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Green defense against thrips: Exploring natural products for early management of western flower thrips

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GREEN DEFENSE AGAINST THRIPS

EXPLORING NATURAL PRODUCTS FOR EARLY MANAGEMENT OF WESTERN FLOWER THRIPS

SANAE MOUDEN

Green Defense Against Thrips

Exploring natural products for early management of western flower thrips

Sanae Mouden

Green Defense Against Thrips – Exploring natural products for early management of western flower thrips
PhD thesis, Leiden University, the Netherlands

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Green Defense Against Thrips

Exploring natural products for early management of western flower thrips

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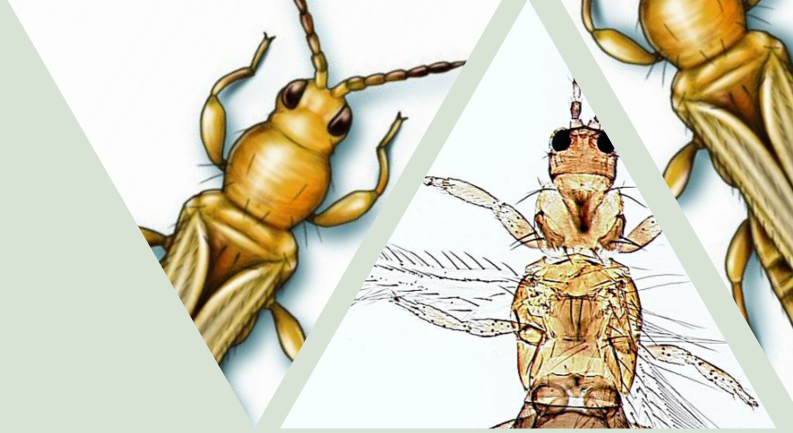
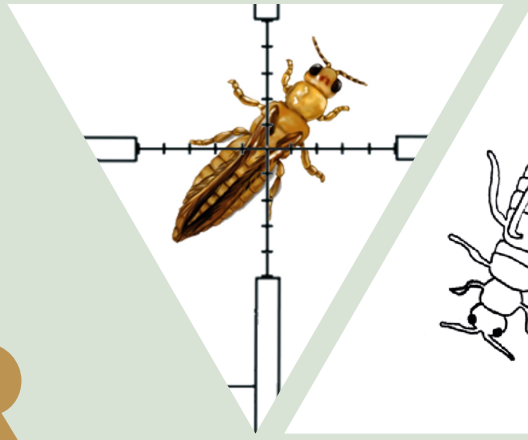
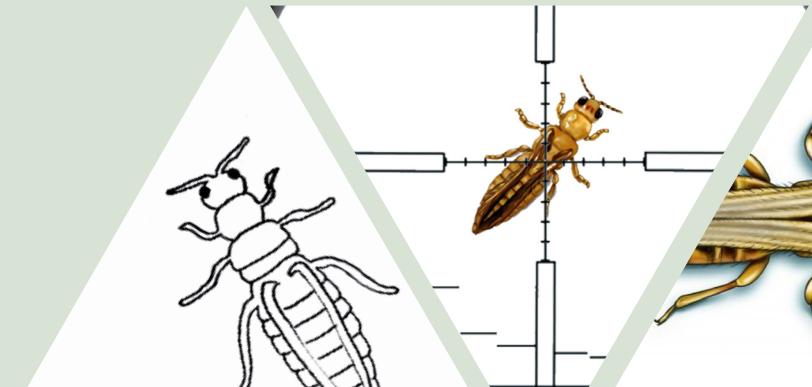




CHAPTER ONE

GENERAL INTRODUCTION



Modern agriculture and the role of pesticides: current state of play

Plant health is of global importance for sustainable agriculture, food security and environmental protection. Particularly, crop protection plays a key role in safeguarding productivity against losses caused by pests (Oerke, 2006). To date, minimizing impairments as a result of pests has, to a large extend, relied on the the use of synthetic pesticides. In fact, the prosperity of modern agriculture owes its success to the discovery and adoption of these chemicals (Cooper and Dobson, 2007). In modern agriculture, crop protection has become increasingly synonymous for pesticide use. Despite the immense benefits in terms of increased commodity production, agriculture has been heavily criticized for its negative impacts on the environment, biodiversity and human health. Societal concerns over significant costs associated with the injudicious use of pesticides (e.g. effects of neonicotinoids on environment; soil fumigants and endocrine disruptors on human health) along with a strong political commitment are nowadays intensively pushing towards more sustainable pest management tools (Coelho, 2009) within the context of an integrated pest management (IPM) system.

European agriculture is at an important juncture and in a period of tremendous change, in which the transition to low impact farming is a key for sustainable agriculture in the second half of this century. Nonetheless, it also presents a huge challenge that prompts reassessment of production practices giving priority, where possible, to non-chemical alternatives. In order to achieve independence from chemical pesticides in the next decades, various political measures at the European level have been undertaken. Ten years ago, the first reduction goals, defined by the EU directive 2009/128/EC, called for the reduction of risks and dependency on pesticides. Chemical pesticides are being progressively withdraw. At the same time, Member States (MS) are encouraged to develop holistic approaches for the much-needed sustainable transition. Fundamental and applied research across Europe are major building blocks for reinventing Europeans plant production systems to ultimately reach pesticide-free agriculture. Biopesticides appear at the horizon as an attractive solution for sustainable plant protection (Villaverde et al., 2013). As a contribution to such efforts, this thesis explores the use of plant derived compounds for early management strategies in tomato (*Solanum lycopersicum*) and *Chrysanthemum morifolium* to control one of the world's most economically important insect species – western flower thrips (WFT). The central question that I seek to answer in my dissertation research is: 'Can we employ plant derived compounds as a strategy to boost the plant's defense system?'

Challenges and dynamic trends shaping pest control of the future

As witnessed over the past decades, plant protection products, as have other technological innovations, have played a major role in driving considerable growth in the global production of agricultural goods and services. However, the ecological costs have been largely underestimated, if not ignored (Horrigan et al., 2002). Over the past decades, concerns have been developed over environmental consequences as well as long-term sustainability. Although environmental concerns

were becoming more prominent in the years following World War II, the modern environmental movement and search for alternatives to the use of chemicals to control pests began in earnest following the publication of Rachel Carson's classic '*Silent Spring'* in 1962. This book highlighted, for the first time, an increasingly widespread belief to regard chemical-based insect control as a menace to society rather than an agricultural miracle. Indiscriminate prophylactic use of synthetic pesticides has given rise to a number of serious problems including pollution of air, soil, surface and ground water, harming both the environment and human health (Handford et al., 2015), including the natural enemies of the pest itself. Deleterious effects on non-target organisms significantly declined the number of beneficial pest predators leading to proliferation of various pests and diseases with adverse impacts on flora and fauna (Hart and Pimentel, 2002). Additionally, overreliance on chemical insect control, exacerbated by continuous and extensive use of few modes of action, has led to the widespread development of insect pest resistance, resurgences and outbreaks of secondary pests (Lamichhane et al., 2016). Over 500 species of arthropod pests, worldwide, have evolved resistance to one or more conventional insecticides (Georghiou and Lagunes-Tejeda, 1991; Whalon et al., 2013) among which WFT, in particular, forms an extremely serious threat for the European horticulture.

Pesticide regulatory framework

A substantial body of legislation on pesticides has been built up over the past decades aiming to ensure high standards of protection for people and for the environment. The individual EU MS countries have a long history of controlling pesticide use through a myriad of country-specific programs. Following a transitional period, some major pieces of national pesticide laws were replaced by new, harmonising European Commission (EC) legislation (Council Directive 76/895/EEC). The main pieces of existing EC legislation on pesticides are briefly outlined here. Directive 91/414/EC, on the placing of plant protection products, was one of the first items of legislation to deal with the authorization of pesticides and established agreed criteria for considering the safety of active substances, as well as safety and effectiveness of formulated products. This directive urged all MS to embark a full review of active substances in pesticides on the EU market for inclusion or exclusion from Annex I (Council Directive 91/414/EEC). This so-called re-registration process significantly reduced the number of approved active substances (SANCO/10796/2003). The waste framework directive (2006/12/EC) and the directive on hazardous waste (91/689/EEC) constitute regulations impacting the establishment of provisions for the safe collection and disposal of empty pesticide packages and unused or expired pesticides. The water framework directive (2000/609/EC) identifies substances that are hazardous for water (including active substances in plant protection products) whereas, regulation (EC) No. (396/2005) addresses the maximum residue levels (MRL) of active substances legally allowed in or on food and feed. The Thematic Strategy on the Sustainable Use of Pesticides completes the overview of the existing pesticide regulations, as it aims to regulate pesticide use. The EU revised its regulation of plant protection products, adopting 'hazard-based' cut-offs for certain categories of active substances under the new Regulation No. 1107/2009 EC. These so called

cut-off criteria banned, a priori, active substances that have been identified as dangerous to human health, animal organisms and the environment, without a risk assessment that considers levels of exposure (Annex II). This applies, among others, to carcinogenic, mutagenic, toxic (for reproduction), endocrine disruptive and persistent substances. Regulation No. 1107/2009 EC and the European framework directive 2009/128/EC on the sustainable use of pesticides, hereinafter referred to as the Sustainable Use Directive (SUD), abrogated the earlier directive 91/414/EC following implementation of Regulation (EU) No. 540/2011 (EFSA, 2013). This new set of legislation, referred to as the 'EU pesticide package' aims at risk reduction for continuance of pesticide use while promoting the use of Integrated Pest Management (IPM) and alternatives (European Union, 2009 a,b). Article 4 of the SUD requires that all MS develop a National Action Plan (NAP), which ensures that a set of eight general principles of IPM are implemented as of January 2014. The general principles of IPM are listed in Annex III of SUD and are discussed in more detail in the next section.

Integrated Pest Management (IPM)

The historical development of crop protection underwent some major evolution, from mainly being dominated by conventional pesticides, to a more comprehensive construct arising in the second half of the twentieth century (Ehler, 2006). This concept, generally referred to as Integrated Pest Management (IPM), applies judicious use of pesticide in consideration of economically, ecologically and socially sound principles (Kogan et al., 1998). The acronym 'IPM' has gained global recognition and is endorsed as the future paradigm for crop protection (Dara, 2019). The holistic concept of IPM emphasizes on systems approaches by integrating preventative and therapeutic intervention measures. IPM constitutes agronomic, mechanical, physical and biological facets and, although pesticides remain an important tool so far, they are no longer a panacea for plant protection. To realize the full potential of IPM, simultaneously implemented pest management actions should be compatible (Stenberg et al., 2017).

The principles of IPM, resulting from a logical sequence of events, are illustrated in Figure 1. The base tier of the IPM pyramid constitutes the first principle and is based on the general proverb "*Prevention is better than cure*". Among these agronomic actions (e.g. cultural control), spatial and temporal diversification through intercropping and crop rotation, respectively are key to minimizing pest pressure (Barzman et al., 2015). Hitherto, pest-tolerant and resistant cultivars significantly contribute to the development of preventative and suppressive strategies. The study of plant resistance to herbivore attack, known as host-plant resistance, involves a large web of complex interactions, mediated by morphological and chemical traits that influence the ultimate amount of damage caused by pests (War et al., 2012). Understanding the nature of plant defensive traits plays a critical role in designing and manipulating crop varieties with enhanced protection against pests. A promising yet, missing aspect in the IPM pyramid are additional measures intended to increase plant resilience. Among these, the concept of plant vaccination, i.e. artificial elicitation of induced defenses, is an underutilized tool to reduce susceptibility to pests (Stout et al., 2002; Stenberg et al., 2017). Host

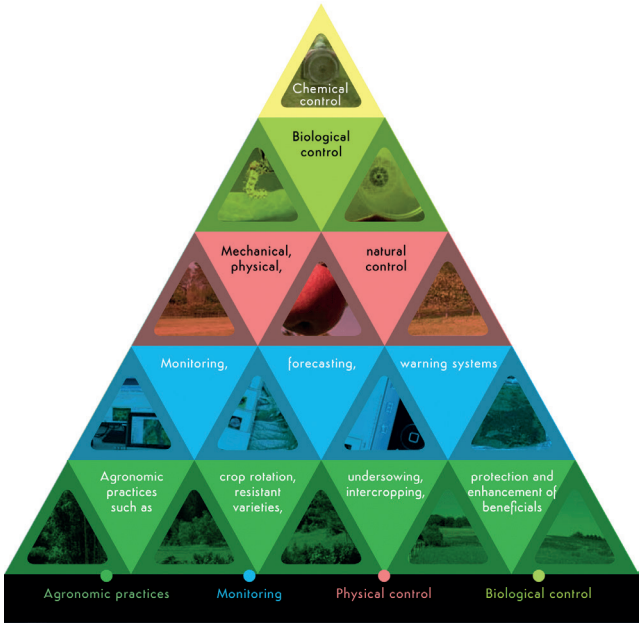


Figure 1. The pyramid of Integrated Pest Management. Jointly developed by International Organization for Biological Control (IOBC), International Biocontrol Manufacturer Association (IBMA) and Pesticide Action Network (PAN) Europe. Figure was adapted with permission.

plant resistance against WFT is covered in detail in **chapter two**, whereas chemical based host plant resistance against a broad range of herbivorous arthropods is reviewed in **chapter three**.

The second tier of the pyramid aims to effectively manage current and anticipate future pest outbreaks and consists of principle 2 on monitoring and principle 3 on decision making. Proper identification of the pest and knowledge of its biology and ecology are key determinants for a successful IPM program. Many IPM programs have centered on threshold-based decision systems. Sampling and monitoring of a pest population size is therefore, often used to evaluate and compare these pressure levels to the 'economic or action' thresholds, to ensure timed treatments when economic thresholds are exceeded. The aim is not to eradicate pests, but to manage levels at which only few interventions need to be applied to control the pest (Weiss et al., 2009).
The following two layers of the pyramid include elements that form the basis for intervention approaches. These aim to seek for synergies by combining an array of compatible protection methods, giving preference to non-chemical methods to reduce reliance on pesticides (principle 4). Biological control, or biocontrol, is one of the oldest non-chemical methods used in agriculture (Smith, 1919). In its strictest sense, it makes use of beneficial natural enemies to reduce the relative abundance and, as such, damage of insect pests (Rebek et al., 2012). Biocontrol is commonly and

more successfully used for control of greenhouse pests (van Lenteren, 2000; Pilkington et al., 2010) as compared field crops (Dara, 2019). Periodical releases of commercially available natural enemies as well as conserving populations allows for both immediate control and a build-up of the population (van Lenteren, 2012). Primary ways by which physical or mechanical approaches can be used for pest management include exclusion by screens and barriers, trapping, removal or destruction. Furthermore, selective plant-based biopesticides represent a promising alternative to chemical pesticides (Chandler, 2011; Villaverde et al., 2013; Campos et al., 2018).

Chemical based intervention approaches, at the top of the IPM pyramid, becomes relevant when prevention and alternative methods fail to provide satisfactory pest control but, should be considered as the last resort. These are guided by three principles that presuppose pesticide use. Principle 5 concerns sound pesticide selection for high target specificity whereas, principle 6 aims to reduce pesticide use by lowering doses and application frequencies. To addresses the growing issue of resistance and prolong the lifespan of pesticides, principle 7 on anti-resistance strategies, includes the rotation of multiple pesticides with different modes of actions. Last but not least, principle 8 regarding evaluation allows to improve the effectiveness of future strategies (Barzman et al., 2015).

How plants protect themselves – constitutive vs induced plant defenses

Plants are physically tied to their environment and are therefore, constantly confronted with multiple attack scenarios involving both abiotic and biotic stresses. To ward off stresses to which they are exposed, plants have evolved highly sophisticated surveillance and dynamic response mechanisms. The phenomenon of plant resistance to herbivore attack, known as host-plant resistance, forms an important cornerstone in IPM for environmental, economic, and social reasons (Stenberg, 2017). In terms of timing and cost two major categories of plant defenses can be distinguished namely: constitutive and induced. Constitutive or ‘static’ defenses are those which are preformed and continuously expressed throughout the lifetime of a plant. These provide direct protection against herbivore attack through a variety of physical and chemical defenses. Morphological features such as cuticles waxes, leaf toughness and spines act as physical barriers (Barton, 2016) whereas, chemical defenses can arise from both primary and secondary metabolites as well as defensive proteins and enzymes (Mouden et al., 2017a,b). Leaf trichomes, on the other hand, may fulfill a dual role by providing chemical defenses in addition to physical defenses, through the production of specialized volatile organic compounds (Glas et al., 2012). Although constitutive defenses can be very successful, they can also be costly when deployed unnecessarily. Most plant defense hypotheses therefore, assume that constitutive defenses are costly as they divert energy and resources away from other plant processes such as growth and reproduction (Cipollini et al., 2014).

Induced or ‘active’ plant defenses, on the contrary, are those which are expressed only in response to, for example, , herbivore and pathogen attack (Agrawal et al., 1999; Karban and Baldwin, 1997; Howe and Jander, 2008). The general consensus, as opposed to constitutive defenses, argues that this type of defense evolved as a cost-saving strategy, particularly when the presence of herbivores

is variable (Maffei et al., 2012; Huot et al., 2014). These responses generally serve as a second line of defense that are activated when constitutive defenses have successfully been evaded. Alternatively, potentiating a plant's defensive capacity without concomitant induction of specific defense-related genes can condition a plant for boosted responses to future attacks, a phenomenon commonly known as priming (Conrath et al., 2006; Mauch-Mani et al.,2017). Typically, host plants mount two types of inducible defense strategies: direct and indirect defense. In addition to direct physical and chemical defenses, plant defenses also work indirectly by recruiting beneficials from the third trophic level (Walling, 2000; Turlings and Ton, 2006; Wu and Baldwin, 2010).

Constitutive and inducible defenses are not necessarily mutually exclusive though, a certain degree of overlap between these two categories is often observed, where herbivore feeding or oviposition activities can reinforce constitutively expressed defenses (Degenhardt et al., 2010; Kempel et al., 2011; Kant et al., 2015). Induced defenses may be more advantageous as they allow tailor-made strategies for each herbivore species after recognition of herbivore-specific cues (Mithöfer and Boland, 2008; Basu et al.,2017). In addition to these herbivore-derived elicitors, the production of endogenous plant-derived elicitors in response to insect attack initiate the first step by provoking plant defenses through a coordinated signaling system (Heil et al., 2009; Erb et al., 2012; Kant et al., 2015). Some of these endogenous plant-derived elicitors, such as jasmonic acid (JA) and salicylic acid (SA), are nowadays recognized as plant hormones.

Hormone-induced plant defense responses

The expression of inducible defenses are primarily mediated by endogenous signaling molecules among which the ubiquitous phytohormone JA and, in particular its bioactive derivative jasmonyl-isoleucine (JA-Ile), are key players in resistance against chewing-biting (i.e. caterpillars) and cell-content feeding (thrips and spider mites) insects as well as necrotrophic pathogens (Walling, 2000; Lazebnik et al., 2014). On the contrary, SA has in general been associated with resistance to (hemi)-biotrophic pathogens and phloem-feeding insects such as aphids and whiteflies (Vlot et al., 2009; Pieterse et al., 2012). Activation of the JA signaling pathway is characterized by induction of several defensive traits including secondary metabolites (Bennet and Wallsgrove, 1994; War et al., 2012), proteins such as polyphenol oxidase (Farmer and Ryan, 1990; Thaler et al., 1996), leaf trichomes (Boughton et a., 2005) as well as indirect induction mechanisms such as the production of plant volatiles (Ament et al.,2004; Arimura et al., 2005). Consequently, the modification of these chemical and/or physical defensive components decreases the preference, performance and abundance of herbivores. Plant signals responsible for inducing resistance are highly conserved among plant species. Accordingly, artificial manipulation of these JA-associated defenses by natural or synthetic elicitors has proven to confer enhanced resistance against multiple insects and diseases and is, therefore, regarded as a valuable component in pest management programs (Thaler, 1999; Vallad and Goodman, 2004; Walters et al., 2013; Steenbergen et al., 2018). The regulation of defenses is quite complicated, and there is abundant evidence for cross-talk among different signaling pathways that allow plants to finely tune immune responses to specific biotic foes (Koornneef and Pieterse, 2008).

In this context, the mechanistic and conceptual crosstalk between JA and auxins is particularly interesting, because at first glance, these two plant hormones possess antagonistic properties (Wang and Irving, 2011). Classically, the phytohormone auxin has been implicated in developmental processes although, recent studies demonstrate they also affect a multitude of plant defense responses through complex interactions among multiple hormone pathways (Kazan and Manners, 2009; Robert-Seilaniantz et al., 2011). However, to date, a unified picture seems to be lacking as both synergistic and antagonistic relationships between JA and auxin have been reported (Lakehal and Bellini, 2019). The role of auxins in plant defense responses was investigated in **chapter six**.

Host plant resistance as a route to sustainable plant protection

'*All the pests that out of earth arise, the earth itself the antidote supplies'* – Lithica (400 BC)

Breeding for host plant resistance is commonly employed in many crops to prevent pests and diseases. It can lead to effective protection, reducing the economic losses while, at the same time, addressing the need of public health concerns in a sustainable way. As the opening quotation implies, the earliest appreciation for the recognition of host plant resistance arose early in the development of agriculture. This line, from a poem attributed to Lithica, indicates that nature provides its own solution against pests. Indeed, many naturally occurring plant species (i.e. wild relatives) display large genetic variations in responsiveness of basal, and induced, resistance mechanisms. However, during the course of domestication, defense traits underlying insect resistance, including the production of defensive secondary metabolites, have been lost to a large extent (Rosenthal and Dirzo, 1997; Chaudhary, 2013; Moreira et al., 2018). As a result of lowered defense levels, domesticated plants are often more susceptible than their wild ancestors (Turcotte et al., 2014; Kempel et al., 2011). The majority of research in the past decades has largely focussed on studying constitutive defenses by screening genotypes with contrasting levels of resistance, classified as resistant or susceptible. A key mechanism underlying host resistance includes the production of a broad range of secondary metabolites which can affect herbivore development, survival and fitness. An example of such a chemical screening, referred to as eco-metabolic approach, was illustrated by Leiss et al. (2011) in order to identify putative plant defensive compounds that mediate resistance against WFT. Nuclear Magnetic Resonance-based metabolic profiles of thrips susceptible and resistant hosts were compared with the aim to identify candidate compounds for constitutive host plant resistance. Subsequent ***in-vitro*** bioassays were performed to validate these compounds (Leiss et al., 2009a,b; 2011; 2013). This wealth of prior research led us explore their application as plant protection agents. **Chapter three** of this thesis describes strategies for screening and selecting plant-derived metabolites as candidates for sustainable agriculture in more detail whereas, **chapter four** explores the external application of such bio-active insectidal metabolites on tomato resistance against thrips.

