultivation are just a few commonly adopted ecological models to food, feed, firewood and medicine production in developing Countries. It is now recognized that these systems can be models of efficiency inasmuch they incorporate careful management of soil, water, nutrients and biological resources. Thus, a main strategy in agroecology is to exploit the complementarities of crops, trees and animals in spatial and temporal arrangements such as polycultures, agroforestry systems and crop-livestock mixtures (Altieri, 1999).

To conclude, the newly coined concept of “sustainable intensification” deserves attention, as it proposes itself as strategy that goes beyond just being a bridge between industrial agriculture (which goal is to assure food security) and the traditional or organic farming practices (which promise an environmentally friendly and socially accepted approach). As Garnett and colleagues state in their manifesto (Garnett et al., 2013), "*both sustainability and food security have multiple social and ethical, as well as environmental, dimensions. Achieving a sustainable, health-enhancing food system for all will require more than just changes in agricultural production, essential though these are. Equally radical agendas will need to be pursued to reduce resource-intensive consumption and waste and to improve governance, efficiency, and resilience*". What this means, in the optic of food security for a growing world population, is that the response at the "demand-side" is just as important as improvements on the "supply-side".

8. Concluding remarks

"You have to start with the truth. The truth is the only way that we can get anywhere. Because any decision-making that is based upon lies or ignorance can't lead to a good conclusion." - Julian Assange

Above I discussed the pros and cons of genetic modification as well as the features necessary for a sustainable agricultural system to exist. I would like to conclude with how these two aspects relate to the work described in the previous chapters. Starting from the point that the use of synthetic pesticides is viewed more and more critically by the major environmental agencies, the consumers and the farmers, we sought to investigate alternative strategies to crop protection that would leave a smaller footprint on the environment, pose less health hazards and reduce the necessary external inputs. Our original aim was to produce a transgenic tomato line that would repel whiteflies through two lines of defence. Firstly, the volatile repellent zingiberene would alert the insects that the plant is unpalatable; subsequently, the pungent antifeedant polygodial would discourage those insects that still landed from feeding and ovipositing. Although this goal has not been achieved, through the identification of two drimane compounds, polygodial and cinnamolide, as powerful antifeedants for whiteflies and aphids, we added a piece to the treasure chest from which integrated pest management can draw. Metabolic engineering of either the drimanes or the volatile zingiberene to confer insect resistance to crops, however, did not prove to be the most straightforward strategy. Through the expression of two genes of the drimane biosynthetic pathway in a model plant and in yeast, no polygodial was produced. What is even more striking is that those genes, when heterologously expressed, produced metabolites that were undetectable in the plants from which the genes were isolated. This phenomenon points at an intrinsic difficulty in predicting the outcome of gene expression in a heterologous host. In my work, this unpredictability also held for overexpression of genes upstream in the biosynthetic pathway of zingiberene; in fact, the co-expression of three genes from that pathway in *Nicotiana spp*. resulted in a decrease in the levels of zingiberene and a massive increase in the levels of the endogenous 5-*epi*-aristolochene, compared with plants expressing only the zingiberene synthase. Interestingly, it was precisely the plants with a high 5EA profile that strongly repelled whiteflies in dual-choice assays. Considering the described uncertainties and practical difficulties in obtaining transgenic plants with the desired metabolic phenotype, and seeing the many conflictual positions on the potentials of GMOs in facing the challenges of modern intensive agricultural practices, I would like to propose, instead, to opt for an ecological and integrated approach to reduce the crop losses due to whiteflies. To that end, I suggest looking into: a) features that could perhaps be achieved through modern breeding, such as the physical-chemical barriers posed by trichomes (in **Chapter 5**, we describe how on *N. benthamiana* whiteflies swiftly die, probably due to the contents of the trichomes, which might differ from those of the congener *N. tabacum*); b) the potential of using microbial or plant-based platforms for the production of the desired natural repellent, which could then be used as sprayables within and IPM framework (polygodial could potentially be produced in a suitable microbial host using PhDS and PhDOX1; c) the vast genetic pool of wild varieties, which are often more resilient though less yielding, and that could be used in combination with intragenic vectors to introduce cisgenes, thereby limiting the ethical opposition of introducing genes into crops from taxonomically distant species (Conner et al., 2007); d) the possibility to employ intercropping or otherwise increase biodiversity it the field/greenhouse; and e) the myriad of ecological interactions that foster plant immunity and resistance to insects, such as tritrophic interactions or interactions with soil-borne antagonists of pests.