Towards a new green revolution – secondary metabolites for plant protection

Annual crop loss estimates of 15 to 20% from 'field-to-fork' are commonly reported, despite the availability of chemical crop protection agents (Schoonhoven et al., 2005). A worrisome development

is in view of the fact that pesticides are currently being restricted at a much faster rate than alternatives are being provided. In the current context of sustainable agriculture, the search for safe and green products has been growing continuously. Plant secondary metabolites appear in the horizon as an eco-friendly alternative to conventional pesticides (Dayan et al., 2009; Adeyemi, 2010). Their implementation in IPM is strongly encouraged as natural products, specifically those with a low-risk profile, display a promising opportunity for commercial utility in agriculture (Lorsback et al., 2019). Breeding programs mostly aim at increasing the levels of desired plant metabolites constitutively. Introgression of ancestral chemical defensive traits, however, may be accompanied by agronomic constraints. Moreover, it is a time consuming process which, additionally, is not always a straight-forward approach as, for example, in polyploid species such as chrysanthemum. Alternatively, exogenous application of putative defense metabolites may represents a promising approach for sustainable plant protection (Mouden et al., 2017b). An increase in chemical defenses mediating resistance against thrips can be achieved by directly administering known putative defense metabolites to seeds and cuttings but, may also be achieved indirectly by triggering the plants' innate defense signaling responses. Crop plants have been grown in a pesticide-dominant background for many decades and thus, have not been bred and cultivated for inducible responses (Stout et al., 2002). Despite being underutilized, induced resistance holds great promise as this approach is thought to be more cost-effective and may prevent auto-intoxication (Steppuhn and Baldwin, 2008). In addition, young plants or tissues are frequently more responsive to defense elicitors than older plants or tissues (Cipollini and Redman 1999; Köhler et al., 2015; Chen et al., 2018).

The experimental system – natural products for early management of thrips in tomato and chrysanthemum

As plants develop, from seedling to reproductive stages, their response to herbivory changes (Boege and Marquis, 2005; Barton and Koricheva, 2010). While some plants are more resistant in the early stage such as brassicas that are known to produce glucosinolates, other plants are, in general, more susceptible during the seedling and young juvenile stages (Barton and Hanley, 2013). Our research group has discovered a new tomato cultivar which is highly resistant to one of the most economically important pest insects worldwide, WFT (Mirnezhad et al., 2009; Bac-Molenaar et al., 2019). However, resistance is not fully developed in the early plant life-stage. Particularly the juvenile stage is vulnerable to WFT damage. Within this context, management of thrips early in the production cycle is extremely important to prevent insect-vectored virus diseases as well as preventing pest populations to build up to economically damaging levels.

The work described in this thesis is part of the program 'Green Defense Against Pests (GAP), funded by the TTW (formerly STW) technology foundation and five agriculture sector companies (Dekker Chrysanten BV, Deliflor Chrysanten, Dümmen Orange, Incotec International BV and Rijkzwaan Nederland BV). This project builds on the considerable progress achieved in our lab towards constitutive chemical host plant resistance against thrips. As a contribution to the changing

legislation and evolving societal attitudes concerning environmental issues, this project aims to enhance and manipulate the plants' own natural defense mechanisms against WFT. A model and a non-model plant species, tomato (*Solanum lycopersicum* L.) and chrysanthemum (*Chrysanthemum morifolium* Ramat), as briefly outlined in the next sections, were used in this project to represent vegetable crops and ornamental plants, respectively. More specifically, an approach based on treatments of seed and cuttings, representing two types of reproductive plant propagation (sexual and vegetative, respectively), was undertaken to protect plants from their early critical young stages onwards. To enhance chemical defenses, known putative defense secondary metabolites were exogenously applied whereas, external application of plant hormones was explored as a means to trigger innate defense responses against WFT. To overcome solubility constraints of plant secondary metabolites, natural deep eutectic solvents (NADES) were explored as an environmentally-friendly carrier solvents (see review in **chapter three**).

Western flower thrips

Thrips belong to the order Thysanoptera, and comprise approximately 6500 species that are classified in two suborders and at least nine families (Tipping, 2008). Less than 100 species, mainly in the family Thripidae, are considered to be pests of commercial cultivated crops. Among these, WFT, also known as *Frankliniella occidentalis* (Pergande), is recognized as one of the most significant agri- and horticultural pests worldwide. This invasive pest insect reached Europe via the Netherlands in 1983, most likely as a result of international movement of horticultural material (Kirk, 2002). Soon after introduction, it rapidly spread throughout Europe, and large outbreaks were observed in most European countries by the nineties (Kirk and Terry, 2003). Nowadays, *Frankliniella occidentalis* is abundant in greenhouses throughout the world. This cosmopolitan and polyphagous invader has developed into one of the most economically important pest insects in many crops due to their damage potential and concurrent lack of viable management alternatives (Morse and Hoddle, 2006).

Biology and ecology

Western flower thrips are small piercing-sucking insects and highly variable in size and colour. The full length of a female WFT is about 1,5 mm, while males are generally smaller (about 1 mm) and paler. Like all thrips, WFT have a haplo-diploid reproductive strategy. Females result from fertilized eggs whereas unfertilized eggs give rise to diploid male offspring (Mortiz et al, 2004). This process is also known as arrhenotokous parthenogenesis or arrhenotoky. The sex-ratio of adults shifts from female- to male-based populations during the season. The life cycle of WFT consists of six developmental stages (Figure 2): egg, two larval instars, two pupal instars and an adult with fully developed fringed wings. Females deposit their elongated, cylindrical eggs into the parenchymal tissue of the leaves, flowers or fruits (Cloyd, 2009). The initial egg stage is smooth and pale in color. Newly hatched instar larvae immediately start feeding on their host. Typically, second instar larvae are more active and feed more than first instars (Tommasini and Maini, 1995). First instar larvae are the only stage capable of viral acquisition (Kritzman et al., 2002). Towards the end of the second

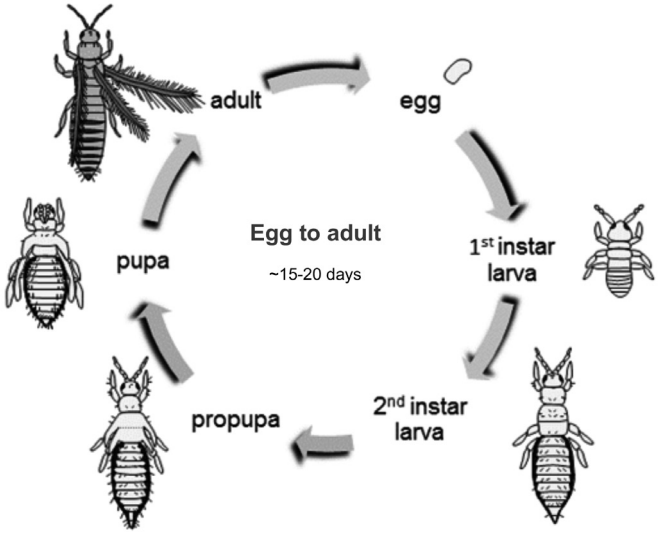


Figure 2. Thrips life cycle at 25°C. Retrieved from Agriculture and Food, Government of Western Australia (https://agric.wa.gov.au/n/2363).

larval stage, larvae enter a quiescent pupal period during which no feeding and little movement occurs. After approximately 6 days adults emerge from the pupal stage. Several studies have focused on the life history characteristics of *F. occidentalis* in different plant species. Environmental factors such as temperature and photoperiod, as well as the quality of the host plants can influence the development of thrips (Ishida, 2003; Zhang et al., 2007). The generation from egg to adult varies in length ranging from 9 to 13 days at optimum temperatures of 25–30° C (77-86° F) (Reitz, 2008). The presence of pollen as food source enhances the various growth parameters (van Rijn and Sabelis, 1993). The short generation time along with a high reproductive capacity leads to multiple generations a year. Adult females may live up to 45 days whereas males generally live half as long. Females can produce up to 7 offspring per day and have average total lifetime fecundities ranging between 150 to 300 (Cloyd, 2009). Both sexes lack diapause, allowing them to remain reproductively active throughout the year (Morse and Hoddle, 2006).

Western flower thrips are highly polyphagous and infest a wide range of hosts plants, including many greenhouse and field crops, vegetables, ornamentals, fruit trees and weeds. Records of WFT host plants are scattered through literature. Western flower thrips have been documented to feed on over more than 250 host species (Jensen, 2000) however, there is a considerable discrepancy in the literature concerning the number of reported hosts (Paini, 2007).

Thrips are cell feeders and both adult and larvae have piercing and sucking mouthparts (Hunter and Ullman 1992; Moritz, 1997). The two well-developed maxillary stylets are joined together to form an elongated sucking tube (Crisholm and Lewis, 1984; Kirk, 1997). According to electrical penetration graph (EPG) monitoring, the feeding behaviour of thrips consists of different phases. Prior to ingesting

the cell content, thrips probe (e.g. puncture) the epidermal layer of the host tissue with their needle-shaped stylets followed by penetration into deeper mesophyll tissue (Harrewijn et al., 1996; Kindt et al., 2003). Western flower thrips are responsible for two major kinds of damage to plants namely, direct and indirect damage. Direct damage results both from feeding and oviposition. Upon feeding, WFT cause two types of plant damage: feeding on actively growing tissue results in growth damage, whereas feeding on older, expanded tissue causes cells to become filled with air, which imparts a silvery appearance. The latter is known as silver damage and noticeably affects the products' cosmetic appearance, thereby reducing market quality (de Jager, 1993). Marketability is also reduced by oviposition. Females lay their eggs within the tissue using their curved ovipositor (Jensen, 2000; Tipping, 2008). Increases in egg size during embryogenesis causes further structural damage to the surrounding plant cells, known as 'flecking' (Moritz, 1995). In addition, WFT can also cause severe indirect damage by transmitting several plant viruses of which the tomato spotted wilt virus (TSWV) ranks among the top 10 of most economically important plant viruses worldwide (Maris et al., 2004; Scholthof et al., 2011). This notion stresses the importance of protecting plants from the early critical stage onwards. Both larval and adult stages can actively feed on a number of host plants. Only first instar larvae acquire the virus, whereas later instar larvae and adults transmit the virus after a latent period. Adult thrips are still capable of acquiring tospoviruses, although these are no longer transmissible (Wijkamp et al., 1996).

Economic impact of western flower thrips

In addition to the direct economic impact resulting from feeding damage and transmission of virus diseases, the mere presence of thrips can also have major implications for export products. Countries, free from major pests and diseases, tend to protect their local agriculture by introducing strict import and border quarantine controls. Considering the broad host range including many important horti- and agricultural plants, the economic impact of thrips is assessed to be huge (Kirk, 2002). However, in reviewing the literature on economic losses caused by WFT, it appears that these estimates are not only scarce and scattered but also outdated. The predicted annual costs from direct damage in greenhouse crops in the Netherlands was estimated to be US$ 30 million. This estimate did not include the effects of tomato spotted wilt tospovirus (TSWV), which adds an additional loss of US$19 million (Kirk, 2002). On the contrary, according to Goldbach and Peters (1994), the projected economic damage from tospoviruses transmitted by western flower thrips could exceed US$1 billion annually. Yet, quantitative data on economic losses caused by thrips are essential in monitoring the effectiveness of current management strategies employed. Obtaining accurate estimates of economic losses attributable to any pest is, in general, difficult as there are many factors involved. The wide range of existing estimates of annual loss, from less than a million dollars to several tens of millions, presented in the few published data, however, reflect several difficulties. Only few national governments have solid programs to systemically evaluate the losses that are caused by insect pests (Yudelman et al., 1998). Moreover, companies quite often tend to conceal their losses by commercial confidentially, which might stem from a fear of publicizing one's pest problems. Not only are the

published economic studies on the impact of WFT relatively scarce, they are also generally limited in scope and methodology. In many cases, the economic impact of thrips have only been measured by the direct costs of damage they cause, whereas expenses related to prevention and controlling pest outbreaks should also be taken into account.

Thrips as successful pest insects

Over the past decades, WFT has developed into a nearly intractable pest problem. It destroys countless acres of agricultural and ornamental crops throughout the world, causing extensive economic losses, establishing itself as a cosmopolitan pest (Kirk and Terry, 2003). Several important factors have contributed to its' success as a highly invasive pest insect. Firstly, their small, minute size and cryptic nature allow them to often remain unnoticed. The tendency to reside in concealed protected locations minimizes the exposure to many foliar applied insecticides, favouring the development of insecticide resistant thrips individuals. Moreover, WFT have a high phenotypic plasticity for morphological traits which makes identification difficult (Morse and Hoddle, 2006). In addition, damage is not immediately apparent and, therefore incorrect diagnoses can lead to inappropriate insecticide application, further enhancing development of pesticide resistance. Along with their polyphagous behaviour, a high reproductive potential and rapid life cycle are also important traits favouring their spread (Jensen, 2000; Cloyd, 2009).

Tomato and chrysanthemum

Tomato (*Solanum lycopersicum* L.) is both an economically important crop, second only to potato as the most grown vegetable, as well as an important model plant species due to its simple diploid genetics, relatively compact genome size, and the availability of a great diversity of genetic resources within the wild germplasm (Arumuganathan and Earle, 1991; Ranjan et al., 2012). Furthermore, its recently sequenced genome has been made publicly available, further enhancing its use as a model for plant research (100 Tomato Genome Sequencing Consortium, 2014). During the course of domestication, however, cultivated tomato suffered a severe genetic bottleneck leading to a serious reduction in its genetic diversity thereby, increasing the susceptibility to a wide array of diseases and pests (Kennedy and Barbour, 1992). Cultivated tomato is a natural host to over 100 arthropod herbivores (Lange and Bronson, 1981). Consequently, it has become an important model species for the study of plant defense mechanisms. Included among the major herbivorous pests of tomato are WFT (Bai and Lindhout, 2007). Defensive strategies of tomato against herbivorous arthropods are fairly well documented and a number of mutants on key defensive pathways are available for research. The tomato mutant *defenseless* 1 (*def1*), deficient in JA production, was shown to be more susceptible to attack by WFT, suggesting that the otadecanoid-mediated host responses are essential for defenses against thrips (Li et al., 2002; Escobar-Bravo et al., 2017). Moreover, a couple of well characterized JA-responsive marker genes including the proteinase inhibitors (PI) wound induced PI-II (WIPI-II) and PI-II (*Pin2*) are upregulated by thrips infestation (Kawazu et al., 2012; Escobar-Bravo et al., 2017). Additionally, the defensive protein polyphenol oxidase (PPO) is another highly reliable

marker for JA-induced defenses in tomato (Stout et al., 1994) and, is more easily measured than gene expression. PPOs serve an anti-nutritive role in response to insect feeding by reducing the digestibility of dietary proteins and the availability of essential amino acids (War et al., 2012).

Seed treatments are routinely applied worldwide and play a vital role in controlling early season insects and diseases, as well as improving seed germination, uniformity of emergence and seedling vigor (Brandl, 2001; Schmitt et al., 2009). In this thesis, I specifically refer to seed treatments as exogenous application of bio-active plant derived chemicals prior to sowing in order to suppress, control or repel above-ground pests from feeding on seedling and juvenile plant (i.e. no longer dependent on maternal storage reservoirs) stages. Seed treatments may offer advantage over early season foliar sprays by virtue of their residual systemic efficacy thereby, potentially reducing exposure of humans and the environment to crop protection products (Sharma et al., 2015). However, in most cases, seed treatments only provide a transient window of systemic protection which often declines as the bioactive compound is degraded or diluted as growth progresses. Alternatively, priming of induced resistance by treating seeds with elicitors enables young plants to mount defenses against pest and pathogen early in the growth cycle (Song et al., 2017). Moreover, many of the identified elicitors are expected to have a low ecotoxicological risk profile, as the responses are mediated by the plant (van der Wolf et al., 2012).

Cultivated chrysanthemum is one of the most economically important ornamental greenhouse crops worldwide and ranks second globally, after rose, in terms of socioeconomic importance (Spaargaren, 2002; Xia et al., 2006). Flower color, shape and size are, without a doubt, the most important visual quality attributes defining the exquisite beauty of chrysanthemums. Owing to the continuous increase in demand for new varieties of flowers, classical breeding programs have mainly focused on improving aesthetic characteristics, leaving few options for altering other agronomic traits. Consequently, many commercial cultivars often lack resistance traits to biotic and abiotic stresses (Teixeira da Silva et al., 2013). Among the most important production constraints are biotic stresses, in particular WFT and leaf miner (*Liriomyza trifolii)* infestations form a prominent hazard during its vegetative state (van Dijk et al., 1994; Leiss et al., 2009a). In contrast to tomato, chrysanthemum (*Chrysanthemum morifolium* Ramat.) is considered to be a non-model plant species as the whole reference genome is lacking, mainly because of the outcrossing polyploid nature and highly heterozygous genetic background. Consequently, seed propagation is not a desirable practice for chrysanthemum. Instead, for such highly heterozygous plants vegetative or asexual propagation is recommended to maintain the same desired genetic characteristics as their parents. Clonal commercial propagation of chrysanthemums cuttings is aided by the use of auxins as root-promoting compounds. Commercial auxin formulations are commonly applied to the basal cut end as dry-dip rooting hormone powder or as rooting solution (i.e. basal quick-dip). Among the available rooting hormones, indole-3-buteryic acid (IBA) is the most widely used auxin because of its high efficiency to promote root initiation and its greater stability in comparison to indole-3-acetic acid (IAA) (Blythe et al., 2007). During the initiation phase of rooting, cuttings are covered with transparent films or propagated under fine water mist sprays to maintain humidity and prevent desiccation of cuttings, which ultimately increases the rooting potential. At

this stage, however, it is not feasible to apply chemical control because residual control from contact insecticides requires thorough coverage of leaves to be effective against target pests. Moreover, the uptake efficiency (i.e. adsorption) of many traditional systemic insecticides is limited due to the lack of a developed root system (Scibaldi et al., 1997). This prompted us to explore dipping treatments of chrysanthemum cuttings as a preventative rather than a curative approach to mitigate pests early in the production cycle and prevent populations to build up to economically damaging levels.

Scope of the thesis

This thesis consist of seven chapters including this general introduction (**chapter one**). This chapter offers an overview of the concept sustainable use of pesticides and reflects on current green developments towards an ever safer agriculture, some of these being discussed in more detail in the following chapters. **Chapter two** reviews the concept of Integrated Pest Management (IPM) in light of the economically important insect species western flower thrips (WFT; *Frankliniella occidentalis*), with focus on biological control and host plant resistance as areas of major progress. Chemical host defenses, from a biopesticidal point of view, may possess anti-insect properties which are either purely insecticidal or act as feeding deterrents, growth inhibitors, growth regulators, repellents or oviposition inhibitors against a variety of insect species. **Chapter three** of this thesis describes how natural plant-derived products, more specifically plant secondary metabolites, can foster and support future sustainable agricultural innovations for management of insect herbivory. To this end, Natural Deep Eutectic Solvents (NADES) are evaluated in this review as environmentally benign solvents to enhance the solubilizing properties of poorly-soluble insecticidal metabolites. Hence, this investigation was further taken up with the final aim to apply this concept for enhancement of tomato defenses against WFT (**chapter four**). The effect of pre-sowing seed treatments on seed germination and plant performance of tomato was studied utilizing naturally occurring plant compounds. One inherent drawback of exogenously applied NADES is its' phytotoxic effect on seed viability, particularly when the duration of tomato seed soaking was increased. The work described in this chapter was therefore, a main driver to shift the focus of follow-up studies to elicitor inducible defenses. Accordingly, **chapter five** seeks to induce defenses against thrips at the seed stage using the ubiquitous plant hormone jasmonic acid (JA). Following a seed soaking treatment with JA, I examined the variation in inducibility of resistance in eight commercial tomato cultivars both phenotypically and biochemically. In search of effective dipping treatments to protect chrysanthemum cuttings during their vegetative stage, a serendipitous observation led us to explore the role of the growth hormone indole-3-buteryic acid (IBA) in relation to thrips defenses (**chapter six**). In the final section, the experimental results of the preceding chapters are put in a broader scope. The main achievements and practical implications are discussed in **chapter seven**. Finally, I elaborate questions raised by some inherent difficulties with regards to plant model systems and repeatability.

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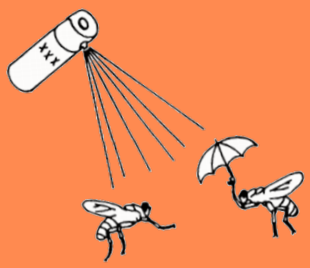
CHAPTER TWO

INTEGRATED

PEST MANAGEMENT

IN WESTERN FLOWER THRIPS

PAST



PRESENT

FUTURE

Abstract

Sanae Mouden, Kryss Facun Sarmiento, Peter G.L. Klinkhamer and Kirsten A. Leiss

Western flower thrips (WFT) is one of the most economically important pest insects of many crops worldwide. Recent EU legislation has caused a dramatic shift in pest management strategies, pushing for tactics that are less reliable on chemicals. The development of alternative strategies is therefore, an issue of increasing urgency. This paper reviews the main control tactics in integrated pest management (IPM) of WFT with focus on biological control and host plant resistance as areas of major progress. Knowledge gaps are identified and innovative approaches emphasized, highlighting the advances in -omics technologies. Successful programmes are most likely generated when preventative and therapeutic strategies with mutually beneficial, cost-effective and environmentally sound foundations are incorporated.

Keywords: thrips; *Frankliniella occidentalis*; integrated pest management; biological control; resistance, -omic techniques

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1. Introduction

Western flower thrips (WFT), *Frankliniella occidentalis* (Pergande), forms a key agri- and horticultural pest worldwide. This cosmopolitan and polyphagous invader is abundant in many field and greenhouse crops. WFT developed into one of the most economically important pests due to their vast damage potential and concurrent lack of viable management alternatives to the pesticide-dominated methods.[1] Direct damage results from feeding and oviposition on plant leaves, flowers and fruits while indirect damage is caused by virus transmission, of which Tomato Spotted Wilt Virus (TSWV) is economically the most important.[2,3] Their small size, affinity for enclosed spaces, high reproductive potential and high dispersal capability cause a high pest pressure.[4] Control of WFT mainly relied on frequent use of insecticides. This overuse of pesticides has led to the development of WFT resistance to major insecticide groups, residue problems on marketable crops, toxicity towards beneficial non-target organisms and contamination of the environment.[5-7] Therefore, in the framework of integrated pest management (IPM) programmes multiple complementary tactics are necessary, including monitoring, cultural, physical and mechanical measures, host plant resistance, biological control, and semiochemicals along with the judicious use of pesticides. IPM programmes for control of WFT have started to develop mainly for protected crops. However, continued injudicious use of pesticides resulted in a resurgence of WFT and associated viruses while depleting its natural enemies and competitive species. As Mors and Hoddle reviewed ten years ago[1], this led to a worldwide destabilisation of IPM programs for many crops. To emphasize the development and implementation of alternative control measures, the EU issued new legislation on sustainable use of pesticides (Directive 2009/128/EC) as well as on regulation of plant protection products (EC N° 1107/2009). Ten years after Mors and Hoddle, we aim at reviewing the current knowledge about WFT control in relation to IPM, stressing biological control and host plant resistance as areas of major progress. Resulting knowledge gaps are identified and new innovative approaches with emphasis on the emerging -omics techniques are discussed. WFT biology and ecology, fundamental to the development of knowledge-based IPM approaches have already been extensively reviewed elsewhere.[1,4,7]

2. WFT control tactics

2. 1 Monitoring

In order to effectively manage current and anticipate future pest outbreaks, early intervention and the development of economic thresholds is critical. However, the assessment of the economic impact of WFT has only recently begun to develop. Therefore, only a few economic damage thresholds for WFT have been established such as in tomato, pepper, eggplant, cucumber and strawberry.[8,9] However, in high-value ornamental crops or in crops with high threat of virus transmission, a near zero tolerance for WFT prevails.[6] Monitoring information on the development of WFT populations levels relative to the economic thresholds are assessed to decide on the employment of control tactics.[7] Monitoring is based on regular visual scouting of WFT adults on flowers and fruits or on the use of sticky traps.[10] Compared to yellow sticky traps, blue traps have shown to catch more WFT

whereby yellow sticky traps can also be used for monitoring aphids, whiteflies and leafminers. The use of monitoring tools has been expanded by the addition of semiochemicals as lures which significantly increase thrips catches.[11] Based on WFT samplings, models for predictions of WFT population growth and spread of TSWV have been developed as potential decision tools for IPM programmes.[12]

2.2 Cultural, mechanical and physical control of WFT

Since ancient time, farmers have been relying on cultural or physical practices for the management of pests. Sanitary practices such as removing weeds, old plant material and debris forms the first line of WFT defense.[13,14] Screening greenhouse openings prevented WFT immigration into protected crops but requires optimization of ventilation.[15] WFT incidence in protected tomato was 20% decreased by greenhouse window screens.[16] A combination of a positive pressure force ventilation system with insect prove screens though did not prevent greenhouse invasion by thrips.[17] UV-reflective mulch repelled WFT colonizing adults through interruption of orientation and host-finding behavior.[18,19] Irrigation, creating a less favorable environment for thrips, decreased numbers of WFT adults.[20] In contrast, high relative humidity favored WFT larval development and stimulated pupation in the plant canopy.[21] Fertilization increases plant development and growth but, also effects WFT abundance. Increased levels of nitrogen fertilization increased WFT population numbers in ornamentals.[22] Similarly, high levels of aromatic amino acids promoted WFT larval development in different vegetables.[23] A positive correlation between phenylalanine and female WFT abundance was observed in one study on field-grown tomatoes, but not in another.[18,24] High rates of phosphorus favored thrips development but did not lead to increased thrips damage.[25] Trap crops draw WFT away from the crop where it can be controlled more easily.[26] Flowering chrysanthemums as trap plants lowered WFT damage in a vegetative chrysanthemum crop.[27] Intercropping French beans with sunflower, potato or baby corn compromised bean yield but reduced damage to the bean pods increasing marketable yield.[28]

2.3 Host plant resistance

Plants and insects have co-existed for more than 350 million years. In the course of evolution, plants have evolved a variety of defense mechanisms, constitutive and inducible, to reduce insect attack and this led to host plant resistance. The study of host plant resistance involves a large web of complex interactions, mediated by morphological and chemical traits that influence the amount of damage caused by pests. Understanding the nature of plant defensive traits plays a critical role in designing crop varieties with enhanced protection against pests.

2.3.1 Morphological defense structures

The surface of a host plant can serve as a physical barrier through morphological traits such as waxy cuticles, and/or epidermal structures including trichomes. WFT damage was negatively correlated with the amount of epicuticular wax on gladiolus leaves.[29] Induction of type VI glandular trichomes

in response to methyljasmonate application trapped higher numbers of WFT.[30] However, other studies did not observe any correlation between WFT feeding damage and morphological traits such as hairiness, leaf age, dry weight and leaf area.[31,32] Instead, the latter provided clear indications that resistance was mainly influenced by chemical host plant composition.

2.3.2 Chemical host plant resistance

Plant chemical defense can arise from both primary and secondary metabolites. Primary metabolites, as nutritional chemicals, are generally beneficial for thrips. However, at low concentrations they can also be involved in WFT resistance. Among different crops, low concentrations of aromatic amino acids were correlated with reduced WFT feeding damage.[23] Nevertheless, these universal compounds do not provide any uniqueness and are not likely to be effective in resistance on their own. Therefore, the majority of studies focuses on the role of secondary metabolites in plant defense. Up to now few studies have investigated chemical host plant resistance to WFT. In a study on different chrysanthemum varieties, isobutylamide was suggested to be associated with WFT host plant resistance.[33] Developing an eco-metabolomic approach comparing metabolomic profiles of resistant and susceptible plants, compounds for constitutive WFT resistance were identified and validated in subsequent *in-vitro* bioassays.[34] Identified compounds included jacobine, jaconine and kaempferol glucoside in the wild plant species *Jacobaea vulgaris,* chlorogenic- and feroluylquinic acid in chrysanthemum, acylsugars in tomato and sinapic acid, luteolin, and β-alanine in carrot.[31,33,35,36] Interestingly, some of these metabolites did not only show a negative effect on WFT, but also receive considerable attention for their antioxidant functions in human health prevention.

2.3.3 Transgenic plants

Plant protease inhibitors (PIs) are naturally occurring plant defense compounds reducing the availability of amino acids for insect growth and development. Transgenic alfalfa, expressing an anti-elastase protease inhibitor, noticeably delayed WFT damage.[37] Purified cystatin and equistatin, when incorporated into artificial diets, reduced WFT oviposition rates.[38] Transgenic chrysanthemums, over-expressing multicystatin, a potato proteinase inhibitor, did not show a clear effect on WFT fecundity.[39] Cysteine PI transgenic potato plants overexpressing stefin A or equistatin, were deterrent to thrips while overexpression of kininogen domain 3 and cystatin C did not inhibit WFT.[40] Expression of multi-domain protease inhibitors in potato significantly improved resistance to thrips.[41] However, the potential interference of these multidomain proteins with basic cell functions has hindered a practical application for pest management so far. Targeting virus resistance, transgenic tomato expressing G_N glycoprotein, interfered with TSWV acquisition and transmission by WFT larvae.[42] The use of transgenic plants, alternated or simultaneously used with additional strategies, is recognized as a promising approach for thrips and tospovirus management by the scientific community. However, highly restrictive political and regulatory frameworks limit the commercialization of genetically modified crops in Europe.

2.3.4 Induced resistance

In addition to constitutive defenses, plants use inducible defenses as a response to pest attack, presumably to minimize costs. Induced defenses are regulated by a network of cross-communicating signaling pathways. The plant hormones salicylic- (SA) and jasmonic acid (JA) as well as ethylene (ET) trigger naturally occurring chemical responses protecting plants from insects and pathogens. The JA-pathway plays an important role in defense against thrips. The JA-responsive genes *VSP2* and *PDF1.2* were strongly stimulated upon exposure of *Arabidopsis* plants to thrips.[43] WFT reached maximal reproductive performance in the tomato mutant *def-1*, deficient in JA, in comparison to the mutant expressing a 35S::prosystemin transgene, constitutively activating JA defense.[44] In contrast to WFT, TSWV infection in *Arabidopsis* induced SA-regulated gene expression.[43] The resulting antagonistic interaction between the JA- and SA-regulated defense systems in response to TSWV infection, enhanced the performance of WFT preferring TSWV infected plants over uninfected ones.[45] Treatments with exogenous elicitors activate the natural defensive response of a plant, thereby enhancing resistance to thrips. Application of JA in tomato resulted in a decreased preference, performance and abundance of WFT.[46] Treatment of tomato with acibenzolar-S-methyl (ASM), a functional analog of SA reduced TSWV incidence, but did not influence WFT population densities.[47] Induced resistance is recently gaining more interest and might particularly be of value in conjunction with other IPM approaches.

2.4 Biological control

Biological control uses the augmentative release of natural enemies as well as conservation approaches to sustain their abundance and efficiency. A large number of natural enemies are known to attack WFT, which can be separated in two groups: macrobials including predators and parasitoids and microbials being subdivided in enthomopathogenic fungi and nematodes. Table 1 summarizes the most commonly commercially available biocontrol agents used against WFT.

2.4.1 Predatory mites

The principal arthropod predators associated with WFT biological control are phytoseiid mites (*Amblyseius* spp.) and pirate bugs (*Orius* spp.). Several species of *Amblyseius* have been recorded as predators of WFT and various species have been assessed for their efficacy. The first predatory mites used for WFT control were *Amblyseius barkeri* and *Neoseiulus* (formerly *Amblyseius) cucumeris* which primarily feed upon first instar larvae. Due to inadequate control achievements a number of other mites have been studied, seeking to find a superior WFT predator. Species such as *A. limonicus, A. swirskii, A. degenerans* and *A. montdorensis* proved to be effective predators of WFT.[48,49] Compared to *N. cucumeris*, *A. swirskii* proved to be a better WFT predator than in sweet pepper since females showed a higher propensity to attack and kill WFT larvae.[50] In chrysanthemum *A. swirskii* provided higher thrips control than *N. cucumeris* in summer, likely due to a better survival while both predators showed similar efficacy in winter.[51] Efficiency of *A. swirskii* as a WFT biocontrol agent is also influenced by host plant species whereby increased trichome densities hinder mite performance.[52] Thrips can also

Table 1. Biological control agents of *F. occidentalis*. Information retrieved from 'Bio-pesticide Database' of University of Hertfordshire (www.herts.ac.uk).

		Classification	Type of agent	WFT stage affected	First use	Commercially available
Predator	Crop-dwellers	Mites (foliar)	*Amblyseius cucumeris*	1st instar larvae	1995	Worldwide
			Amblyseius barkeri	1st instar larvae	1981	Worldwide
			Amblyseius degenerans	Larvae	1993	Worldwide
			Amblyseius californicus	Larvae	1985	Europe
			Amblyseius swirskii	1st and 2nd instar larvae	2005	Europe
			Amblyseius andersoni	Larvae	2007	Netherlands
			Amblyseius montdorensis	Larvae	2010-2011	Netherlands
			Amblydromalus limonicus	Larvae	2010-2011	Netherlands
		Minute bugs	*Orius insidious*	Larvae and adults	1900s	North- America
			Orius laevigatus	Larvae and adults	1900s	Worldwide
			Orius albidipennis	Larvae and adults	1991	Europe
			Orius majusculus	Larvae and adults	1993	EU and US
			Orius armatus	Larvae and adults	2008/2009	Australia
	Soildwellers	Mites	*Macrocheles robustulus*	Pupae	2008	Europe
			Hypoaspis aculeifer	Pupae	1995	Europe
			Hypoaspis miles	Pupae	1994	Europe
		Rove beetle	*Atheta coriaria*	Pupae	2002	Canada
Parasitoids		Parasitic wasp	*Ceranisus menes*	Parasitizes larvae	1996	Netherlands
			Ceranisus americensis	Parasitizes larvae	1996	Netherlands
Entomopathogen		Nematodes	*Steinernema feltiae*	Pupae, pre-pupae and larvae	2005	Worldwide
		Fungi	*Lecanicillium lecanii*	Adults most susceptible	2012	Europe
			Metarhizium anisopliae	Adults most susceptible	2012	Netherlands
			Beauveria bassiana	Adults most susceptible	2012	Europe and America
			Isaria fumosorosea	Larvae	2012	Netherlands

consume *A. swirskii* eggs and female predators were observed to preferentially oviposit at sites without thrips, or to kill more thrips at oviposition sites, presumably to protect their offspring.[53] Thrips are not the best food source for mites. Therefore addition of supplemental food to *A. swirskii* has recently been investigated. Supplying pollen improved performance of *A. swirskii* in control of WFT in chrysanthemum as did the addition of decapsulated brine shrimp cysts (*Artemia* sp.).[54] Next to being an efficient predator of WFT, *A. swirskii* is easily reared which allows economic mass production.[49] Since its commercial introduction in 2005 *A. swirskii* has, therefore, become the main predator used

for biological control of WFT in vegetables and ornamentals worldwide.[49] In addition to control of WFT, *A. swirskii* also provides control of whiteflies. Although the presence of whitefly can lead to a short-term escape of thrips from predation, thrips control is not negatively affected by the presence of whitefly, while in contrast *A. swirskii* is a better predator on whitefly in the presence of thrips.[55,56]

2.4.2 Predatory bugs

Orius, commonly known as pirate bugs, are known to be generalist predators, preying on adults and larvae of a wide range of insect species such as aphids, whiteflies, spider mites and thrips. Several species of *Orius* have been tested to evaluate their use against WFT. Observations from field and glasshouse experiments in sweet pepper demonstrated that *O. insidious* suppressed WFT to almost extinction, but failed to control WFT properly under short day conditions in autumn as they enter diapause.[57] In contrast, *O. laevigatus* has been successful in all year round biological control of WFT in vegetables and ornamentals.[59,59] Success of *Orius* in ornamentals depends on the complexity of flower structure.[59] Oviposition of *O. laevigatus* has been shown to induce WFT resistance in tomato through wound response.[60] Although a key natural enemy in biocontrol of WFT, *Orius* spp. are relatively expensive to mass rear.[59]

2.4.3 Soil-dwelling predators

Most research on WFT biocontrol focused on adult and larval stages. However, WFT spend one-third of their life as pupae in the soil. Different soil-dwelling predatory mites have been investigated of which *Macrocheles robustulus, Stratiolaelaps scimitus* (formerly *Hypoaspis miles*) and *Gaeolaelaps aculeifer* as well as the rove beetle *Dalotia coriaria* (formerly *Atheta coriaria*), are commercially produced as biocontrol agents against WFT pupae.[61-63]

2.4.4 Parasitoids

To date, *Ceranisus menes* and *C. americensis*, are the only two parasitoid wasps investigated for their potential to control WFT.[64] Under laboratory conditions, these parasitic wasps oviposit into first-instar larvae, resulting in death of the pre-pupal stage. However, slow wasp development time hinders efficient WFT control.

2.4.5 Entomopathogens

Entomopathogens used as WFT biocontrol agents consist of nematodes and fungi. The use of various nematode species and strains in the nematode genera *Steinernema* and *Heterorhabditis* against soil-inhabiting WFT pupae produced low and inconsistent control results.[65,66] While foliar application of *S. feltiae,* in the presence of a wetting agent, has not been shown to successfully control WFT adults and larvae in chrysanthemum [67,68], repeated applications successfully reduced thrips damage in cucumber.[69] Treatment with *Thripinema* nematodes, infecting WFT residing within flower buds and foliar terminals, was non-lethal and caused sterility of female WFT. This treatment was insufficient for control of WFT.[67]

Entomopathogenic fungal conidia infect thrips by penetrating their cuticle to obtain nutrients for growth and reproduction. In general, adult thrips are more susceptible than larval and pupal stages possibly because molting avoids contact with fungal inoculum. In addition, larvae have thicker cuticles, which may delay penetration of fungus. Foliar applications of different fungal strains belonging to *Beauveria bassiana*, *Metarhizium anisopliae* and *Lecanicillium lecanii* (formerly *Verticillium*) significantly reduced thrips populations in greenhouse vegetable and floral crops.[70,71] Besides the direct effects, *B. bassiana* showed sublethal effects on the progeny of treated WFT adults.[72] Several formulations of entomopathoghenic fungi are now available for foliar applications but their efficacy has been inconsistent likely due to varying ambient humidity and temperature. Formulations targeting the soil stage have shown promising results in potted chrysanthemum.[73] A major constraint to the use of entomophatogenic fungi as augmentative biological control agents remain difficulties in mass production, storage and formulation.[73] Recently, the use of endophytic fungi, developing within plant tissues without causing disease symptoms, has been explored for WFT control. So far no negative effects on WFT preference or development have been observed.[75,76]

2.4.6 Combinatorial use of biological control

Combinatorial treatments of natural enemies with different arthropods or arthropods with entomopathogens are used as alternative or back-up treatments. This requires careful timing and compatibility of treatments. Application of *A. swirskii* together with *N. cucumeris* in laboratory trials led to negative interactions on WFT control through intra-guild predation.[77] Simultaneous use of predatory mites and pirate bugs did have a negative effect on WFT in greenhouse crops but the effect was not greater than using one predator alone.[58,78] In contrast, a combination of *O. laevigatus* and *Macrolophus pygmaeus*, a generalist predator to control aphids, achieved enhanced control of both thrips and aphids in sweet pepper.[79] Combinations of the entomopathogenic fungus *B. bassiana* with predatory mites did not inhibit nor enhance the control of WFT, because fungal dissemination seemed to be hindered by mite grooming.[70,80]

Thrips generally complete their life cycle within two weeks causing several generations to overlap during a single crop production cycle. Hence, combinations of foliar and soil-dwelling biocontrol agents targeting all WFT life stages have been investigated. Simultaneous treatment of different mites or pirate bugs as foliage predators with the soil predators *G. aculeifer*, *D. coriaria* or the nematode S. *feltiae* did not reduce thrips numbers in ornamentals beyond that caused by foliage predators alone.[81] In contrast, the use of *Heterorhabditis* nematodes with the foliar-dwelling mite *N. cucumeris* provided superior control in green bean compared to individual releases.[82] Combinations of different predatory mites with the nematode *S. feltiae* achieved good WFT control in cyclamen, while combinations of *O. laevigatus* with the respective nematodes failed to control thrips.[59] Likewise, laboratory combinations of different soil dwelling predators with *S. feltiae* did not improve thrips control, while combinations of these predators with the entomopathogenic fungi *M. brunneum* and *B. bassiana* achieved higher control of WFT compared to single treatments.[83] Concurrent use of the soil dwelling mite *H. aculeifer* with the nematode *S. feltiae* increased mortality of WFT pupae in green bean.[84] It is

apparent that combinations of biocontrol agents for control of WFT are promising but require careful management and fine-tuning suiting the crop in question.

2.5 Behavioral control

An important focus in applied pest control is the manipulation of adult insect behavior using semiochemicals functioning as signal compounds. Pheromones serve for intraspecific communication between arthropods while allelochemicals mediate plant-insect interactions. Semiochemicals are used as lures for monitoring as well as control purposes.

2.5.1 Pheromones

Two key pheromones in male WFT were identified: (R)-lavandulyl acetate and neryl (S)-2-methylbutanoate.[85] The latter is a sexual aggregation pheromone attracting both male and female WFT. The synthetic analogues, Thripline AMS (Syngenta Bioline) and ThriPher (Biobest), are commercially in use. Decyl and dodecyl acetate, 10- and 12-AC respectively, are produced as alarm pheromones in anal larval droplets. Synthetic equivalents caused WFT to increase movement and take-off rates, reduce oviposition and decrease landing rates, suggesting its function as an alarm pheromone.[86,87] More recently, 7-methyltricosane, a WFT male specific cuticular hydrocarbon was suggested to inhibit mating.[57]

2.5.2 Allelochemicals

Volatiles used to locate plant hosts for feeding and oviposition can be applied as lures. Various volatile scents, including benzenoids, monoterpenes, phenylpropanoids, pyridines and a sesquiterpene attracted adult female *F. occidentalis* in a dose-dependent way.[89] While WFT were attracted by pure linalool as well as linalool emitted by engineered chrysanthemum plants, they were deterred by linalool glycosides.[90] The latter may represent a plant defense strategy against WFT as a floral antagonist, balancing attractive fragrance with poor taste. Methyl isonicotinate, the active ingredient of Lurem-TR (Koppert Biological Systems), is an attractant for both male and female WFT as well as other thrips species and is used to locate host plants.[91] Recently, a new potential active ingredient for thrips lures, volatile (S)-verbenone, was described from pine pollen.[92] Volatiles with repellent activities can be utilized for disruption of host finding. Applications of methyl-jasmonate and cis-jasmone deterred WFT larvae from feeding and settling although repeated exposure resulted in a dose-dependent habituation.[93,94] The monoterpenoid phenols thymol and carvacrol exhibited both a feeding as well as a oviposition deterrent effect to WFT.[95,96]

Currently the three commercially available WFT semiochemicals are mainly used as lures in conjunction with sticky card traps. Adult thrips constantly explore their host range for feeding and reproduction by utilizing different cues including volatiles. Therefore, semiochemicals hold great promise for thrips mass trapping as well as "lure and kill' strategies.[97,98] Combination of dodecyl acetate with maldison, an organo-phosphorous insecticide, increased larval mortality of WFT.[99] Use of LUREM-T together with the WFT predator *O. laevigatus* increased the abundance of the latter.[100]

The 'lure and infect' strategy employs LUREM-T for autodissemination of the entompathogenic fungus *M. anisopliae* by attracting thrips to particular traps provided with fungal inoculum.[101]

2.6 Chemical control

Chemical control is among one of the most frequently used methods to suppress WFT, particularly for ornamentals, where an almost zero damage tolerance encourages intensive application of insecticides. Commonly used insecticides for management of thrips, approved at European level, are listed in Table 2.