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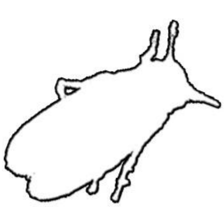

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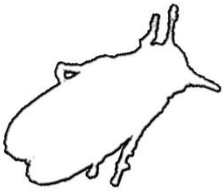
Summary



Summary

Whitefly is an insect pest that has systematically spread into colder latitudes for the past two decades and it poses a serious threat to crops, mainly due to the viruses for which it acts as a vector. As the application of synthetic pesticides is often less effective due to development of resistance or restricted by crop- and country-specific regulations, Integrated Pest Management (IPM) strategies to combat insect pests become attractive. In this thesis, I discuss the potential of the use of secondary metabolites, particularly sesquiterpenoids, of plant origin, both as sprayed repellents or antifeedants and as part of host plant resistance against whitefly.

In **Chapter 1**, I give a detailed description of the silverleaf whitefly, *Bemisia tabaci*, its ecology and its effect on crop yield, taking both direct and indirect damage (caused by the viruses the insect transmits) into account. This chapter also provides a detailed account of pest management strategies, both traditional and emergent, with their advantages and disadvantages. The chapter introduces the reader to plant secondary metabolites, and specifically terpenes, discussing their role in plant ecology and their potential as pest management tools with a low environmental impact. Finally, a short overview of the following chapters is given.

Chapter 2 focusses on the antifeedant activity of the drimane sesquiterpene polygodial. This dialdehyde had been described before as antifeedant to a number of insects, such as the Colorado potato beetle, *Leptinotarsa decemlineata*, the African cotton leafworm, *Spodoptera littoralis,* and the green peach aphid, *Myzus persicae*. In this chapter, the effect of polygodial on the feeding preference of whitefly is reported for the first time. The effect of polygodial was benchmarked against that of the more widely used natural pyrethrins, and both were also tested against *M. persicae*. From the results, we conclude that pyrethrins were effective against whiteflies at 18-fold lower concentrations than polygodial (ED_{50} of 1.4 and 25 µg gFW^{-1} respectively), while in the case of aphids this difference in efficacy was only two-fold (ED_{50} of 28 and 54 µg gFW^{-1}, respectively).

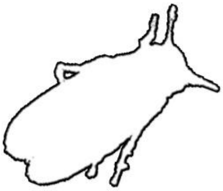
To adopt polygodial as a more persistent and easy to implement pest management strategy, we set out to isolate the genes responsible for its biosynthesis, which could then be used to transform crops. As a basis for the selection of the right species and plant tissue to achieve this objective, **Chapter 3** describes the chemical composition of one of the sources of polygodial – *Persicaria hydropiper* (water pepper), as well as of two other congeners (*Persicaria minor* and *Persicaria maculosa*). For all three species, GC-MS analysis of extracts of leaves and flowers was performed, which gave insight into the interspecific differences and similarities as well as into the differences between the two tissues. *P. hydropiper* was the species with the biggest variety and the greatest abundance of secondary metabolites, while *P. maculosa* had the fewest. The flowers of all species were richer in secondary metabolites compared with the leaves of the same species. Furthermore, the accumulation pattern of the identified compounds throughout the development of flowers and leaves is described. Finally, in this chapter, the possible ecological role of polygodial is also briefly addressed.