Management of thrips has relied on application of insecticides as has been described in previous reviews to which we refer to for further detail.[4,7] The use of broad spectrum insecticides including pyrethroids, neonicitinoids, organophospates and carbamates kills native outcompeting thrips species and natural enemies disrupting WFT management. [1,4-7,102] Spinosad, a natural reduced-risk insecticide derived from an actinomycete bacteria is compatible with natural enemies and, currently, provides the most effective chemical control of WFT.[4] New, narrow-spectrum insecticides, for WFT control include pyridalyl and lufenuron. However, frequent applications of broad and narrow spectrum insecticides, including spinosad, have led to the development of WFT resistance to active ingredients of most chemical classes as has been extensively revised elsewhere.[5-6,103] Management of WFT insecticide resistance, as reviewed in other publications, comprises resistance monitoring coupled with rotations among different classes of insecticides.[5-6] However, development of rotation schemes does not necessarily focus on reducing overall insecticide use. Therefore, insecticides should only be used if economic damage threshold are reached whereby applications should be accurate and precise while conserving natural enemies. Rotation schemes need to be complemented with other compatible control approaches.[5] Rotation programs including entomopathogenic organisms successfully controlled WFT under greenhouse conditions.[103] Various insecticides have been shown to be compatible with WFT predatory mites, bugs, and other competing thrips species.[104,105]

Table 2. Overview of synthetic and natural compounds used against thrips based on commercial spray advice cards 2015.

		Type of compound	Trade name	Target	Crops
Natural origin		Pyrethrins	Spruzit/Raptol	Sodium Channel	Lettuce, cutflowers, strawberry
		Azadirachtin	NeemAzal	Ecdysone receptor	Rose, chrysanthemum, cutflowers
Synthetic origin	Selective chemicals	Pyridalyl	Nocturn	Protein Synthesis	Rose
		Lufenuron	Match	Chitin biosynthesis	Rose, cutflowers
	Broad chemical spectrum	Spinosad	Conserve	Nicotinic acetylcholine receptor	Capsicum, rose, cutflowers, lettuce, cucumber, strawberry
		Abamectin (Avermectin, Milbemycin)	Vertimec	Glutamate-gated chloride channel	Capsicum, Chrysanthemum, rose, cutflowers, lettuce, strawberry
		Thiametoxam	Actara	Nicotinic acetylcholine receptor	Chrysanthemum, rose, cutflowers
		Methiocarb	Mesurol	Acetylcholinesterase	Chrysanthemum, rose, cutflowers
		Esfenvaleraat	Sumicidin	Sodium channel	Chrysanthemum, rose, cutflowers
		Deltamethrin	Decis EC	Sodium channel	Capsicum, Chrysanthemum, rose, cutflowers, lettuce, cucumber, strawberry
		Spirotetramat	Movento	Acetyl CoA carboxylase	Chrysanthemum

3. Future directions of WFT control: 'Omics' technologies

Pest management programs are constantly searching for innovative approaches advancing prevention and management of pest insects. The development of non-targeted analytical methods, from genomes to metabolites, has been a major driver for the adaptation of systems-based approaches. Such integrative approaches enable a comprehensive view of defense mechanisms. The emergence of omic-based techniques as well as advances in computational systems provide a powerful tool to drive innovation in crop protection. Understanding plant-insect interactions, genetic variations among insect populations and resistant crop varieties, generates valuable information that provide new opportunities and technologies by improving our knowledge of complex resistance traits.

3.1 Plant genomics

While domestication of wild plants through selection improved yield and palatability, it greatly reduced phenotypic and genetic diversity leading to loss of insect resistance. Wild ancestors, therefore, provide a promising source for breeding of WFT resistance traits.[32,35] Besides, the presence of considerable variation in resistance to WFT between accessions, as observed in various vegetables and ornamentals, can be exploited as well.[32,35,36,106] Identifying sets of genes or metabolites as biomarkers enables the introduction of novel insect resistance traits into breeding lines. In a highly

resistant pepper accession, a quantitative trait locus (QTL), mapped to chromosome 6, confers resistance to WFT by affecting the larval development of thrips.[107] This approach, however, might be less suitable for polyploid ornamentals. At present, successful breeding of resistant cultivars is limited to TSWV control. Genes known to confer resistance against TSWV isolates include: Sw-5 (*L. peruvianum*), Sw-7 (*L. chilense*) and Tsw (*C. chinense*).[108,109]

3.2 Insect genomics

Despite their economic importance as world-wide crop pests, the 'i5k' (5000 insect genome) project has only recently developed genomic and proteomic tools for WFT including a collection of assembled an annotated sequences.[110,111] The availability of the thrips genome will open new powerful possibilities to elucidate thrips gene function and develop alternative control strategies based on the molecular interaction of thrips with plants as well as viruses.[112] An RNA interference tool has been developed using microinjection for delivery of double-stranded RNA into adult thrips.[113] Targeting the vacuolar ATP synthase subunit-B gene resulted in increased WFT mortality and reduced fecundity of surviving females. Alternatively, symbiont mediated RNAi, down-regulating an essential tubulin gene, resulted in high mortality of WFT larvae.[114] For transmission of TSWV a suit of WFT candidate proteins reacting to viral infection have been identified but no RNAi approach for disruption has yet developed.[110] Sequencing the salivary gland transcriptome of TSWV-infected and non-infected WFT lead to the putative annotation of genes involved in detoxification and inhibition of plant defense responses.[111] The availability of WFT genome and transcriptome sequence data will facilitate the development of approaches identifying thrips effectors suppressing or inducing plant defense responses.

3.3 Metabolomics

Metabolomics has a great potential to detect a wide range of compounds in an unbiased or untargeted fashion. So far, metabolomics has mainly been restricted to comparative approaches using genotypes with contrasting levels of resistance, classified as resistant or susceptible.[34] Addressing the metabolome, however, allows investigating the complex and integrated network underlying defense mechanisms. Combined with genetic approaches, metabolomics analyses provide powerful opportunities identifying metabolic markers for resistance to thrips and opens opportunities for 'metabolite breeding'. Identification of compounds conferring resistance to different herbivores, i.e. cross-resistance, could form a basis for a multi-resistance breeding program. An overlap of resistance to WFT and celery leafminer (*Liriomyza trifolii*) has been described in chrysanthemum.[106] Manipulation of environmental factors may increase concentrations of resistance related metabolites within plants thereby, enhancing WFT control. Rutin and chlorogenic acid, two phenolic compounds involved in thrips resistance are enhanced upon UV-B exposure.[115] In addition, plant secondary metabolites involved in WFT resistance could be used to develop new protection agents which enhance or activate the plants' own defense mechanisms or which may provide new mode of actions with improved selectivity, minimizing the effects on non-target organisms.

Next to plants, microbials offer a huge source of metabolites to be used for insect resistance. Assembly of microbial communities may influence performance of thrips through plant chemistry or volatile emission. Colonization of onion seedlings by fungal endophytes induced resistance to *Thrips tabaci* likely due a repellent effect of volatiles.[116] Investigations into endophytes increasing resistance to WFT have not been successful so far.[74,75] Rhizobacteria are known to play an important role in plant growth, nutrition and health in general. Genetic variation in response to the capacity of plants in reacting to these beneficial bacteria opens the way for breeding of plants maximizing bacterial benefits. The effect of soil microbial communities on plant above ground defense directed against insects, such as thrips, still need to be explored. Similarly, the effect of the bacterium *Pseudomonas syringae* producing the JA analogue coronatine and thus triggering herbivore defense has a potential to be explored for plant defense to WFT.[117]

3.4 High-throughput screening

Employing genomic as well as metabolomics techniques however, requires a high-throughput screening (HTS) system for thrips resistance. Screening large numbers of plants for identification of resistance sources is vital for resistance breeding programmes.[118] Recently, a high-throughput phenotyping method has been described using automated video tracking of WFT behaviour.[119] However, a reproducible high-throughput method assessing thrips damage is still lacking. Similarly, HTS systems testing for active metabolites against WFT deriving from plants or microbials are absent. Development of stable thrips derived cell lines, beyond primary cell cultures, has been unsuccessful until now.[120] However, the availability of the thrips genome sequence provides an unprecedented opportunity to identify gustatory or olfactory receptors to form the basis of HTS development.

4. Conclusions

As from 2014, farmers in the EU are obliged to implement the principles of integrated pest management. However, despite the various benefits expected from IPM, there seems to be little evidence that IPM has been largely adopted. Many studies seek to develop their respective methods as single-solution approaches to pest problems rather than integrating these into an 'IPM toolbox'. Besides, vertical integration of control measures looking at IPM of different pests in one cropping system is scarce.[7] Developing and implementing IPM remains a complex knowledge-based task. Integrating different control tactics is fundamental to achieve successful control of WFT, yet, it presents significant challenges. Clearly, research into the integration of methods involves cooperative, jointly planned activities that cannot be pinned down into a single methodological blueprint. How can scientists in different groups develop protocols and tests that allow the combination of multiple approaches in sustainable pest management, while retaining the capacity to determine the individual contributions and, hence, modify and improve these? For optimal effectiveness and progress, strategies should not only be integrated at inter- and multidisciplinary research levels but, should be driven through applied outcomes in co-operation with commercial partners by transdisciplinary research.

Significant research progress in control of WFT has been made. Host plant resistance to WFT becomes increasingly important. Some breeders already have varieties with different resistance ratings, however, for certain crops such as polyploid ornamentals this approach is not as straightforward. Recently, more emphasis has been put on biological control of WFT in protected crops. Nevertheless short crop cycles and low thresholds for ornamentals in particular, make biological control challenging. Another promising approach is the use of semiochemicals, not only for monitoring but also for thrips control. Looking to the future, there are many exciting (bio)-technologic advances that will undoubtedly boost the control of thrips. With the 'omics' revolution, we have the tools at hand to fully grasp this potential. Nevertheless, much remains to be learned about plant-insect interactions to make further important contributions for developing biologically, environmental friendly, sustainable crop protection strategies against thrips. Molecular modifications, genetic engineering and the development of novel biological products, including microorganisms and metabolites, will allow development of improved cultivars that are able to respond to WFT attack by enhancing resistance. However, not only new strategies need to be explored but existing ones should be viewed in the context of IPM programs with emphasis on compatibility as well as on ecological, environmental and economic consequences. Looking at different crops it becomes even more complex. In crop protection, as in life, one size does not fit all. In order to achieve successful control, strategies should be tailored to fit the requirements of different production systems. Controlling pests is not a trivial issue, and has never been. The basic question remains of how one gets consistent long-term control. Most importantly remains the need for transdisciplinary approaches integrating different practices for control of thrips.

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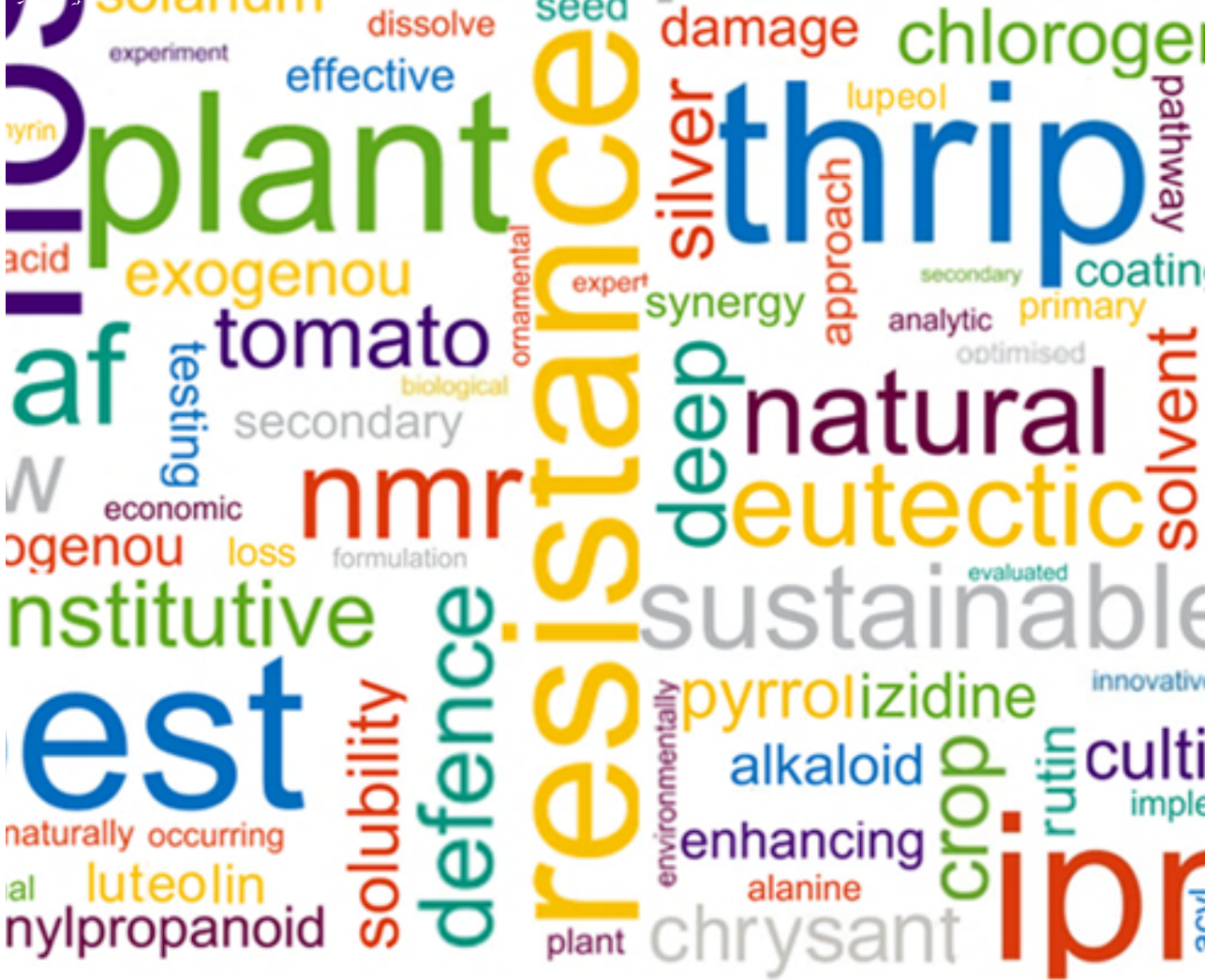
CHAPTER THREE

TOWARDS ECO-FRIENDLY CROP PROTECTION

NATURAL DEEP EUTECTIC SOLVENTS AND DEFENSIVE METABOLITES



Abstract

Sanae Mouden, Peter G.L. Klinkhamer, Young Hae Choi and Kirsten A. Leiss

With mounting concerns over health and environmental effects of pesticides, the search for environmentally acceptable substitutes has amplified. Plant secondary metabolites (PSM) appear in the horizon as an attractive solution for green crop protection. This paper reviews the need for changes in the techniques and compounds that, until recently, have been the mainstay for dealing with pest insects. Here we describe and discuss main strategies for selecting plant-derived metabolites as candidates for sustainable agriculture. The second part surveys ten important insecticidal compounds, with special emphasis on those involved in human health. Many of these insecticidal metabolites, however, are crystalline solids with limited solubility which might potentially hamper commercial formulation. As such, we introduce the concept of Natural Deep Eutectic Solvents (NADES) for enhancing solubility and stability of such compounds. The concept, principles and examples of green pest control discussed here offer a new suite of environmental-friendly tools designed to promote and adopt sustainable agriculture.

Keywords: insectidal, metabolomics, NADES, plant protection compounds, solubility

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Introduction

One of the greatest challenges that agriculture faces in the 21st century is the need to feed the world's rapidly growing population (Hertel, 2015). Selection of high-yielding crop varieties have immensely benefitted mankind. However, along with the success of this 'green revolution', severe outbreaks of pests and diseases occurred. Agronomic improvements, as a result of domestication, have often been accompanied by limitations such as loss of resistance traits (Wink, 1988; Rosenthal & Dirzo, 1997). Minimizing crop impairments due to pests has mainly been addressed by the use of synthetic pesticides. Modern agriculture partially owes its success to the discovery and adoption of these chemicals (Cooper & Dobson, 2007). Over the past decades, however, concerns have been developed over environmental consequences as well as long-term sustainability. Indiscriminate use of synthetic pesticides has given rise to a number of serious problems, including the widespread development of resistance to pesticides, crop residues, non-target effects, environmental contamination and public concerns about potential health risks (Handford et al., 2015). Furthermore, increased pressure through recent EU legislation (Sustainable Use Directive 2009/128/EC) caused a dramatic shift in pest management strategies, which is not only pushing for tactics that are less reliable on chemicals but, in addition, significantly restrict the application of several important active ingredients (Plant Protection Products Regulation 1107/2009) (European Commission, 2009; European Union, 2013a; 2013b).

The above facts necessitate the urge for safer, environmentally friendly approaches which, preferably, also exhibit new biochemical modes of action to minimize development of pesticide resistance. Consequently, natural compounds have increasingly become the focus among those interested in discovery of sustainable crop protection agents. This review focuses on plant-derived insecticides and discusses their perspectives and challenges as sustainable alternatives in pest management approaches. These naturally occurring bioactive compounds produced by plants, also referred to as secondary metabolites, elicit different insecticidal effects which act as feeding deterrents, growth inhibitors, growth regulators, repellents or oviposition inhibitors against a variety of economically important insect species. Providing a full overview of all plant secondary metabolites with insecticidal potency is beyond the scope here. We will therefore be deliberately selective, taking a few classes of secondary metabolites of plant origin as examples for natural crop protection, in particular those known to have beneficial health effects on humans.

Though seemingly useful as green insecticides, from a practical perspective secondary metabolites can be a double-edged sword. An inherent problem of many secondary metabolites is their low aqueous solubility, which might hamper commercial formulation. Consequently, organic solvents are often used in large quantities. The need to replace these harmful solvents by safer, non-toxic, inexpensive and easily available ones has significantly increased over the past decades, partially in response to the stringent environmental regulations (Smith et al., 2008). Natural Deep Eutectic Solvents (NADES), a new innovative class of green media, have now come to the fore as such major endeavor.

Here, we discuss several approaches for selecting plant secondary metabolites as candidates for crop protection. Next, we will briefly focus on the insecticidal properties of a selected sample of plant defense compounds. Finally, NADES are introduced as environmentally benign solvents, which brings a new dimension to the agrochemical industry. In developing this concept, we review the unique solvent properties of NADES and explore their potential as solubilization vehicles for plant derived crop protection agents.

Secondary metabolites for crop protection

Plants have evolved a variety of defense mechanisms to reduce insect attack, both constitutive and inducible. A key mechanism by which plants defend themselves against attack is through the production of a broad range of secondary metabolites. These represent a large and varied reservoir of chemical structures with many potential uses, including their application as pesticides (Adeyemi, 2010; Isman et al., 2011; 2006). Throughout history, numerous plants have been successfully exploited for their pesticidal properties (Thacker, 2002). Today, phytochemicals are used to develop commercial insecticides and serve as models for new crop protection agents (Cantrell et al., 2012). Although plant-derived biopesticides are generally considered to present lower risks to consumers (Dayan et al., 2009) some plant metabolites such as alkaloids (pyrrolizidines, tropane) as well as certain glucosinolates and saponins are known for their adverse and, possibly even toxic effects (Wiedenfield and Edgar, 2011; Dorne and Gremmels, 2013). On the other hand, there are numerous phytochemicals, particularly phenolic compounds, are associated with human health benefits such as antioxidant, antimutagenic, anti-inflammatory, antimicrobial, antiviral, anti-allergic, immunoprotective and ultraviolet (UV) filtering properties (Dillard and German, 2000; Yao et al., 2004). The above benefits in combination with a growing concern about synthetic pesticides make plant secondary metabolites, when carefully selected, highly valuable compounds for crop protection.

Strategies to identify defensive metabolites

The search for insecticidal biopesticides requires screening of naturally occurring bioactive compounds in plants. Plant secondary metabolites have a wide spectrum of activity against pest insects and are known to affect them at cellular, tissue and organism levels. Thus, it is of utmost importance to study the behavioural patterns in insects to unravel the underlying mechanism responsible for the proclaimed insecticidal activity. Secondary metabolites can have direct implications on insect populations by acute toxicity however, they may also affect population dynamics by impairing important biological traits through physiological and behavioural sublethal effects such as reduced fecundity, malformations and delayed development.

Metabolomics

Secondary plant metabolites represent a diverse group of low-molecular mass structures which makes comprehensive analysis a difficult analytical challenge (Barah and Bones, 2015). Bio-assay guided

fractionation is a well-established platform to isolate and characterize active constituents present in plant extracts. However, besides the tedious and time consuming process, another important drawback of this approach is the potential loss of synergistic functions of metabolites during the purification steps. Until recently, studies of phytochemicals were mainly restricted by methods allowing only such reductionist approaches (Hall, 2006). In the past decades, a new field of science, known as 'system biology', emerged. This holistic approach, collectively placed under the umbrella metabolomics, stands in contrast to the traditional reductionist approach. Metabolomics aims to comprehensively identify and quantify metabolites in a high-throughput, non-biased manner, rather than focusing on a pre-determined small set of metabolites or a specific class of chemical molecules (Kuhlisch and Pohnert, 2015).

The significant advances in a variety of analytical platforms have enabled the detection and characterization of such chemically diverse structures. Among them, mass spectrometry (MS) in combination with liquid (LC) and gas chromatography (GC), as well as nuclear magnetic resonance spectroscopy (NMR) are most widely used (Aliferis and Tokousbalides, 2011). Each method has its own advantages and limitations in terms of sensitivity, selectivity and reproducibility however, none of them is capable to detect all metabolites within a given biological sample (Verpoorte et al., 2008). Details on analytical technologies used in metabolomics have been reviewed elsewhere (Weckwerth, 2003; Allwood et al., 2011; Wolfender et al., 2013).

Eco-Metabolomics

The last decade has seen an increasing number of applications of metabolomics and has evoked considerable interest in ecological studies including the study of plant-herbivore interactions (Allwood et al. 2008; Macel et al., 2010; Leiss et al., 2011; Maag et al., 2015). Untargeted metabolomics, also known as 'metabolic fingerprinting', is well-suited for the discovery of chemical metabolic markers related to plant resistance. Commonly, phenotypic screening is used by analyzing genotypes with contrasting levels of resistance (Jansen et al., 2009; Kuzina et al., 2009, Leiss et al., 2009a,b; 2013; Mirnezhad et al., 2010; Capitani et al., 2013). The next challenge is to make sense of the wealth of data that has been generated during metabolite analysis. Therefore, computational, in conjunction with chemometric or bio-informatic tools, are crucial to process and interpret these results in a biological context (Worley and Powers, 2013). The majority of the eco-metabolomic approaches, however, are often correlative in nature. Ultimately, once metabolites are structurally elucidated, their contribution to the observed resistant phenotype needs to be demonstrated in subsequent bioassays. An example of the latter has been provided by Leiss et al. (2009a; 2013), who experimentally addressed the role of several defense metabolites to support the claim of insecticidal activity.

Insecticidal metabolites

There has been a remarkable interest in the use of biopesticides, specifically plant-based products. This paper presents a critical review of insecticidal metabolites from plant origin, identifying existing challenges as well as opportunities with regards to their use in sustainable crop protection. A literature

search was conducted to survey secondary metabolites for their insecticidal properties. After evaluating the available literature over the past two decades, 47 metabolites were selected based on their insecticidal activities. Searches were then carried out for metabolites with proven health-promoting effects. The dual activities of these compounds are highly valuable, both from an ecological and a pharmaceutical perspective. The chosen representatives discussed here include ten metabolites belonging to the phenylpropanoids and flavonoids. Table 1 summarizes their insecticidal activities on various economically important target insects and, where known, their potential mode of action.

Phenylpropanoids

Phenylpropanoids, are among the most common and widespread plant secondary metabolites involved in plant defense (Dixon, 2002). The biosynthesis of phenylpropanoids originates from phenylalanine. Followed by sequential hydroxylation and methylation reactions of *trans*-cinnamic acid, several substituted derivatives such as *p*-coumaric acid, caffeic acid, ferulic acid, chlorogenic acid and sinapic acid have frequently been implicated in plant defense against insect herbivores, including, Hemiptera, Lepidoptera, Orthoptera, Coleoptera, Thysanoptera and Diptera.

Phenylpropanoids often function as feeding deterrents and digestibility reducers (Table 1). Cell wall modifications, mainly established by hydroxycinnamic acid derivatives, may directly pose physical barriers to various insect species incorporating and cross-linking with carbohydrates (Abdel-Aal et al., 2001; Santiago et al., 2006; Leiss et al., 2013). Phenols act as pro-oxidants (Summers and Felton, 1994) whereby their oxidative products covalently bind to amino acids and proteins decreasing the digestibility of dietary proteins (Felton, 1992). In addition, the insecticidal activity of phenols also arises from inhibition of vital insect gut proteases as has been shown for caffeic, ferulic, sinapic, chlorogenic and *p*-coumaric acid (Johnson, 2005; Joshi et al., 2014).

Flavonoids

Flavonoids represent one of the most studied classes of phenylpropanoid-derived metabolites and are found ubiquitously in plants (Harborne, 2001; Simmonds, 2001). Structurally, flavonoids consist of several classes such as flavones (e.g., luteolin, rutin), flavonols (e.g., kaempferol, quercetin), flavanones (e.g., naringenin) and others.

Flavonoids have many complex roles in plant-insect interactions (Simmonds, 2001). A number of flavonoids and some glycosides have been investigated as feeding deterrents, digestibility reducers or as metabolic toxins against many insect pests (Treutter, 2006; Mierziak et al., 2014). Negative effects of flavonoids on herbivore survival as well as performance including growth and fecundity have been demonstrated by artificial diet experiments or *in planta*. Rutin and quercetin represent model phenolics in the study of plant defense compounds due to their abundant occurrence and well documented toxicity to numerous insect herbivores. However, despite the importance of flavonoids in plant-insect interactions, detailed understanding of how they modulate resistance at the biochemical and molecular levels remains largely unknown (Simmonds, 2003).

As with many chemicals, the dosage often determines the degree of effect it produces. Depending on the insect species, rutin and quercetin, at varying doses, elicited variable behavioral responses and provoked both negative as well as stimulating effects on herbivore feeding (Simmonds, 2003; Jadhav et al., 2012; Goławska et al., 2012; Diaz Napal et al., 2015). Another level of complexity is posed by the fact that defense responses to a specific compound can often be modulated by the presence of other compounds. For example, methanol extracts of *Lonicera maackii*, dominant in the flavonoid luteolin, deterred feeding of the generalist herbivore *Spodoptera exigua*. However, when offered as individual compound in diet plugs, no anti-herbivory activity was observed. Instead, luteolin was marginally stimulating feeding (Cipollini et al., 2008). Onyilagha et al. (2012) provided strong evidence of defensive synergies among different flavonoids. While individual metabolites minimally deterred flea beetle feeding, combined flavonoid fractions were more effective in feeding deterrence. This highlights the importance of complex matrices of plant extracts in which active substituents might act additively or synergistically.

Table 1. Plant defense secondary metabolites identified for their potential contribution to resistance against various target insect pests.

Class	Secondary metabolite	Subclass	Target insect	Main observed effect	Reference
Phenylpronanoid	Caffeic acid	Hydroxycinnamic acid	*Acanthoscelides obtectus* Coleoptera: Bruchidae	Knockdown effect: reduced mobility and increased mortality in adults (*in-vitro* bioassay)	Regnault-Roger et al., 2004
			Helicoverpa zea Lepidoptera: Noctuidae	Prooxidant : oxidative stress in midgut proteins Development rate, survivorship and pupal weight negatively influenced at 4 mM	Summers and Felton, 1994
			Sitobion avanae Homoptera: Aphididae	Reduction of phloem sap ingestion at 2.5 mM	Leszczynski et al. 1995
			Helicoverpa armigera Lepidoptera: Noctuidae	Protease inhibitor. Body weight and survival significantly reduced in the range of 50-200 ppm	Joshi et al., 2014
	Chlorogenic acid	Monolignols	*Dalbulus maidis* Hemiptera: Cicadellidae	Antifeedant activity in adults at 400 ppm (*in-vitro* bioassay)	Dowd and Vega, 1996
			Plagiodera versicolora Coleoptera: Chrysomelidae	Antifeedant activity above 50 µg/ disk	Jassbi, 2003
			Agelastica alni Coleoptera: Chrysomelidae	Feeding deterrent at concentration of 2.48% by weight (dual choice bioassay)	Ikonen et al., 2002
			Trichoplusia ni Lepidoptera: Noctuidae	Reduced larval growth and development at 100 ppm (2.8 mM) and 1000 ppm (28 mM)	Beninger et al., 2004
			Lymantria dispar Lepidoptera: Lymantriidae	Reduced larval growth and development at 100 ppm (2.8 mM) and 1000 ppm (28 mM)	Beninger et al., 2004
			Spodoptera litura Lepidoptera: Noctuidae	Developmental retardation and increased larval mortality (>60 %) at 0.7-0.8 µg/ml	Mallikarjuna et al., 2004
			Frankliniella occidentalis Thysanoptera: Thripidae	Reduced relative growth rate and survival of first instar larva at 5% (5 mg/ml)	Leiss et al., 2009a
			Helicoverpa zea Lepidoptera: Noctuidae	Prooxidant: chronic exposure at 4 mM inducedoxidative stress in midgut proteins. Development rate and pupal weight are negatively influenced	Summers and Felton, 1994
	Ferulic acid	Monolignols	*Acanthoscelides obtectus* Coleoptera: Bruchidae	Knockdown effect: reduced mobility and increased mortality of adults at day 8 (*in-vitro* bioassay)	Regnault-Roger et al., 2004
			Choristoneura fumiferana Lepidoptera: Tortricidae	Oviposition deterrent 7.8 nmol/cm^2 (dual choice bioassay)	Grant and Langevin, 2002

			Sitodiplosis mosellana Diptera: Cecidomyiidae	Correlation study: positive correlation to resistance. Floret infestation lower in resistant cultivar (Arin cv contains 70.5 mg/100 g DW ferulic acid)	Abdel-Aal et al., 2001
			Sesamia nonagrioides Lepidoptera: Noctuidae	Correlation study: ferulic acid concentration was correlated with the resistance level in the genotypes Structural resistance: cell wall fortification and lignification	Santiago et al., 2006
			Rhopalosiphum padi Homoptera: Aphididae	Constitutive defense of resistant cultivar Regina and induced	Havlickova et al., 1996; 1998
			Sitobion avanae Homoptera: Aphididae	Reduced sieve element salvation at 2.5 mM	Leszczynski et al. 1995
				Prolonged reproductive period and reduced daily fecundity	Chrzanowski et al., 2012
			Sitodiplosis mosellana Diptera: Cecidomyiidae	Constitutive levels exceeding 0.35 µg/g fresh weight (1.57 µg/g dry weight) increased mortality of newly hatched larvae	Ding et al., 2000
			Dalbulus maidis Hemiptera: Cicadellidae	Antifeedant activity in adults at 400 ppm (*in-vitro* bioassay)	Dowd and Vega, 1996
Phenylpropanoid	p-Coumaric acid	Monolignols	*Etiella zinckenella* Lepidoptera: Pyralidae	Oviposition deterrent at 0.5 mg	Hattori et al., 1992
			Rhopalosiphum padi Homoptera: Aphididae	Constitutive Defense; higher concentration in resistant cultivar Regina	Havlickova et al., 1996; 1998
			Sesamia nonagrioides Lepidoptera: Noctuidae	Structural resistance: cell wall fortification and lignification	Santiago et al., 2006
			Sitobion avanae Homoptera: Aphididae	Reduction ingestion of phloem sap	Leszczynski et al. 1995
			Dalbulus maidis Hemiptera: Cicadellidae	Antifeedant activity in adults at 400 ppm (*in-vitro* bioassay)	Dowd and Vega, 1996
	Scopoletin	Coumarin	*Zygogramma exclamationis* Coleoptera: *Chrysomelidae*	Phytoalexin: feeding deterrent. Topical application of 100 µg/ml reduces preference	Roseland and Olson, 1997
			Sitobion avanae Homoptera: Aphididae	Reduced ingestion of phloem sap at 2.5 mM	Leszczynski et al. 1995
			Plutella xylostella Lepidoptera: Plutellidae	Antibiosis > 2500 µg/g diet growth inhibitor $LD_{50} \approx 8300$ µg/ ml	Peterson et al., 2003
			Spilarctia obliqua Lepidoptera: Noctuidae	Antifeedant and growth inhibitor. At 250 µg/g deterrence and growth inhibition was similar to that of azadirachtin	Tripathi et al., 2011

Table 1. Continued

			Coptotermes formosanus Isoptera: Rhinotermitidae	Termicidal and antifeedant effects at 5 µmol	Adfa et al., 2010
			Anopheles arabiensis *Diptera*: Culicidae	Repellent activity at 100 µg/ml	Narayanaswamy et al., 2014
Phenylpropanoid	Sinapic acid	Monolignols	*Choristoneura fumiferana* Lepidoptera: Tortricidae	Oviposition deterrent at 78.6 nmol/cm^2	Grant and Langevin, 2002
			Delia radicum Diptera: Anthomyiidae	Oviposition deterrent; 10 mM in buffered solution reduced number of eggs by 60-70%	Jones and Finch, 1988
			Rhopalosiphum padi Homoptera: Aphididae	Constitutive defense; higher expression correlated with resistance in cultivar Regina (lower reproduction rate). Induction upon feeding on resistant cultivar	Havlickova et al., 1996; 1998
			Sitobion avanae Homoptera: Aphididae	Reduced ingestion of phloem sap at 2.5 mM	Leszczynski et al. 1995
			Frankliniella occidentalis Thysanoptera, Thripidae	Significant increase in mortality of first instar larvae at 25% of natural plant concentrations (0.53 mg/g)	Leiss et al., 2013
	t-Cinnamic acid	Monolignols	*Mechoris ursulus* Coleoptera: Attelabidae	Contact toxicity: mortality of 86.7 % at a rate of 5 mg/paper	Park et al., 2000
Flavonoid	Luteolin	Flavones	*Acyrthosiphon pisum* Hemiptera: Aphididae	Probing and feeding deterrent at 100 µg cm^{-3}	Goławska and Łukasik, 2012
			Frankliniella occidentalis Thysanoptera: Thripidae	Significant increase in mortality of first instar larvae at 25% of natural plant concentrations (0.53 mg/g)	Leiss et al., 2013
	Luteolin-7-glucoside		*Acanthoscelides obtectus* Coleoptera: Bruchidae	Toxin: Knockdown effect: reduced mobility and increased mortality	Regnault-Roger et al., 2004
	Luteolin 7-O- β -D-apiofranosyl-(1→2)- β-D-glucopyranoside		*Liriomyza trifolii* Diptera: Agromyzidae	Ovipositional deterrent at 4.90 µg/cm^2	Kashiwagi et al., 2005
	Methyl-luteolin glycoside 2		*Phratora vulgatissima* Coleoptera: Chrysomelidae	Constitutive defense: Negative correlation with oviposition in *Salix* F2 hybrids	Torp et al., 2013
	Rutin	Flavones	*Acanthoscelides obtectus* Coleoptera: Bruchidae	Knockdown effect in bioassay: reduced mobility and increased mortality of adults	Regnault-Roger et al., 2004

Flavonoid			*Anticarsia gemmatalis* Lepidoptera: Noctuidae	Larval development, amount of food consumed, and pupal weight were negatively influenced at 0.65% (mg/ml)	Salvador et al., 2010
			Phyllotreta cruciferae Coleoptera: Chrysomelidae	Feeding deterrent of 1 month old flea beetle (binary feeding bioassay)	Onyilagha et al., 2012
			Spodoptera litura Lepidoptera: Noctuidae	Developmental retardation and increased larval mortality (>60 %) at 0.7-0.8 µg/ml	Mallikarjuna et al., 2004
			Lymantria dispar Lepidoptera: Lymantriidae	Larval growth inhibitor. Larval weight reduced by 45% at 0.05% at day 15	Beninger and Abou-Zaid, 1997
			Helicoverpa armigera & *Helicoverpa* zea Lepidoptera: Noctuidae	Feeding deterrent at 10^{-3} M	Simmonds, 2003
			Helicoverpa armigera Lepidoptera: Noctuidae	Cessation of feeding inhibits larval growth (1 µg/ml)	Jadhav et al., 2012
			Spodoptera litura Lepidoptera: Noctuidae	Larval development arrested, increased pupal mortality and malformation of adults at 1 µg/ml	Jadhav et al., 2012
	Kaempferol glycosides	Flavonols	*Frankliniella occidentalis* Thysanoptera: Thripidae	Constitutive defense. Resistance in *Senecio* F2 hybrids correlates with kaempferol-glucoside	Leiss et al., 2009b
			Mamestra configurata Lepidoptera: Noctuidae	Kaempferol-3,7-diglucoside acts as feeding deterrent when applied at concentration > endogenous levels ; 0.05–0.45 µmol per leaf disk(choice feeding assay)	Onyilagha et al., 2004
			Pieris brassicae Lepidoptera: Pieridae	Reduced levels of kaempferol 3-O-rhamnoside 7-O-rhamnoside, mediated by overexpression of MYB75, increased susceptibility to feeding. Growth negatively affected when applied exogenously at 100 µM.	Onkokesung et al.,2014
	Quercetin	Flavonols	*Spodoptera litura* Lepidoptera: Noctuidae	Developmental retardation and increased larval mortality (>60 %) at 0.7-0.8 µg/ml	Mallikarjuna et al., 2004
			Lymantria dispar Lepidoptera: Lymantriidae	Larval growth inhibitor. Larval weight reduced by 33% at 0.05% at day 15	Beninger and Abou-Zaid, 1997
			Acyrthosiphon pisum Hemiptera: Aphididae	Probing and feeding deterrent at 100 µg cm^{-3}. At higher concentration mortality and development time are increased whereas fecundity is decreased	Goławska et al., 2014

Table 1. Continued

Flavonoid	*Spodoptera frugiperda* Lepidoptera: Noctuidae	Feeding deterrent (57 %) at 100 µg/cm^2	Diaz Napal et al., 2015
		78% Larval mortality at 0.1 mg/g	Gallo et al., 2006
	Helicoverpa armigera Lepidoptera: Noctuidae	Negative effect on growth reducing larval weight by ~50 % at 16 mg/g development reducing pupation by 50% at 8 mg/g) and survival (>80% mortality at 32 mg/g)	Li et al., 2016
		Deleterious effects growth, pupation and adult emergence, and survivalat 3 and 10 mg/g	Liu et al., 2015
	Bactrocera cucurbitae Diptera: Tephritidae	Growth regulator and or deterrent: reduction in food assimilation as well as larval and pupal weight at 125 ppm	Sharma et al., 2013
	Aphis fabae Hemiptera: Aphididae	Inhibitory activity on progeny deposition 0.1 M	Lattanzio et al., 2000

Secondary metabolites: when poor solubility becomes an issue

Plant secondary metabolites represent a new generation of green insecticides with potential opportunity for commercial utility in agriculture (Adeyemi, 2010; Dayan et al., 2009). However, the majority of secondary metabolites are poorly water soluble, thus limiting their application as crop protection agents. The results of our literature search indicate that 78% of the selected insecticidal metabolites are poorly water soluble. For products such as pharmaceuticals, efficacy often depends on the effective solubility of the active ingredient to ensure proper dispersion. Solubility, therefore, presents a key prerequisite for ensuring successful formulation. Numerous candidates fail to reach commercialization due to solubility problems (Lipinski, 2002). The literature on solubility issues regarding agrochemicals is relatively scarce, however, we can safely assume that the same dilemma exists there. To illustrate this statement, we summarized the aqueous solubilities of the ten representative metabolites in Table 2. Discrepancies of water solubility measurements reported in the literature may be attributed to one or more of the following: compound purity, particle size, analytical method employed or time allowed for equilibrium conditions to be reached. As such, the reported water solubilities were retrieved from a database using ALOPGS 2.1 as a modeling software program predicting aqueous solubility.

Descriptive terms are often used to designate solubility and usually refer to ranges of solubility rather than providing detailed information on true solubilities. According to the United Stated Pharmacopeia (USP30), the water solubility of a 'slightly soluble' compound ranges from 10 mg/ml down to 1 mg/ml whereas 'very slightly soluble' compounds are defined as 1 mg/ml to 100 µg/ml. In addition, interpretation of the term 'poorly-soluble compound' can vary, depending on an individual's definition. Therefore, the term low solubility in this review is defined as the aqueous solubility of a compound that falls into the range of 'slightly soluble' and below (i.e. < 10 mg/ml). There are various

Table 2. Solubility data of plant secondary metabolites in water. Data retrieved from ALOPGS 2.1. According to the United Stated Pharmacopeia (USP30), the water solubility of a 'slightly soluble' compound ranges from 10 mg/ml down to 1 mg/ml whereas 'very slightly soluble' compounds are defined as 1 mg/ml to 100 µg/ml.

Designation	Secondary metabolite	Solubility [mg/ml]
Very slightly soluble	Ferulic acid	0.906
	Kaempferol	0.178
	Luteolin	0.138
	Sinapic acid	0.631
	Quercetin	0.261
Slightly soluble	Caffeic acid	1.61
	Chlorogenic acid	3..4
	t-Cinnamic acid	1.15
	p-Coumaric acid	1.02
	Rutin	3.54
	Scopoletin	2.35

methodologies available to enhance the aqueous solubility of chemical compounds (Savjani et al., 2012). In this paper, we discuss the potential of a recently developed green solvent, known as NADES, to improve the solubility of poorly soluble defensive metabolites.

Deep Eutectic Solvents

As with many conventional pesticides, a low aqueous solubility often requires large amounts of organic solvents (e.g., alcohols, chlorinated hydrocarbons, arenes, and nitriles) to be used in agrochemical formulations (Anjali et al., 2010). In most cases, active ingredients of biopesticides are formulated in a similar way (Gasic and Tanovic, 2013). Considerable attention has been directed towards the reduction or elimination of organic solvents for safer handling (Knowles, 2008; EEA, 2013). This increasing environmental consciousness has led to the development of greener formulations as alternatives to hazardous organic solvents. The motivation to develop solvents that are less harmful to the environment became more apparent with the development of ionic liquids. Ionic liquids (ILs) are liquids that are entirely composed of ions with melting points lower than 100°C (Ruβ and König, 2012). For a long time ILs were hailed as green solvents for the future . ILs, however, encounter several drawbacks such as toxicity and low-biodegradability questioning their 'greenness' (Paiva et al., 2014). In the search for green alternatives, deep eutectic solvents (DES), emerged as a promising substitute for both ILs as well as organic solvents (Alonso et al., 2016). The term DES was first introduced by Abbott more than a decade ago (Abbott, 2003). His pioneer work led to the discovery of liquids with unique physicochemical properties that were obtained by mixing two solids. A classical example is the mixture of urea (melting point 133°C) with choline chloride (melting point 302 °C). Figure 1 illustrates the phase diagram of the urea-choline chloride system. At a molar ratio of 2:1 (urea-choline chloride) a eutectic mixture is formed at 12°C.

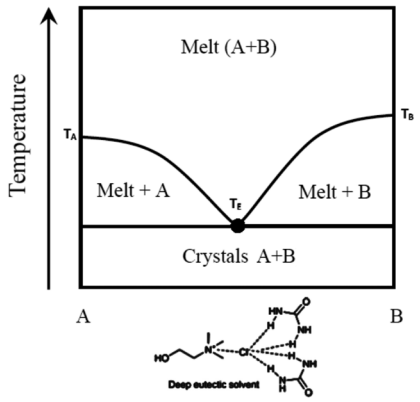

Figure 1. Schematic phase diagram of binary chemical mixture. Urea (A), at the far left of the diagram has melting temperature (T_A) of 133°C. Choline chloride (B), at the far right of the diagram has a melting temperature (T_B) of 302 °C. The liquidus lines slope downwards the melting points of the pure components and meet at a point known as the eutectic point (indicated by the black dot). Deep eutectic liquids (T_E) have melting points far below any of the starting materials.