Chapter 4 focuses on the discovery and characterization of genes involved in the biosynthesis of drimane sesquiterpenoids. Based on the findings of Chapter 3, we used 454 sequencing of a cDNA library constructed from young flowers of *P. hydropiper* and *P. maculosa*, for comparison, to identify a drimenol synthase (*PhDS*) and a drimenol oxidase (*PhDOX1*) which can convert farnesyl diphosphate (FPP) into drimenol and an array of other sesquiterpenoids when working in concert. Of the compounds produced in the heterologous systems used (*Saccharomyces cerevisiae* and *Nicotiana benthamiana*), two were identified as drimendiol and cinnamolide. The latter was purified and tested against whiteflies and aphids as described for polygodial in Chapter 2. Cinnamolide also displayed antifeedant activity against both insects, although with at slightly lower efficacy than polygodial. In the heterologous hosts used, no polygodial was detected amongst the products of the enzymatic activity of the two genes studied in this chapter. The potential reasons for this are explored in the discussion section of this chapter.

Chapter 5 focuses on the volatile zingiberene, member of the bisabolane family of sesquiterpenes. This compound and its enantiomer, 7-*epi*-zingiberene have both been previously identified as repellent to whiteflies. In this chapter, we used Alaska yellow cedar (*Callitropsis nootkatensis*) as a possible source of a zingiberene synthase, which we wanted to use a primary line of defence against whitefly next to polygodial, which would be a secondary line of defence. Alaska yellow cedar produces curcumene in some of its tissues, and this metabolite is known to be a dehydrogenation product of zingiberene. An EST from a *C. nootkatensis* cDNA library with homology to sesquiterpene synthases was cloned and expressed in *E. coli.* The resulting protein converted FPP to zingiberene as the sole product. This enzyme was therefore named zingiberene synthase (CnZIS). Although no strict correlation was found between the expression levels of *CnZIS* in Alaska yellow cedar tissues and the accumulation of curcumene in the same tissues, those with high *CnZIS* expression such as leaves also produced high amounts of curcumene, while heartwood neither expressed the gene nor had detectable levels of curcumene. Subsequently, we tested the effect of transiently expressed *CnZIS* in tobacco (*Nicotiana tabacum*) on whiteflies. Except for one time point, the effect of zingiberene on whitefly feeding was negligible, likely due to the fact that only trace amounts of zingiberene were emitted. When genes upstream of *CnZIS* in the biosynthetic pathway – 3-hydroxy-3-methyl-glutaryl-CoA reductase (*HMGR*) and farnesyl diphosphate synthase (*FPS*) – were co-expressed in tobacco, the antifeedant effect on whiteflies became very strong; however, there was still no detectable level of zingiberene emitted. Instead, the emission of the tobacco sesquiterpene, 5-*epi*-aristolochene, was almost 100-fold higher than in the control, not expressing *HMGR* and *FPS*. We discuss potential explanations for the high repellence against whiteflies of those plants, despite the absence of zingiberene, as well as the uncommon outcome of having an endogenous sesquiterpene boosted by the co-expression and redirection to the mitochondria of the three genes of the zingiberene biosynthetic pathway.

Chapter 6 brings all the findings together, discussing their place within a wider scientific perspective and their potential in the frame of IPM. Advantages as well as drawbacks of the use of GM crops are addressed. Finally, the emerging new agricultural paradigm, of a sustainable way of growing crops with less environmental impact compared with the current intensive industrial approach, is brought forth as a broad-spectrum solution to most agricultural problems that arose in parallel to the intensification of agriculture stemming from the Green revolution.

Samenvatting



Tabakswittevlieg (*Bemisia tabaci*) is een insect dat zich in de afgelopen decennia steeds noordelijker heeft weten te verspreiden. Tabakswittevlieg vormt een serieus gevaar voor landbouwgewassen, ook in Nederland, doordat het drager is van plantenvirussen. Aangezien het gebruik van synthetische bestrijdingsmiddelen aan effectiviteit verliest door de ontwikkeling van resistenties en door de strengere regelgeving, wordt de *Integrated Pest Management* (IPM) benadering steeds aantrekkelijker voor de bestrijding van insecten. In dit proefschrift bestudeer ik de mogelijkheden voor het gebruik van plantenstoffen, in het bijzonder sequiterpenoïden, voor de bestrijding van tabakswittevlieg. Deze bestrijding kan zowel gebeuren door middel van bespuiting als door ingebouwde resistentie in de plant. Daarnaast kijk ik naar de invloed die deze stoffen hebben op het eetgedrag van tabakswittevlieg.

In **Hoofdstuk 1** beschrijf ik in detail de tabakswittevlieg, zijn ecologie, en zijn effecten op de oogst, zowel direct (door vraat) als indirect (door overdracht van virussen). Vervolgens geef ik een gedetailleerd overzicht van zowel traditionele als nieuwe insectenbestrijdingsmethoden, met hun voor- en nadelen. Daarnaast biedt dit hoofdstuk de lezer een introductie in de secundaire plantenstoffen, in het bijzonder de terpenoïden, door middel van een beschrijving van de rol die deze stoffen spelen in de plantenecologie, en hun toepassing als gewasbeschermingsmiddelen met een beperkte invloed op het milieu. Aan het einde van het hoofdstuk wordt een kort overzicht gegeven van de inhoud van de eropvolgende hoofdstukken.

Hoofdstuk 2 gaat dieper in op de *antifeedant*-eigenschappen van de drimane sesquiterpenoïde polygodial. Deze dialdehyde is eerder beschreven vanwege zijn activiteit als *antifeedant* tegen verschillende insecten, waaronder de coloradokever (*Leptinotarsa decemlineata*), de katoenuil (*Spodoptera littoralis*) en de groene perzikluis (*Myzus persicae*). In dit hoofdstuk zijn de effecten van polygodial op de eetvoorkeur van de tabakswittevlieg *Bemisia tabaci* voor het eerst beschreven. Om het effect van polygodial te kwantificeren zijn pyrethrinen, een bekend natuurlijk bestrijdingsmiddel, gebruikt als standaard. Beide stoffen zijn ook getest op hun activiteit tegen *M. persicae*. Onze experimenten hebben aangetoond dat pyrethrinen effectiever zijn tegen tabakswittevlieg dan polygodial; de benodigde concentratie pyrethrinen was 18 keer lager dan polygodial om een vergelijkbaar effect te krijgen (ED_{50} van 1.4 voor pyrethrinen vergeleken met een ED_{50} van 25 µg gFW^{-1} voor polygodial). In het geval van de groene perzikluis was de benodigde concentratie polygodial slechts tweemaal hoger (ED_{50} van 28 en 54 µg gFW^{-1}, respectievelijk).

Om polygodial als een gemakkelijk te implementeren en langdurig werkzame bestrijdingsstrategie te gebruiken zijn we ertoe overgegaan om de genen die verantwoordelijk zijn voor de synthese ervan te isoleren, zodat deze kunnen worden gebruikt om gewassen te transformeren. Om de juiste selectie te maken van de relevante plantensoorten en -weefsels waaruit de benodigde genen geïsoleerd kunnen worden, hebben we de chemische samenstelling van één van de bekende polygodial-producerende plantensoorten, waterpeper (*Persicaria hydropiper*), en twee gerelateerde soorten (*Persicaria minor* and *Persicaria maculosa*) geanalyseerd. Deze experimenten zijn beschreven in **Hoofdstuk 3**. Een GC-MS analyse van de extracten van bladeren en bloemen van alle drie de soorten werd uitgevoerd. Deze analyse heeft aangetoond dat er verschillen en overeenkomsten in chemische samenstelling zijn tussen de drie *Persicaria* soorten, en ook tussen de twee verschillende weefsels. De grootste hoeveelheid en diversiteit van secundaire metabolieten werd gevonden in *P. hydropiper*. *Persicaria maculosa* was in chemisch opzicht de armste soort, met de kleinste hoeveelheid plantenstoffen. Bloemenweefsels van alle drie de soorten hadden meer secundaire metabolieten dan de bladeren van dezelfde soort. Naast deze experimenten hebben we de chemische samenstelling van bloemen en bladeren in verschillende fasen van ontwikkeling verder geanalyseerd. Ter afsluiting bespreekt dit hoofdstuk in het kort de mogelijke ecologische rol van polygodial.