As the research interests in deep eutectic solvents grew in the past decade, many innovative applications of DES emerged including, among others, in analytical chemistry (Karimi et al., Zheng et al., 2014) biotechnology (de Maria et al., 2011), extraction and separation processes (Bi et al., 2013; Dai et al., 2013), nanotechnology (Abo-Hamad et al., 2015), drug delivery (Morrison et al., 2009; Aroso et al., 2016), polymerization (del Monte et al., 2014) and electrochemistry (Nkuku and LeSuer, 2007). The field of DES is growing at a fast rate, with an increased interest in natural and bio-renewable compounds (Espino et al., 2016). Following the pioneering work of Abbot, the concept of DES was extended to numerous combinations of natural origin (Abbott et al., 2004; Imperato et al., 2005; Choi et al., 2011; Ruβ and König, 2012). More recently, a wide range of bio-based compounds for constructing a new class of innovative green solvents, known as natural deep eutectic solvents (NADESs) were discovered (Choi et al., 2011). This revolutionary class of non-toxic media provides a novel biotechnological solution to deal with scarcely water soluble metabolites.

Natural Deep Eutectic Solvents

NADES are commonly based on naturally occurring plant compounds, in particular primary metabolites. It has been hypothesized that, in analogy to synthetic ionic liquids, living organisms contain a third liquid medium as an alternative to water and lipids. This hypothesis arose from the observation that many solid primary metabolites became liquid when mixed in a certain ratio, suggesting that DES had long been invented by nature itself. The occurrence of NADES, intracellularly, helps to explain certain biochemical processes such as the biosynthesis, storage and transport of compounds which otherwise would be difficult to solubilize (Choi et al., 2011). More than 100 stable combinations of NADES were designed, based on particular molar ratios of two compounds such as amino acids, organic acids, sugars or choline derivatives (Dai et al., 2013). Water is often incorporated as a third component and is strongly retained in the solvent (Choi et al., 2011). Due to the generation of intramolecular hydrogen bonds the resulting NADES displays a high melting point depression causing the solids to liquefy. This eutectic mixture, which is characterized with a melting point temperature that is far below its individual precursors, remains fluid at room temperature.

NADES as designer solvents

Modifying the nature and molar ratio of the compounds allows to customize properties in order to meet specific requirements hence, the accolade ‘designer solvents’. The plethora of possible combinations can therefore, be seen as a huge opportunity to find a suitable solvent for any application (Francisco et al., 2013). Choline chloride, an inexpensive, non-toxic, and biodegradable quaternary ammonium salt, is by far one of the most dominant constituents used in the field of deep eutectic solvents. Previously known as vitamin B_4, it has some important key functions in the human body. Choline chloride serves as building block for membrane phospholipids and as precursor of the neurotransmitter acetylcholine (Ueland, 2011).

Among the available preparation methods, the heating and stirring method is the most common one for preparing eutectic solvents (Espino et al., 2016). This easy method simply requires mechanical stirring of solid starting materials while heating at moderate temperature until a homogenous liquid is formed. Besides the ease of preparation, NADES offer several other advantageous qualities as solvents such as a wide liquid range, water compatibility, low toxicity, non-flammability, biocompatibility and low vapor pressure (Paiva et al., 2014).

Physicochemical properties of NADES

Being designer solvents, the physicochemical properties of NADES, e.g. melting point, density, viscosity, acidity, and hydrophobicity, are highly tunable (Dai et al., 2015). Solvent polarity is an important parameter in chemistry that characterizes how a solvent interacts with solutes. Typically, solvents can be classified into three main categories: non-polar solvents (hydrocarbons), polar protic solvents (e.g. water, alcohols) and polar aprotic solvents (e.g. DMSO, acetone). The solvation properties of NADES cover a wide range of polarity. Mixtures of organic acids were most polar, followed by amino acids based NADES whereas polyalcohol based NADES are least polar, displaying a polarity similar to that of methanol (Dai et al., 2013). Nonetheless, the majority of NADES are generally hydrophilic due to their hydrogen bonding ability. Consequently, hydrophobic eutectic mixtures based on menthol and fatty acids have now come to the fore (Ribeiro et al., 2015; Osch et al., 2015). Another important physical property is viscosity. NADES tend to be fairly viscous when compared to traditional organic solvents, which forms a disadvantage for practical applications. The strong hydrogen interactions, which are the key to NADES formation, promote these high viscosities. Both properties, polarity and viscosity, may be modulated by the addition of water (Dai et al., 2013). The viscosity of NADES is significantly decreased upon dilution with water, while still maintaining its supermolecular structure (Dai et al., 2015).

Green Defense Against Pests

Driven by legislation and evolving societal attitudes concerning environmental issues, the search for safe and green products has been increasing continuously. As a contribution to such efforts, we present an alternative green approach which involves the use of insecticidal crop protection agents and solvents from plant-origin.

Green formulations: Improving solubility with NADES

Chemical formulations, especially during earlier phases of research and development, mostly start with the evaluation of their general suitability prior to launching into full development (Sasson et al., 2007). One of the most frequently asked questions that scientist face in technical fields relating to chemical formulation of compounds concerns the solubility of a specific active ingredient. While at first glance the answer seems to be just a simple number, it is one of the most critical pre-formulation parameters that has a significant impact on the performance of a molecule. In this paper, we

demonstrated the potential of NADES as a promising sustainable solvent for improving the solubility of several resistance related secondary metabolites.

NADES has several important advantages, particularly the high solubilizing capacity of both polar and non-polar compounds. The solubility of the poorly soluble insecticidal metabolites rutin, quercetin and *trans*-cinnamic acid was significantly increased as compared to the aqueous solubility (Dai et al., 2013; Dai et al., 2015). The strong hydrogen bonding between NADES and the solutes did not only cause this huge increase in solubility but also contributed to the stability of secondary metabolites under various conditions such as high temperature, light and storage time (Dai et al., 2014).

As an extension of these studies, we have investigated the solubility of six insecticidal metabolites in a variety of NADES. The solvent selection framework consisted of the following steps:

1. Preliminary solvent screening: a pre-selected set of 20 NADES was used as a starting point to identify and rank potential NADES candidates (Table 3). Selection constraints were imposed on important properties such as viscosity and stability.
2. Secondary screening: solubility tests were performed to determine the best solvent for each of the metabolites. Saturated solutions, generated by adding an excess amount of each compound to different NADES, were kept under constant stirring (1100 rpm) for 24 hours at 50 °C. The 20 candidates were ranked in decreasing order of solvent power.
3. Solubility verification: For the verification of solubilities, samples were centrifuged for 5 min at 2000 rpm and subsequently diluted suitably with methanol for spectrophotometric analysis (UV-1800 UV-VIS spectrophotometer, Shimadzu Europe GmbH, Duisburg, Germany). Five different concentrations, in the range of 3 – 35 µg/ml, were prepared in triplicate to construct a standard curve. Corresponding solubilities, analysis wavelengths, linear range and correlation coefficient (r^2) are presented in Table 4.

Among the pre-screen solvents, lactic acid: 1,2-propanediol in a molar ratio of 2:1 (abbreviated by LAP 2:1) and 1,2-propanediol: choline chloride: water in molar ratios of 1:1:1 and 1:1:3 (abbreviated PCH 1:1:1 or 1:1:3) have demonstrated considerable improvement in solubility. Results of solubility studies indicated that, enhancements with NADES, as compared to aqueous solubility, were more than 29, 34, 35, 95 and 195 fold in cases of chlorogenic acid, sinapic acid, ferulic acid, luteolin and quercetin, respectively.

A major advantage of the high solubilizing power is that it allows a high degree of flexibility in tailoring dosage treatments. However, an important drawback of NADES that might constrain the applicability, is its' high viscosity. While modifiers such as water can be used to reduce the high viscosity, it also significantly affects the solubility (Dai et al., 2014). The solubility of rutin, for example, was increased by 5-fold in PCH (1:1:3) compared to water (Table 2 and 4). For non-polar compounds, the highest solubility is achieved in pure NADES, whereas solutes with a medium polarity such as rutin display a higher solubility when diluted with 5-10% of water. Increasing the water content to 25% or 50% (v/v) drastically reduced the solubility, which presumably is attributed to the loss of the supermolecular

structure of NADES (Dai et al., 2013) and provides an explanation of the less pronounced enhancement of rutin in PCH (1:1:3). Interestingly, the insecticidal metabolites *trans*-cinnamic acid, caffeic acid and *p*-coumaric acid can also be included as hydrogen bond donors for the formation of NADES (Maugeri and de Maria, 2012).

Concluding remarks: the way forward for sustainable agriculture

The pest control industry is constantly searching for innovative approaches that advance the way we manage pest insects. This emerging need has created a significant market opportunity for alternative and bioactive products such as plant-derived metabolites. The interest in phytochemicals extends beyond their natural function as defensive weapons against insect attack as many appear to provide numerous desirable health benefits. Central to the control of pest insects is the question of how these green substituents can be formulated and promulgated. The current review, therefore, presents a simplified guide from plants to practice. This approach comprises the following three

Table 3. Different combinations of natural deep eutectic solvents.

	NADES components	Molar ratio		NADES components	Molar ratio
1	1,2-propanediol: choline chloride: water	1: 1: 1	11	Lactic acid: 1,2-propanediol	2: 1
2	1,2-propanediol: choline chloride: water	1: 1: 3	12	Lactic acid: glucose: water	5: 1: 3
3	β-Alanine: citric acid: water	1: 1: 3	13	Lactic acid: β-Alanine: water	1: 1: 3
4	Betaine: citric acid: water	1: 1: 5	14	Malic acid: sorbitol: water	1: 1: 3
5	Fructose: chloline chloride: water	1: 1: 3	15	Malic acid: L-serine: water	1: 1: 3
6	Glucose: choline chloride: water	2: 5 : 5	16	Malic acid: choline chloride: water	1: 1: 2
7	Glucose: citric acid: water	1: 1: 5	17	Proline : malonic acid : water	1: 1: 6
8	Glycerol: choline chloride: water	2: 1: 1	18	Xylitol: choline chloride: water	1: 1: 2
9	Lactic acid: choline chloride	1 : 1	19	Xylitol: choline chloride: water	1: 2: 3
10	Lactic acid: 1,2-propanediol	1 : 1	20	Xylitol: citric acid: water	1: 1: 3

Table 4. Solubility of insecticidal metabolites (mg/g) in different Natural Deep Eutectic Solvents. Data represented as mean ± SD, n = 3.

Secondary metabolite[a]	NADES composition[b]	Water %	λ max	Linearity range	Correlation coefficient	Solubility [mg/g]
Sinapic acid	LAP 2:1	–	325 nm	3 – 12.5 ug/ml	0.9981	21.59 ± 0.24
Chlorogenic acid	LAP 2:1	–	325 nm	3 – 20 ug/ml	0.9994	100.92 ± 0.57
Luteolin	LCW 1:1:1	7.71%	348 nm	3 – 15 ug/ml	0.9941	13.08 ± 0.86
Rutin	LCW 1:1:3	20.04%	359 nm	3 – 35 ug/ml	0.9961	18.46 ± 0.67
Quercetin	LCW 1:1:1	7.71%	375 nm	3 – 20 ug/ml	0.9975	44.34 ± 0.76
Ferulic acid	LAP 2:1	–	322 nm	3 – 12.5 ug/ml	0.9901	31.92 ± 0.70

[a] Luteolin was purchased from Chengdu pharmaceutical co ltd (Chengdu, China), whereas all other metabolites were obtained from Sigma (MO, St. Louis, USA). [b] NADES abbreviations followed by molar ratio. LAP: lactic acid: 1,2-propanediol and LCW: 1,2-propanediol: choline chloride: water

essential elements: i. a robust, reliable and quantitative eco-metabolic approach to screen for bioactive metabolites, ii. a rigorous validation process to study and verify the insecticidal activity, and iii. a strategy for improving the solubility of sparingly soluble compounds.

With increasing pressures on product performance, formulation is a key technology for agrochemical companies to differentiate their products and add significant value. As such, NADES are introduced as environmentally benign solvents presenting a promising solution to enhance the solubilizing properties of poorly-soluble insecticidal metabolites.

Drug delivery systems based on eutectic mixtures have been described to increase drug bioavailability (Aroso et al., 2015; 2016). As for the implementation of these green alternatives, efforts should be made in evaluating NADES as a solvent carrier system for the delivery of these insecticidal compounds.

The application of insecticidal plant secondary metabolites as a pre-sowing treatment for seeds (e.g., coating) and cuttings (e.g., dipping) presents a promising approach to protect plants from their most vulnerable stage onwards. Rather than shying away from unknown challenges presented by new technologies, we should take the opportunity to use and develop them to improve pest control strategies. Those concerned with developing sustainable crop protection agents are therefore, highly encouraged to assess the applicability of these plant-derived alternatives.

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CHAPTER FOUR

Effect of NADES seed treatments on tomato growth and thrips resistance

Abstract

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Crop protection delivered via the seed is a promising approach to reduce the need for synthetic pesticides. Plant secondary metabolites can be harnessed for this purpose by incorporating these bioactive compounds onto the seed coat. However, an inherent problem of many of these metabolites is their low solubility, which might hamper commercial application. Natural Deep Eutectic Solvents (NADES) were used to improve the solubility of defensive secondary metabolites. This study investigated the effect of pre-sowing seed treatments on seed germination and plant performance of tomato utilizing naturally occurring plant compounds that are environmentally safe, with the final aim to apply this concept for enhancement of plant defenses against Western flower thrips (WFT). Sugar based NADES negatively affected germination and seedling survival whereas, plant dry mass and number of leaves were not influenced. Seed germination gradually decreased with increased duration of soaking. Seed treatment with sinapic acid, chlorogenic acid or beta-alanine did not reduce silver damage symptoms, despite an attempt to increase the duration of soaking. Only minimum traces of sinapic acid and chlorogenic acid were in detected in the seed by LC-MS, suggesting limited uptake through the seed coat or metabolism by the embryo. The present work indicated that the main focus of follow-up studies should shift from constitutive to inducible defenses.

Keywords: NADES, seed soaking, permeability, western flower thrips, phenolic acid

Introduction

Plants are regularly exposed to herbivore attack, and in response, they have acquired a vast array of defense mechanisms to protect themselves. Perhaps one of the most outstanding aspects of plant defense is that they vary tremendously within and among populations and species (Karban and Baldwin, 1997; Endara and Coley, 2011). Although plant defenses are very successful, they can also be costly when deployed unnecessarily. Defenses are, therefore, carefully regulated. As plants develop, from seedling to reproductive stages, their response to herbivory changes (Boege and Marquis, 2005; Barton and Koricheva, 2010). While some plants are more resistant in the early stage such as brassicas that are known to produce glucosinolates, other plants are, in general, more susceptible during the seedling stages (Barton and Hanley, 2013). Our research group has discovered a new tomato cultivar which is highly resistant to one of the most economically important pest insects worldwide, western flower thrips (WFT) (Bac-Molenaar et al., 2019). However, resistance is not fully developed in the early plant life-stage. Particularly the seedling stage is vulnerable to WFT damage. Thrips are polyphagous insects and cause extensive damage in agriculture through feeding, oviposition as well transmission of tospoviruses. The use of synthetic pesticides to control pests and pathogens is becoming increasingly less desirable. EU policies have limited the range of active substances and prompted to adopt integrated pest management (IPM) (European Commission, 2009; European Union, 2013a; 2013b). In response to the decreasing number of available pesticides, increased emphasis is placed upon the discovery of natural metabolites that could potentially serve as novel crop protection agents (Lorsbach et al., 2019).

Plants defend themselves against attack through the production of a broad range of secondary metabolites. This mechanism, known as host plant resistance, forms an important strategy in IPM programs. An eco-metabolomic approach to study chemical host plant resistance to thrips, has led to the identification of several plant secondary metabolites involved in constitutive defences (Leiss et al., 2009a,b; 2011; 2013; Mirnezhad et al., 2009). Secondary plant metabolites display a number of desirable properties useful for many commercial and industrial applications. One potential application is that of an agricultural seed treatment. Biological seed treatments are among the fastest growing sectors in the global crop protection market. The use of natural compounds having a low risk profile offers a simple and a cost-effective opportunity for sustainable pest management (Brandl, 2001; Pickett et al., 2014; Sharma et al., 2015). Utilizing these metabolites for treatments to soak seeds represents a promising approach to protect plants from the early critical young stage onwards. Interestingly, many of these compounds have received considerable attention for their positive effects in human health. As such, they are of great relevance for practical implementation in sustainable crop protection programs (Mouden et al., 2017). However, exogenous application of many of these metabolites is strongly limited by their poor aqueous solubility. We, therefore, introduce Natural Deep Eutectic Solvents (NADES) to improve the solubility of defensive secondary metabolites with the final aim of applying this concept to enhance plant defenses against WFT. NADES are bio-based green solvents composed of naturally occurring plant compounds and have proven to significantly

enhance the solubility of a wide range of secondary metabolites (Dai et al., 2013; Dai et al., 2015; Mouden et al., 2017). The approach of this study builds on the considerable progress made in our lab towards chemical host plant resistance and involves exogenous application of putative defense metabolites dissolved in NADES as a pre-sowing seed treatment for tomato (*Solanum lycopersicum*). The present study was undertaken to evaluate the effect of several NADES on tomato seed germination and plant growth performance. Subsequently, we explored the potential of NADES as solubilization vehicles for plant-derived crop protection agents against thrips using sinapic acid, beta-alanine and chlorogenic acid as model compounds. The chosen representatives were included in this study based on their proven insecticidal properties (Leiss et al., 2009a; Leiss et al.,2013; Mouden et al., 2017).

Materials and Methods

Seed treatment with NADES

Tomato seeds (*Solanum lycopersicum*, cv Moneymaker) were purchased from Intratuin (Leiden, the Netherlands). The requirements for pre-selection of NADES were related to the physicochemical solvent properties (e.g., stability, viscosity), the operational properties (e.g., solubility of metabolites) as well as the solvent function properties (phytotoxicity) of NADES. Moreover, treatments were chosen to represent the different NADES subgroups (Mouden et al., 2017). The selected NADES for seed soaking treatments were based on previous work examining the solubility of secondary metabolites as well as pilot experiments on germinabilty. The eight NADES tested here included: (1) lactic acid: 1,2-propanediol (LAP molar ratio 1:1) – (2) LAP molar ratio 2:1 – (3) 1,2-propanediol: choline chloride: water (PCW molar ratio 1:1:1) – (4) PCW molar ratio 1:1:3 – (5) glucose: choline chloride: water (GUCW molar ratio 2:5:5) – (6) glycerol: choline chloride water (GYCW molar ratio 2:1:1) – (7) lactic acid: glucose: water (LGW molar ratio 5:1:3) and (8) xylitol: choline chloride: water (XCW molar ratio 1:2:3). Tomato seeds were fully immersed in an excess amount of one of these NADES at a ratio of seeds to solution 1:8 (w:w). After 10 minutes of soaking at room temperature (RT) excess solution was drained off by sieving the seeds.

Germination

Seeds were placed in a 90 mm plastic petri dish (Fischer Scientific, Landsmeer, the Netherlands) on two layers of filter paper moistened with 3 ml MilliQ water. The petri dishes were covered with a lid and kept in a plastic bag in order to keep moisture loss minimal and were then randomly placed in a controlled climate room at 20 °C (16h light, 8h dark, 70% RH) for germination. Untreated tomato seeds served as control seeds. Germination was recorded daily for two weeks and calculated as a percentage of the number of seeds sown. The final germination percentage (G_{max}) was calculated with the following formula: (G_{max}) (%) = n / N × 100 where, n is number of germinated seeds and N is number of total seeds. The time to reach 50% germination (T_{50}) was calculated according to the following formula of Coolbear et al. (1984) modified by Farooq et al. (2005):

$T_{50} = t_i + [(N / 2 - n_i) (t_i - t_j)] / (n_i - n_j)$ where, *N* is the final number of seeds that germinated and n_i and n_j the cumulative number of seeds germinated by adjacent counts at times t_i and t_j, respectively when $n_i < N / 2 < n_j$. Seeds were considered to have germinated after examination for radicle protrusion through the seed coat under a dissection microscope. Seeds that developed fungal infection were removed and considered non-viable. Germinated seeds were also removed from the petri dish on each observation date and transplanted to 6-cell plastic tray containers. Survival percentage of the seedlings was counted when transplants were in the first leaf stage and calculated based on the number of germinated seeds (Maguire, 1962). Additionally, germination was also evaluated on soil using 90 mm plastic petri dishes containing 30 g air-dried soil moistened with 10 ml MilliQ water. For each treatment 100 seeds in four replicates of 25 seeds each were treated separately. Regular watering was done as per requirement to maintain adequate moisture necessary for germination and seedling growth.

Time dependent soaking

To determine the effect of soaking duration on germination, 100 seeds subdivided into four replicates were soaked in lactic acid: 1,2-propanediol (LAP molar ratio 2:1) at different time intervals ranging from 5 minutes to 12 hours. After these different time intervals, excess NADES was drained off and seeds were placed on pre-moistened filter paper. Based on the considerable improvements in solubility of several plant secondary metabolites with lactic acid: 1,2-propanediol (LAP molar ratio 2:1), the time-course experiment was performed only with LAP 2:1. Incubation conditions and measurement of germination were performed as stated above.

Growth

For experiments with potted plants, seeds were treated with eight different NADES as described above and grown in 11 cm diameter plastic pots filled with potting soil. Each treatment was replicated two times in a randomized block design and each replicate comprised five plants (i.e. 10 plants per treatment). Four weeks post-treatment, plant height (from the cotyledon to the top of the main plant stem), total number of leaves and dry mass were measured as an indication of plant growth.

Seed soaking and thrips bioassay

In several separate experiments the effect of the defensive secondary metabolites on thrips resistance was evaluated by soaking tomato seeds (cv Moneymaker) for 10 minutes, 30 minutes or 15 hours in 100 mg/g β-alanine, 20 mg/g sinapic acid or 80 mg/g chlorogenic acid dissolved in lactic acid: 1,2-propanediol (LAP molar ratio 2:1). The time course experiment revealed that the maximum acceptable duration of seed soaking, without adversely lowering germination percentages, was 30 minutes. However, for commercial purposes seed treatments usually attain saturation moisture content after a soaking period of 12 to 16 hours (H. Bruggink, pers. comm.). Untreated seeds and NADES soaked seeds were included as controls. Four-week old plants were individually placed in thrips-proof cages consisting of a Perspex cylinder (80 cm height, 20 cm diameter) closed at one end

with a displaceable ring of nylon gauze of 120 µm mesh size, as described in Leiss et al. (2009). Plants were randomly placed in a growth chamber at a temperature of 25 °C, a photoperiod of 16L:8D and 70% relative humidity. For thrips infestation, 20 adult thrips (18 females and 2 males) were collected in glass jars using a mouth-operated aspirator and released inside the cage. Western flower thrips were obtained from a colony reared on chrysanthemum flowers (cultivar Euro Sunny) maintained in a climate room at 25 °C and 70% RH.

Seed coat permeability of secondary metabolites

In order to study the uptake of secondary metabolites through the seed coat, the seed coat permeability for sinapic acid (SA), chlorogenic acid (CGA) and beta-alanine was investigated in permeable snap beans (*Phaseolus vulgaris*) and in semi-permeable tomato seeds (*Solanum lycopersicum*, cv Moneymaker) (Taylor and Salanenka, 2012). Twenty seeds were soaked in an excess amount of aqueous methanol or NADES for a period of 10 minutes or 15 hours. Sinapic acid and CGA were dissolved in 20% aqueous methanol at a final concentration of 2.24 and 3.54 mg/ml, respectively. For seed soaking in NADES, SA and CGA were dissolved in lactic acid: 1,2-propanediol (LAP molar ratio 2:1) at a concentration of 20 mg/g and 70 mg/g, respectively whereas beta-alanine was dissolved in water at a final concentration of 100 mg/ml. All compounds were obtained from Sigma Aldrich. After soaking, seeds were rinsed three times with aqueous methanol and sterile water to remove surface coated metabolites. Subsequently, seeds were flash frozen in liquid nitrogen, ground to a fine powder and defatted twice with hexane. A hundred mg of finely ground seed powder was ultrasonically (Branson Ultrasonics, Danbury, CT, USA) extracted with 3 ml methanol for 10 minutes. The supernatant was collected after centrifuging at 11 000 rpm for 5 minutes. The residue was further extracted twice and combined extracts were concentrated in a rotary evaporator (Büchi, Flawil, Switzerland). The resulting residue was dissolved in 1 mL aqueous methanol and subsequently filtered through a 0.45 µm PTFE syringe filter. The filtrate was stored at 4 °C prior to LC-MS-analysis. LC-MS analysis was performed using a MicrOTOF-QII (Bruker Daltonics GmbH, Germany) coupled to an Ultimate 3000 RS (ThermoScientific, Usa) UHPLC quaternary pump. Detection was carried out using electrospray ionization in either positive or negative mode over a mass range of m/z 120–1200. Extracted ion chromatograms (XIC) were created to display the intensity of pre-selected masses of m/z 255, 355, 90.1 for sinapic acid, chlorogenic acid and beta-alanine respectively.

Statistical analyses

All statistical analyses were performed using the SPSS software package (version 24; SPSS Inc., Chicago, IL, United States). All data were first analyzed using Kolmogorov–Smirnov and Levene's tests to determine normality and heteroscedasticity of variance, respectively. The effect of NADES soaking on the total number of germinated seeds, dry biomass and silver damage were analyzed by one-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc test. To evaluate the effect of seed soaking with β-alanine, sinapic acid and their combination on thrips-associated feeding symptoms, silver damage data were square root transformed prior to analysis to meet ANOVA

assumptions. When data transformations failed to reduce heteroscedasticity or normalize residuals, the nonparametric Kruskal-Wallis-test or Welch's ANOVA was used. Welch's ANOVA and Games-Howell's post hoc were used in case of unequal variances (T_{50} and number of leaves). The non-parametric Kruskal–Wallis, followed by Dunn's post-hoc test with Bonferroni correction, was used to assess significant differences in seedling survival and plant height between plants grown from NADES treated seeds and control seeds.

Results

Germination responses to NADES soaking

The time course of cumulative germination of seeds treated with eight different NADES and control on filter paper and soil are shown in figs. 1 and 2, respectively. The percentage of seeds that germinated during the 14 day period on filter paper varied significantly among treatments as determined by one-way Anova ($F(8,27) = 2.420$, $p = 0.041$). Tomato seed soaking with glucose: choline chloride: water (GUCW molar ratio 2:5:5) and lactic acid: glucose: water (LGW molar ratio 5:1:3) significantly decreased the maximum percentage of germinating seeds (G_{max}) in the filter paper experiment as compared to non-treated control seeds (Table 1). Additionally, exogenous application of each of all evaluated NADES caused a significant delay in germination (Welch's $F(8, 10.96) = 68.02$, $p < 0.001$). A right shift of the germination curve denotes an increase in the time to reach 50% of germination (T_{50}). Compared to control seeds, the T_{50} was increased by at least 0,94 days for seed soaking with lactic acid: 1,2-propanediol (LAP molar ratio 2:1), whereas a maximum significant delay of 2,86 and 3,00 days were observed for LGW and GUCW, respectively (Table 1).

Table 1. Effect of NADES seed soaking on the cumulative germination percentage of tomato seeds at day 14 (G_{max}) and time to reach 50% germination (T_{50}) comparing filter paper and soil. Data are expressed as mean ± S.E.M.

Treatment	G_{max}		T_{50}	
	Filter[a]	Soil	Filter[b]	Soil
Control	96±1.63	99.0±1.00	4.69±0.06	3.73±0.03
LAP 1:1	90±3.46	97.0±1.91	5.90±0.19	3.83±0.19
LAP 2:1	91±3.00	99.0±1.00	5.60±0.09*	3.74±0.09
PCW 1:1:1	91±5.26	100.0±0.00	6.63±0.52	3.93±0.21
PCW 1:1:3	93±3.00	98.0±1.15	6.66±0.54	4.17±0.10
GUCW 2:5:5	83±4.12*	93.0±3.42	7.22± 0.36*	4.32±0.19
GCW 2:1:1	93±1.91	98.0±1.15	7.68± 0.11*	3.99±0.25
LGW 5:1:3	75±8.06*	95.8±1.78	7.55±1.00	4.54±0.22
XCW 1:2:3	89±1.91	97.0±1.91	6.86±0.94	4.07±0.18

[a] one-way Anova following Fisher's least significant difference, LSD, test. [b] Welch ANOVA followed by Games–Howell. *P<0.05 as compared with the control group.

Furthermore, a strong inhibition of radicle elongation was observed in seeds treated with sugar based NADES (supplementary figure S1). GUCW and LGW treated seeds were covered by far more fungal hyphae and, consequently, exhibited more malformed seedlings. The delayed germination and in particular the inhibition of G_{max}, however, were far less pronounced in the soil experiment (Table 1). For tomato seeds grown on soil there were no significant differences among treatments in the final percentage of seed germination ($F(8,27) = 1.457$, $p = 0.219$) and T_{50} (Welch's F (8, 19.67) = 2.36, $p = 0.058$).

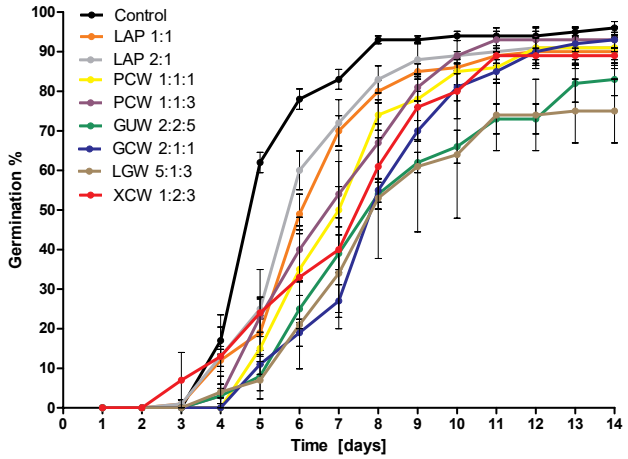

Figure 1. Seed germination curves after NADES soaking on filter paper. The black line shows the germination curve of non-treated control seeds. Line colors orange, grey, yellow, purple, green, blue, brown and red correspond to the following NADES treatments; LAP 1:1 – LAP 2:1 – PCW 1:1:1 – PCW 1:1:3 – GUCW 2:5:5 – GCW 2:1:1 – LGW 5:1:3 – XCW 1:2:3. Data points are expressed as the mean ± S.E.M.

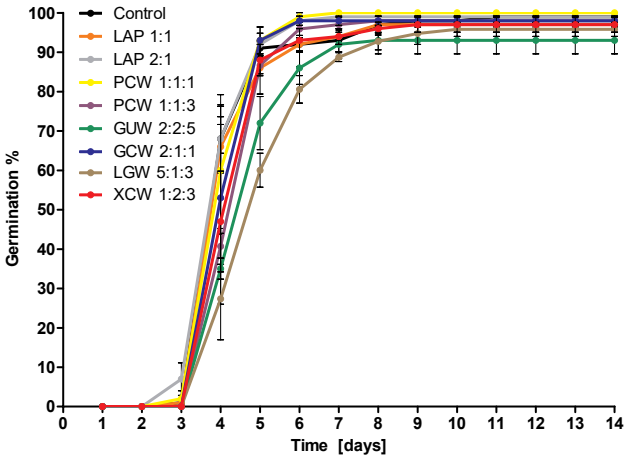

Figure 2. Seed germination curves after NADES soaking on soil. The black line shows the germination curve of non-treated control seeds. Line colors orange, grey, yellow, purple, green, , blue, brown and red correspond to the following NADES treatments; LAP 1:1 – LAP 2:1 – PCW 1:1:1 – PCW 1:1:3 – GUCW 2:5:5 – GCW 2:1:1 – LGW 5:1:3 – XCW 1:2:3. Data points are expressed as the mean ± S.E.M.

Time dependent seed soaking

Various durations of soaking along with non-treated control seeds were used to determine the critical soaking duration (Figure 3). Soaking duration had a significant effect on total germination after 14 days ($F(8,27) = 148.322$, $p < 0.001$). A clear delay in the time required to achieve 50% of germination (T_{50}) was already observed after 5 minutes of soaking. Strong negative effects on germination were particularly evident when seeds were subjected to a minimum soaking duration of 30 min. Seed germination decreased dramatically with the duration of NADES treatment up to 12 hours. Germination percentages ranged between 10% for 6 and 12 hours soaking and 100% for 10 minutes soaking (Table 2).

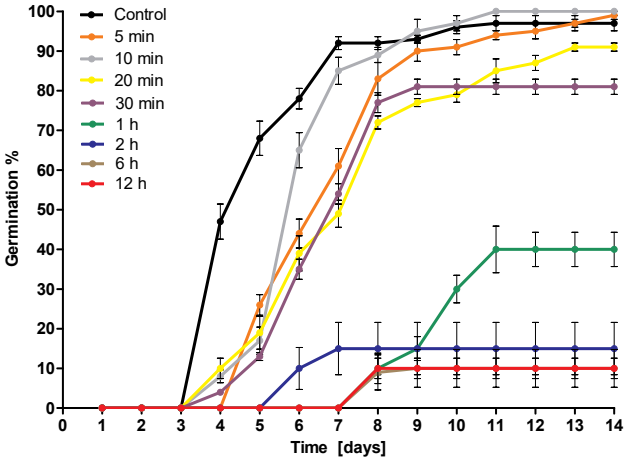

Figure 3. The effect of duration of soaking in lactic acid: 1,2-propanediol (LAP molar ratio 2:1) on germination of tomato seeds, cv. Moneymaker. Data points are expressed as the mean ± S.E.M.

Table 2. Effect of lactic acid: 1,2-propanediol (LAP molar ratio 2:1) soaking duration on the maximum germination percentage after 14 days . Data are expressed as mean ± S.E.M.

Soaking duration	Germination %
Control	97±1.91
5 min	99±1.00
10 min	100±0.00
20 min	91±1.00
30 min	81±1.91*
60 min	40±4.32*
2 hours	15±6.61*
6 hours	10±4.76*
12 hours	10±2.58*

*P<0.05 as compared with the control group (one-way Anova following Fisher's least significant difference, LSD, test).

Influence of NADES on growth parameters

Significant differences in percentage of seedling survival after NADES seed treatment were observed (Table 3). A significant effect was observed among the mean ranks (H = 24.876, p = 0.002, df = 8). Plants grown from seeds treated with GUCW 2:5:5, LGW 5:1:3 and XCW 1:2:3 had significantly lower seedling survival than control plants.

The results for plant growth parameters of surviving seedlings are summarized in table 4. Dry mass and total leaf number were not significantly affected by the different NADES soaking treatments (F(8,81) = 1.369, p = 0.223; H = 12.8965, p = 0.113, df = 8), respectively). On the contrary, a significant effect on plant height was observed for GUCW seed-treated plants (Welch's F (8, 33.57) = 18.98, p < 0.001). Plant height was significantly reduced by 21,3% as compared to the control.

Table 3. Effect of NADES seed soaking on the seedling survival percentage. Data are expressed as mean ± S.E.M.

Treatment	Seedling survival%
Control	96.92±1.03
LAP 1:1	97.86±0.62
LAP 2:1	96.73±0.55
PCW 1:1:1	92.74±1.28
PCW 1:1:3	97.86±0.62
GUCW 2:5:5	57.71±3.95*
GCW 2:1:1	97.87±0.61
LGW 5:1:3	63.70±0.77*
XCW 1:2:3	89.92±1.68*

Differences between seedling survival of NADES seed treated plants and control plants were evaluated by the Mann-Whitney U test (two-tailed). *P<0.05 was considered to indicate a statistically significant difference from control.

Table 4. Effect of NADES seed treatments on plant performance parameters. Data are expressed as mean ± S.E.M.

Treatment	Dry mass [g]	No. of leaves	Height [cm]*
Control	1.69±0.11	7.4±0.27	15.19±0.69
LAP 1:1	1.71±0.14	7.6±0.43	15.25±1.11
LAP 2:1	1.72±0.16	7.7±0.21	15.27±0.51
PCW 1:1:1	1.82±0.12	7.7±0.15	17.44±0.37
PCW 1:1:3	1.69±0.10	7.5±0.27	15.01±0.53
GUCW 2:5:5	1.33±0.12	6.6±0.22	9.97±0.45*
GCW 2:1:1	1.71±0.10	7.5±0.27	15.41±0.42
LGW 5:1:3	1.69±0.10	7.2±0.29	15.61±0.85
XCW 1:2:3	1.63±0.06	7.4±0.34	14.47±0.71

*P<0.05 as compared with the control group (Welch ANOVA followed by Games–Howell).

Thrips resistance and permeability for different metabolites

Plants grown from sinapic acid, chlorogenic acid and beta-alanine treated seeds were subjected to whole plant bioassays as a proof of concept that these secondary metabolites will be effective in enhancing thrips resistance. No significant differences in silver damage between control and treated plants were observed. At a duration of 30 minutes seed soaking with chlorogenic acid, beta-alanine and a mixture of both in LAP 2:1 tomato resistance was not improved (Figure 4A; (F(4,25) = 0.497, p = 0.738). Likewise, treatment of tomato seeds with sinapic acid, beta-alanine and their combination did not significantly affect silver damage (Figure 4B; (F(4,77) = 0.414, p = 0.798). Increasing the duration of soaking from 10 minutes to full saturation of seeds after 15 hours had no effect on silver damage either (Figure 4C; (F(3,36) = 0.298, p = 0.826) .

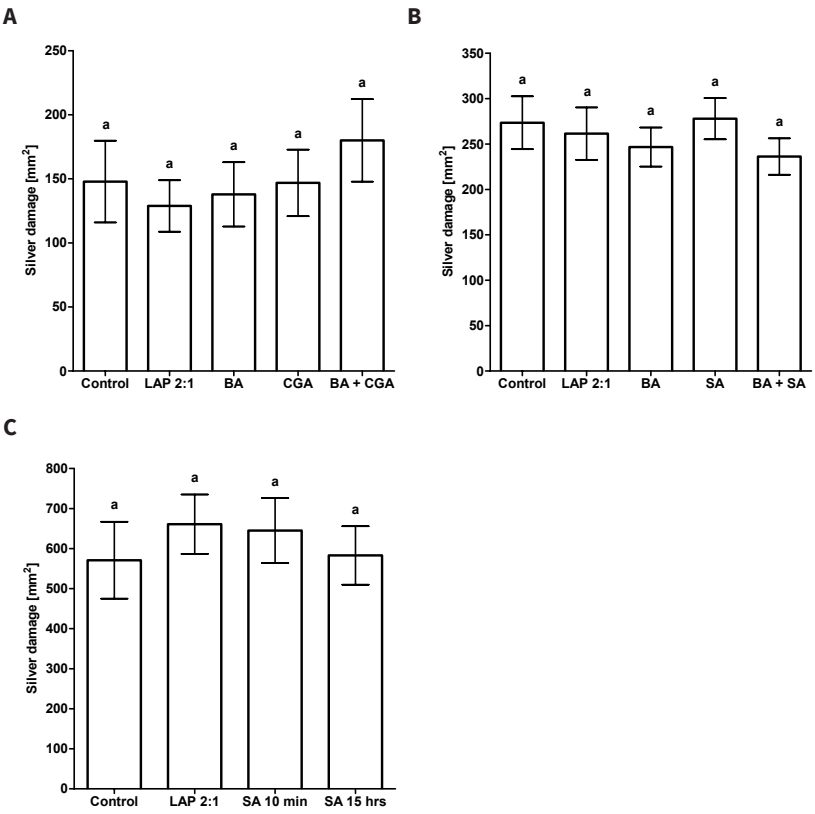

Figure 4. Effect of seed soaking with secondary metabolites on tomato resistance against western flower thrips. Four week old plants were infested with 20 thrips and thrips-associated silver damage was evaluated 7 d after thrips infestation. **(A)** Effect of 30 minutes soaking with 100 mg/g beta-alanine (BA), 80 mg/g chlorogenic acid (CGA) and a mixture of BA and CGA (mean ± SEM, n=6). **(B)** Effect of 30 minute seed soaking with 20 mg/g sinapic acid (SA) with or without BA (mean ± SEM, n=16. **(C)** Effect of duration of soaking with sinapic acid on silver damage (Mean ± SEM, n=10).

To investigate the diffusion of sinapic acid, chlorogenic acid and beta-alanine, seeds were extracted before visible germination in order to determine the actual uptake by the embryo. The uptake of these compounds was compared in semi-permeable tomato seeds and permeable snap bean seeds. After soaking the seeds in sinapic acid or chlorogenic acid, we observed no differences in the amount present in embryos when comparing permeable snap beans to semi-permeable tomato seeds. Both compounds were only detected in trace amounts. When duration of soaking was increased, uptake was not improved as similar endogenous amounts were detected (not quantified). Furthermore, no differences were detected for solvent type (NADES versus methanol) in both species. No quantitative approach was used to determine the exact concentration of metabolites because only minimum trace amounts were observed in both plant species.

Discussion

Seed treatments are routinely applied worldwide to protect seeds and early developmental stages from attack by insect pests and pathogens (Brandl, 2001). The present investigation was conducted to study the effect of various NADES seed treatments on germinability and plant performance of tomato, with the final aim of improving thrips resistance using plant-derived secondary metabolites. Seed germination and early seedling growth phases are considered critical for raising a successful crop. Germination tests showed that sugar based NADES (lactic acid: glucose: water; LGW molar ratio 5:1:3 and glucose: choline chloride: water; GUCW molar ratio 2:5:5) significantly reduced the final germination percentage in the filter paper experiment but, not when seeds were allowed to germinate on soil (Table 1). Seed germination is a key process in the plant life cycle that is regulated by both phytohormones as well as sugar signaling cascades (Koornneef et al. 2002; Rolland et al., 2006). Glucose has been well documented to play a regulatory role in seed germination and post-germination development (Smeekens, 2003; Rolland et al., 2006). Exogenous glucose application exerts a profound influence and has been shown to delay seed germination and inhibit seedling development (Gibson et al, 2005). Our observations on reduced germinability are in agreement with those reported by Gibson and coworkers (2005). A concentration-dependent delay in seed germination occurs at concentrations as low as 0.5% glucose. A high, non-physiological concentration used in NADES is, therefore, most likely explaining our experimental observation on seed germination inhibition. Intermediate concentrations (1.5–3%) of sugar alcohols such as sorbitol and mannitol have very little effect on germination, suggesting that the effect of glucose is not only explained by osmotic stress (Price et al., 2003; Dekkers et al., 2004). In contrast, NADES composed of the sugar alcohol xylitol (XCW molar ratio 1:2:3) delayed germination. Again, this result is likely explained by a far higher molar concentration used in our experiments (Figure 1).

Phytotoxicity is a significant factor in the selection of appropriate solvents. Soaking with sugar based NADES (LGW 5:1:3, GUCW 2:5:5 and XCW 1:2:3) did not only negatively influence germination, it also significantly reduced seedling survival (Table 3). Explaining these negative responses involves several considerations. Seeds in the filter paper experiment were covered by far more fungal hyphae

than seeds that were allowed to germinate on soil. Fungal exudates can reduce seed germination (Abdalla, 1970). Primary roots were generally shorter than those of control seedlings. In particular, GUCW and LGW treated seeds exhibited malformed seedlings which may have led to difficulties of seedling establishment thereby, explaining the reduction in the percentage of seedling survival (Supplementary Figure S1). Despite the favourable solubility features, NADES does show some disadvantage due to its' high viscosity. The viscous NADES film surrounding the seed could potentially limit oxygen transfer thereby, inhibiting rapid oxygen access to the surface of the seed. Oxygen availability is known to influence speed, uniformity and total percentage of germination (Kaufman, 1991). The question naturally arises as to whether duration of soaking plays a significant role and what the optimum soaking time is to leave both seed and seedlings uninjured. Seed germination gradually decreased with increasing duration of soaking. The critical duration of seed soaking with LAP 2:1 corresponds to an acceptable level of reduction in germination and was set to a threshold of maximum 30 minutes (Table 2).