Hoofdstuk 4 richt zich op de ontdekking en de karakterisering van de verscheidene genen die betrokken zijn bij de biosynthese van drimaan-sesquiterpenoïden. Gebaseerd op de bevindingen omschreven in Hoofdstuk 3 hebben we de cDNA-bank van de jongste bloemmonsters van *P. hydropiper* en *P. maculosa* (ter vergelijking) *gesequenced* door middel van 454-*sequencing*. Hiermee wisten we een drimenol synthase (*PhDS*) en een drimenol oxidase (*PhDOX1*) te identificeren die farnesyl difosfaat (FPP) omzetten naar drimenol en vele andere sesquiterpenoïden wanneer ze gezamenlijk actief waren. Na overplaatsing en expressie van deze genen in twee heterologe systemen (*Saccharomyces cerevisiae* (bakkersgist) en *Nicotiana benthamiana*) konden twee van de stoffen die werden aangemaakt geïdentificeerd worden als drimendiol en cinnamolide. Cinnamolide is vervolgens geïsoleerd en op dezelfde wijze getest tegen tabakswittevlieg en bladluis als is omschreven voor polygodial in Hoofdstuk 2. Hieruit bleek dat ook cinnamolide een *antifeedant* effect heeft tegen beide insectensoorten, hoewel minder sterk dan polygodial. In bakkersgist en *N. benthamiana* werd geen polygodial gedetecteerd na transformatie met PhDS en/of PhDOX1. In de discussie van dit hoofdstuk worden de mogelijke reden daarvan verder geanalyseerd.

Hoofdstuk 5 richt zich hoofdzakelijk op de vluchtige bisabolaan-sesquiterpeen zingibereen. Eerder was een afstotende werking tegen tabakswittevlieg aangetoond van zingibereen en zijn enantiomeer 7-*epi*-zingibereen. Om zingibereen en diens enantiomeer *in planta* te produceren als primaire afweer tegen tabakswittevlieg, naast polygodial als secundaire afweer, is het enzym zingibereen synthase nodig. Als mogelijke bron van dit enzym hebben we de nootkacipres (*Callitropsis nootkatensis*) geselecteerd, aangezien deze soort in sommige weefsels de stof curcumeen aanmaakt, een dehydrogenatieproduct van zingibereen. In een *C. nootkatensis* cDNA-bank werd een *exspressed sequence tag* (EST) geselecteerd die homoloog was aan andere sesquiterpeensynthasen. Deze werd gekloond en tot expressie gebracht in *E. coli*. Het enzym dat op deze manier geproduceerd werd catalyseerde de omzetting van FPP in uitsluitend zingibereen. Aangezien zingibereen het enige product was, kreeg het enzym de naam zingibereen synthase (CnZIS). Er was geen sterk verband aan te wijzen tussen het expressieniveau van *CnZIS* in de verschillende weefsels van de nootkacipres en de hoeveelheid curcumeen in deze weefsels. Echter, weefsels met een hoge genexpressie, zoals bladeren, bevatten ook een hoog gehalte curcumeen, terwijl in het houtweefsel geen *CnZIS* expressie waarneembaar was en ook geen curcumeen werd gevonden. In een volgend experiment hebben we het effect van de transiënte expressie van *CnZIS* in tabak (*Nicotiana tabacum*) op tabakswittevlieg getest. Met uitzondering van één tijdvak was het effect van zingibereen op tabakswittevlieg gedurende het experiment verwaarloosbaar, waarschijnlijk ten gevolge van de lage gehaltes zingibereen die uit de plant vrijkwamen. Toen twee genen die voor *CnZIS* in the biosynthetische route actief zijn, 3-hydroxy-3-methyl-glutaryl-CoA reductase (*HMGR*) en farnesyl difosfaat synthase (*FPS*), tegelijkertijd met *CnZIS* in de tabaksplant werden geïntroduceerd, was het effect op tabakswittevlieg zeer sterk. Er werd echter geen zingibereen gevonden in de mix van vluchtige plantenstoffen die uit de tabaksplant vrijkwam. In plaats daarvan bleek dat de emissie van de tabaksstof 5-*epi*-aristolocheen (5EA) bijna 100 keer hoger was dan in het controlemonster, genomen van een plant die geen HMGR en FPS bevatte. De mogelijke verklaringen voor de uitzonderlijke uitslag van de *boosting* van de genexpressie door middel van co-expressie en doorverwijzing naar de mitochondria van de drie genen uit de biosynthetische route van zingibereen, waarna de hoeveelheid van de endogene sesquiterpeen 5EA verhonderdvoudigd bleek, worden besproken, evenals het sterke *antifeedant* effect van de *gebooste* planten ondanks de afwezigheid van zingibereen.

In **Hoofdstuk 6** zijn alle bevindingen bij elkaar gebracht en beschouw ik ze in een breder wetenschappelijke perspectief. Ook analyseer ik wat hun mogelijkheden zijn in het kader van *Integrated Pest Management* (IPM). Daarnaast worden in dit hoofdstuk de voor- en nadelen van genetisch gemanipuleerde (GG) gewassen bediscussieerd. Ten slotte presenteer ik het nieuwe opkomende landbouwparadigma, dat uitgaat van een duurzame wijze van telen, met minder gevolgen voor het milieu dan de huidige intensieve industriële teeltwijze. Deze nieuwe landbouwmethode wordt gezien als een brede oplossing voor de vele problemen die zijn ontstaan tijdens en na de Groene Revolutie en de daaruit voorkomende intensifiëring van de landbouw.

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The making of a PhD thesis is far from being an individual achievement. The front page might be listing only one person, and the research chapters a few, but in truth there is a whole team that worked hard and/or believed in the PhD student to assure they'd make it thus far.

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List of publications

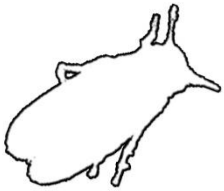
Published:

Prota N, Bouwmeester HJ, Jongsma MA (2013) Comparative antifeedant activities of polygodial and pyrethrins against whiteflies (*Bemisia tabaci*) and aphids (*Myzus persicae*). Pest Manag Sci **70:** 682-688

Prota N, Mumm R, Bouwmeester H, Jongsma M (2014) Comparison of the chemical composition of three species of smartweed (genus *Persicaria*) with a focus on drimane sesquiterpenoids. Phytochemistry **108:** 129-136

To be published:

Henquet M, Prota N, van der Hooft JJJ, Varbanova M, Hulzink RJM, de Vos M, Prins M, de Both MTJ, Bouwmeester HJ and Jongsma MA. Identification of a drimenol synthase and drimenol oxidase from Persicaria hydropiper, involved in the biosynthesis of insect deterrent drimanes (*in preparation*)



Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Neli Prota**
Date: **15 January 2015**
Group: **Laboratory of Plant Physiology**
University: **Wageningen University & Research Centre**

1) Start-up phase	*date*
• **First presentation of your project**	
Molecular breeding of insect resistance in vegetable crops	Jan 30, 2008
• **MSc courses**	
Introduction to Communication and Innovation Studies CPT-23804	2013

Subtotal Start-up Phase *5.5 credits**

2) Scientific Exposure	*date*
• **EPS PhD student days**	
1st European PhD retreat, Wageningen University	Sep 02-03, 2008
• **EPS theme symposia**	
EPS Theme 3 symposium 'Metabolism and Adaptation', Wageningen University	Nov 06, 2007
EPS Theme 3 symposium 'Metabolism and Adaptation', Utrecht University	Feb 18, 2009
EPS Theme 2 symposium 'Metabolism and Adaptation', Utrecht University	Jan 22, 2009
EPS Theme 3 symposium 'Metabolism and Adaptation', Leiden University	Feb 16, 2010
• **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
• **Seminars (series), workshops and symposia**	
Workshop Plant-Insect interactions, Amsterdam	Oct 26, 2007
LIFE SCIENCE symposium - Bio-imaging, Leiden	Mar 06, 2008
Plant systems biology symposium, Leiden	Apr 24-25, 2008
EPTA Conference (Technology Assesment), The Hague	Oct 27-28, 2008
Workshop Plant-Insect interactions, Leiden	Oct 29, 2008
Lecture Pamela Hines - editor Science magasine, Wageningen	Nov 06, 2008
Symposium Epigenetics, KNAW, Amsterdam	Sep 18, 2009
Workshop Plant-Insect interactions, Utrecht	Nov 11, 2009
VIBes symposium, Leuven, Belgium	Oct 13-15, 2010
ExPectations 2010 Career Day Event, Wageningen	Nov 19, 2010
Life science momentum (NGI) – Science for society, Rotterdam	Nov 22, 2011
Crafting your career CWTS, Utrecht	Oct 30, 2013
Lecture 'It's the food my friend' by Joel Salatin, Wageningen	May 9, 2014
All Inclusive Breeding symposium, Wageningen	Oct 16, 2014
• **International symposia and congresses**	
International conf. on Cytochrome P450, Nice, France	Jun 08-12, 2008

• **Presentations**	
Poster presentation board of directors PPH, Wageningen University	Nov 05, 2007
Oral presentation Plant insect interactions workshop Utrecht	Nov 11, 2009
Oral presentation TTI-GG meeting at Keygene Wageningen	Oct 2008
Oral presentation TTI-GG meeting at Keygene Wageningen	Mar 2009
Oral presentation TTI-GG meeting at Keygene Wageningen	Oct 2009
Oral presentation TTI-GG meeting at Keygene Wageningen	Mar 2010
Oral presentation TTI-GG meeting at Keygene Wageningen	Oct 2010
Oral presentation TTI-GG meeting at Keygene Wageningen	Mar 2011
Oral presentation TTI-GG meeting at Keygene Wageningen	Oct 2011
Oral presentation TTI-GG meeting at Keygene Wageningen	Mar 2012
• **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Dec 04, 2009
Subtotal Scientific Exposure	*19.7 credits**

3) In-Depth Studies	date
• **EPS courses or other PhD courses**	
Postgraduate course 'Natural variation in plants', Wageningen	Aug 26-29, 2008
Winter school on plant VOCs, Wageningen	Nov 11-14, 2008
Summer school 'Environmental Signalling, Utrecht	Aug 24-26, 2009
Course 'Plants and Metabolomics', Leiden	Apr 12-16, 2010
• **Journal club**	
Participant in literature discussion group at Plant Physiology	2007-2011
Subtotal In-Depth Studies	*8.1 credits**

4) Personal development	date
• **Skill training courses**	
Course Career Orientation, Wageningen	May-Jun 2008
Workshop on scientific publishing, Wageningen	Nov 05, 2008
Course Interdisciplinarity in research, Wageningen	Jun 11, 16, 23, 2009
Course Science, the press and the general public: communication and interaction, Wageningen	May 07-08 & Jun 04, 2009
Scientific Writing, Wageningen	Jan- Mar 2010
• **Membership of Board, Committee or PhD council**	
Member of Science Café Wageningen board	Nov 2013-present
Subtotal Personal Development	*6.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**39.4**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits ** A credit represents a normative study load of 28 hours of study.*	

COLOPHON

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