Interestingly, the delayed germination and inhibition of final germination percentages (G_{max}) were far less pronounced in the soil experiment (Table 1). The physical nature of substrates may be important in explaining differences in germination response among substrate types (Blank and Young, 1992). Soil could exert a buffering effect by absorbing the viscous film layer due to a greater seed-substrate contact.

While all three sugar based NADES (LGW 5:1:3, GUCW 2:5:5 and XCW 1:2:3) reduced the maximum germination percentage and seedling survival, dry mass and total number of leaves were unaffected by NADES soaking. Treatment of seeds with glucose: choline chloride: water (GUCW molar ratio 2:5:5) significantly reduced plant height (Table 4). It can be speculated that the reduction in plant height upon GUCW seed application, while maintaining dry mass and total number of leaves, potentially induces morphological leaf changes (i.e. thicker leaves). This may form an interesting strategy to enhance resilience against biotic and abiotic stresses by reinforcement of mechanical resistance in leaves (Arif et al., 2004; Hanley et al., 2007; Caldwell et al., 2016). On the contrary, 1,2-propanediol: choline chloride: water (PCW molar ratio 1:1:1), although not significant, had a marginally stimulatory effect on plant height. Plant height is known to be an important parameter for seed dispersal, as taller plants are predicted to disperse their seed further (Thomson et al., 2011). However, this does not necessarily apply for commercially grown crops and could, perhaps, even be a disadvantage due to space restrictions for growth.

Solubility represents an important parameter, nevertheless, not one that finally matters for a compound to exert its efficacy. Active ingredients do not have to be highly soluble, as long as the formulation provides suitable bioavailability. Chlorogenic acid and sinapic acid have frequently been implicated in plant defense against insect herbivores, including, Hemiptera, Lepidoptera, Orthoptera, Coleoptera, Thysanoptera and Diptera (Mouden et al., 2017). Seed treatment with sinapic acid, chlorogenic acid or beta-alanine, however, did not improve silver damage symptoms, despite an attempt to increase the duration of soaking from 10 min to 15 hours. The observed lack of efficiency in protecting tomato plants against thrips, following seed soaking with plant secondary metabolites,

could be attributed to their limited uptake. Compounds applied as seed treatment may be taken up via three pathways: uptake through the seed structures, root absorption or uptake through the coleoptile (Quérou et al., 1998; Dias et al., 2014). Transport of an active ingredient to the site of action is a complex process where different mechanisms take part, but the essential step is the diffusion. Previous studies suggested that this phenomenon may be related to the seed coat permeability (Niemann et al., 2013). Addressing the fate of these secondary metabolites could shed light on the lack of response in thrips resistance. The existence of a semi-permeable seed coat has been widely reported in several species, yet its importance in crop protection has not often been addressed. The semi-permeable layer is an important structure for restricting or impeding the penetration of some solutes into embryos during imbibition while being permeable to water and gases (Salanenka and Taylor, 2011a). In order to test this assumption three defensive metabolites were selected to study seed diffusion in species with a permeable seed coat (snap bean) and semi-permeable seed coat (tomato). The two plant species, snap bean and tomato (cv. Moneymaker) were evaluated based on their previous use in relation to uptake studies and, as such, their permeability classification (Salanenka and Taylor, 2011a; Taylor and Salanenka, 2012). Comparison of the uptake of sinapic acid and chlorogenic acid in NADES by LC-MS revealed that there were no differences between snap bean and tomato. Increasing the duration of soaking from 10 minutes to 15 hours as well as replacing NADES by methanol did not enhance the uptake in both seed species. Sinapic acid ranged from no detectable amounts to low trace amounts. Semi-quantitative analyses were not possible due to these minimum trace amounts. Tomato seed has selectively permeable characteristics, with non-ionic compounds being able to diffuse through the seed coat, while ionic compounds are blocked (Taylor and Salanenka, 2012). Although β-alanine detection by LC-MS failed due to issues with the ionization spray, the barriers present in tomato seed coats restrict diffusion of water-soluble compounds such as amino acids (Taylor et al., 1995) and may, therefore, provide an important explanation for the lack of defense responses.

Furthermore, penetration of active ingredients is related to the physicochemical properties, especially molecular size and lipophilicity (Yang et al., 2018). How each of these factors influence the uptake is only partially understood. It is generally believed that for weak acid chemicals, the uptake increases with decreasing medium (carrier) pH. The lower the carrier pH, the greater the proportion of molecule in a non-dissociated form. Although the exact pH for LAP 2:1 has not been determined, the presence of lactic acid as a main component suggests that the majority of SA and CGA should be present in their non-ionic form. Nevertheless, only trace amounts of sinapic acid and chlorogenic acid were detected in tomato. Intriguingly, only trace amounts of the phenolic acids were present in snap beans despite their permeable character. Thus, it cannot be ruled out that the lack of detection of phenolic compounds is due to the their limited uptake. In fact, the lack of detection, as well as the lack of improvement with plant secondary metabolites on trips resistance, could be attributed to metabolism of the parent compound, dilution of the compound during seedling growth or retention by the substrate (Quérou et al., 1998). The metabolic fate however, has not been adequately evaluated and could greatly improve our knowledge on seed permeability. Further detailed inspections of seed

biochemistry, distribution and metabolism of seed applied secondary metabolites are necessary to further explore the potential of these compounds. Increasing the dose of a seed treatment, however, does not necessarily improve embryonic uptake. Consequently, saturated uptake could rapidly exhaust the seed reservoirs leading to dilution of an active ingredient during growth. Additionally, various other factors, including soil moisture, water solubility, treatment dose and formulation all influence the translocation and the final efficacy of an active ingredient applied as seed treatment (Quérou et al., 1998).

Furthermore, another causal explanation for the lack of enhanced defenses upon β-alanine seed treatment may arise from its' mechanistic action. Buswell and co-workers (2018) reported that β-alanine, as a structural analogue of β-aminobutyric acid (BABA), induced partial resistance against the biotrophic oomycete *Hyaloperonospora arabidopsidis*. Elicitor induced resistance is differentially effective against pests and diseases (Thaler et al., 2012). BABA-induced defenses are generally associated with the salicylic acid pathway. Consequently, treatment with β-alanine could downregulate JA-associated defenses through an antagonistic interaction and, hence, result in suppressive effects on plant defense against herbivory.

To circumvent differences in the metabolic fate of the phenolic acids, foliar application could offer a potential solution. Phenylpropanoids, including sinapic acid and chlorogenic aid, often function as feeding deterrents and digestibility reducers. Cell wall modifications, mainly established by hydroxycinnamic acid derivatives, may directly pose physical barriers to various insect species incorporating and cross-linking with carbohydrates (Abdel-Aal et al., 2001; Santiago et al., 2006). Interestingly, negative effects of sinapic acid, chlorgenic acid and beta-alanine on thrips mortality have been demonstrated by artificial diet experiments (Leiss et al., 2009a; Leiss et al., 2013). Additionally, sinapic acid and beta-alanine were observed to significantly reduce thrips oviposition (Rakhmawati et al., unpublished). However, external foliar application of sinapic acid as well as beta-alanine, at naturally occurring plant concentrations, did not improve thrips-associated damage nor preference (Ramdayal, unpublished).

In summary, natural deep eutectic solvents have proven to be an interesting solvent to significantly improve solubility. The observed phytotoxicity clearly depends on the type of NADES as well as the duration of soaking. The plethora of possible combinations allows us to select only those carrier solvents without adverse effects in order to meet specific requirements. However, in exploring its use as a carrier solvent for defensive secondary metabolites, both phenolic compounds as well as beta-alanine did not improve thrips resistance in terms of silver damage. Therefore, the focus of our research has shifted from constitutive defenses to inducible defenses exploring the use of elicitors such as jasmonic acid.

Acknowledgements

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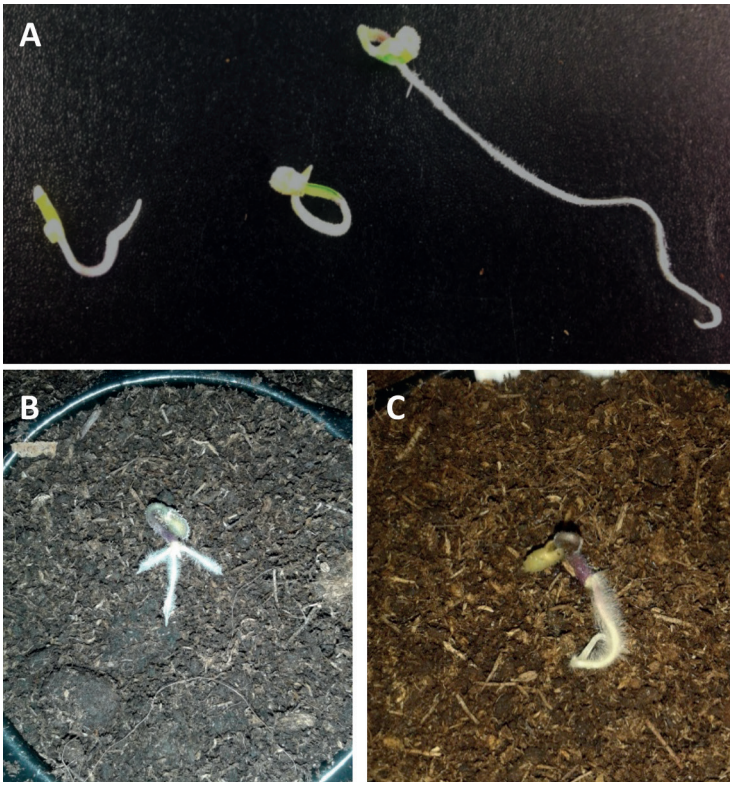
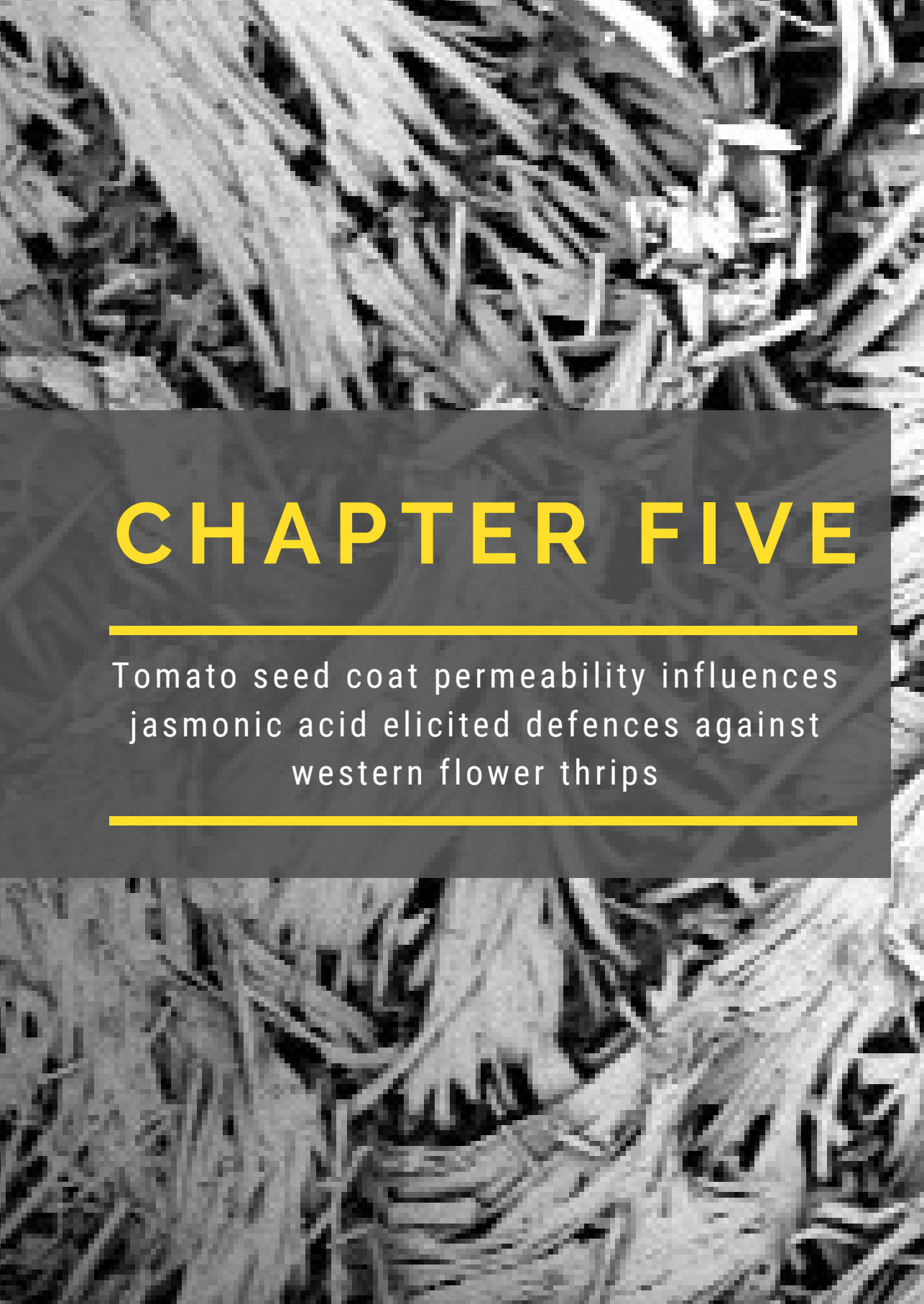
Supplementary figures



Supplementary Figure S1. Representative photographs of root development in tomato (cv. Moneymaker). **(A)** Radicle elongation at day 7. From left to right represent seedlings derived from seeds treated with lactic acid: glucose: water (LGW molar ratio 5:1:3), glucose: choline chloride: water (GUCW molar ratio 2:5:5) and control seedlings. Malformed seedlings treated with LGW **(B)** and GUCW **(C)**.



CHAPTER FIVE

Tomato seed coat permeability influences jasmonic acid elicited defences against western flower thrips

Abstract

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Induction of defenses is one of the most widely accepted eco-friendly approaches for management of pests and diseases. Seeds are receptive to resistance-inducing chemicals and could offer broad-spectrum protection at the early stages of development. However, seed treatment with elicitors has previously been shown to differentially influence induced defense responses and thus, could hamper commercial exploitation. In this context, the objective of the present study was to evaluate jasmonic acid (JA) for its ability to induce resistance against western flower thrips (WFT) at the seed stage. We examined the extent of variation in inducibility of resistance in eight commercial tomato cultivars. Causal factors accounting for discrepancies in JA-induced responses at the seed stage were phenotypically and biochemically evaluated. Seed receptivity to exogenous JA appeared to be cultivar dependent. Thrips associated silver damage was only reduced in JA seed-treated plants of cultivar Carousel. Enhancement of resistance, however, was not associated with activation of defense-related traits such as polyphenol oxidase activity (PPO), trichomes or volatiles. Sulfuric acid scarification, prior to JA seed incubation, significantly augmented the embryonic responsiveness in cv. Moneymaker without an adverse effect on growth. Hence, these results suggest that the lack of defense induction in non-responsive tomato cultivars were likely caused by seed coat impermeability rather than genetic variation in inducibility. The outcome of our study is of translational value as it creates opportunities for the seed industry to perform pre-treatments on non-responsive cultivars as well as for tomato breeding programs to select for genetic traits that affect seed permeability.

Keywords: induced defense, seed receptivity, JA, *Frankliniella occidentalis*, scarification

Introduction

Plants are physically tied to their environment and are, therefore, regularly under attack by a variety of pests and pathogens. Besides constitutive defenses, plants may also deploy inducible defenses that are activated in response to an attack. The expression of inducible defenses is primarily mediated by endogenous signaling molecules, among which the phytohormone jasmonic acid (JA) is a key player in resistance against herbivorous arthropods and necrotrophic pathogens (Howe and Jander 2008; Pieterse et al., 2012). For several decades it is known that activation of the JA signaling pathway is characterized by induction of multiple defensive traits including secondary metabolites (Bennet et al., 1994; War et al., 2012), proteins (Farmer and Ryan, 1990; Thaler et al., 1996), leaf trichomes (Boughton et al., 2005) as well as indirect induction mechanisms such as the production of plant volatiles (Ament et al.,2004; Arimura et al., 2005). Plant signals responsible for inducing resistance are highly conserved among plant species. Accordingly, artificial manipulation of these JA-associated defenses by natural or synthetic elicitors has proven to confer enhanced resistance against multiple insects and diseases and is, therefore, regarded as a valuable component in pest management programs (Thaler, 1999; Vallad and Goodman, 2004; Walters et al., 2013; Steenbergen et al., 2018).
In spite of the long-standing importance of jasmonates in plant resistance, commercial applications have met with rather limited success to date, as induced defences are often associated with substantial yield penalties (Cipollini et al., 2003; Walters and Heil., 2007). Defense mechanisms can be activated directly, however, potentiation of a plant's defensive capacity without concomitant induction of specific defense-related genes can condition a plant for boosted responses to future attacks, a phenomenon commonly known as priming (Conrath et al.,2002;2006; Mauch-Mani et al.,2017). The prospects of broad-spectrum enhanced resistance provided by priming has led to increased interest in the development of agents which can mimic natural inducers of resistance, particularly because priming is associated with minimal allocation costs (Van Hulten et al., 2006).
The vast majority of studies on JA induction focused on foliar application and root drenches, whereas the use of elicitors in seed treatments has been investigated less vigorously despite the fact that the seedlings and young juvenile plants can be particularly more vulnerable to herbivory (Moles and Westoby, 2004; Barton and Hanley, 2013; Ochoa-López et al., 2015). Worrall and co-workers (2012) have demonstrated that tomato seeds are receptive to plant defense activators and, thus, are able to prime herbivore resistance responses by stimulating the plant's own defense mechanisms. Plants grown from JA-treated seeds exhibited long-lasting increased resistance against herbivores of multiple arthropod taxa, persisting over eight weeks, without concurrent impact on plant growth. These findings were corroborated by Paudel et al. (2014) and Strapasson et al. (2014), who demonstrated that tomato seed treatment with methyl jasmonate (MeJA) induced durable resistance against herbivory by *Helicoverpa zea* and *Tuta absoluta*, respectively. Hence, seed treatments can be particularly useful in activation of induced resistance, especially for crops which are likely to face pest and pathogen attack early in the growing season. Intriguingly, however, JA seed treatment of the tomato cultivar 'Moneymaker' failed to induce resistance against *Tetranychus urticae*, whereas

a significant reduction in oviposition on JA-seed treated plants was observed in the tomato cultivar 'Carousel' (Smart et al., 2013). Whilst the mechanisms behind these differential responses remain unclear, differences in the availability of inducible defense genes have been suggested to play an important role. The phenomenon of genetic variation in inducibility of resistance has been demonstrated in numerous studies, however, the majority of these considered variation in plant responsiveness caused by pests and pathogens rather than defense responses elicited by chemicals (Sharma et al., 2010; Degen et al., 2004; Bruce, 2014). An alternative explanation for the variation across cultivars is that they differ in seed coat permeability. The existence of a semi-permeable layer has been extensively reported in several species, including tomato, yet its importance for seed treatments has rarely been addressed (Salanenka and Taylor, 2012). Seed uptake of JA is pivotal, and insufficient penetration into the seed would fail to activate defenses. For potential inclusion of elicitors in IPM programs it is, therefore, of outmost importance to understand how these factors potentially affect plant resistance mechanisms.

To date, little work has been conducted to explain differences in elicitor-mediated induced resistance among cultivars. This prompted us to evaluate several key questions concerning the inducibility of resistance against western flower thrips (*Frankliniella occidentalis*) by exogenous application of JA at the seed stage. Western flower thrips is one of the most economically important pest insects worldwide (Mouden et al., 2017). This polyphagous insect causes extensive damage in agriculture through feeding, oviposition as well as through the transmission of tospoviruses. Artificial induction of JA-mediated defenses has been reported to increase resistance to thrips in cotton (Omer et al., 2001), Chinese cabbage (Abe et al., 2009) and tomato (Escobar-Bravo et al., 2017).

In the present study, we addressed the following questions: i. Can tomato seed treatments with JA confer resistance to WFT? ii. if so, are the defense responses against thrips elicited by JA seed soaking cultivar dependent? iii. what mechanisms underlie this elicitor-mediated resistance? and iv. can tomato seed scarification, prior to exogenous JA seed treatment, augment thrips resistance in cultivars that are non-responsive?

Materials and Methods

Seed treatments and growth conditions

Seed treatments were performed as previously described by Worrall et al. (2012) with slight modifications. Briefly, a jasmonic acid stock solution (1.19 M) was prepared by dissolving 2.5 mg in 1 ml absolute ethanol. Prior to use, the stock solvent was diluted to appropriate concentrations that were adjusted to a final ethanol concentration of 0.5%. Tomato seeds were soaked in an excess amount of freshly prepared dilutions of the JA stock solvent and incubated at 4 °C in a rotary shaker for 24 h to facilitate the penetration of JA into the seeds. Control seeds were soaked in a mock solution consisting of 0.5% aqueous ethanol under the same conditions. After incubation, seeds were thoroughly washed in aqueous ethanol followed by sterilized MilliQ water. Subsequently, seeds were randomly sown in plastic trays filled with potting soil (Horticoop, Lentse Potgrond, the Netherlands)

and placed in a climate room provided with 113.6 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR) and a photoperiod of 16/8 h light/dark, at 20 °C and 70% RH. Ten days later, seedlings were transplanted into individual plastic pots (11 x 11 x 12 cm) and grown under the same conditions for a period of 4 weeks. Pots were watered when needed.

Non-choice whole plant thrips bioassay

A non-choice whole plant bioassay was used to evaluate tomato resistance against WFT (Leiss et al., 2009). Four-week old tomato plants were individually placed in thrips-proof cages consisting of a plexiglass cylinder (50 cm height, 20 cm diameter) closed at one end with a displaceable ring of nylon gauze of 120 µm mesh size. Plants were randomly placed in a climate-controlled growth chamber at a constant temperature of 25 °C, a photoperiod of 16L:8D and 70% For thrips infestation twenty adult thrips (eighteen females and two males) were collected using a mouth-operated aspirator and were then released inside the cage. Western flower thrips were obtained from a colony reared on chrysanthemum flowers (cultivar Euro Sunny) maintained in a climate room at 25 °C and 70% RH. Seven days after infestation, thrips feeding damage, hereafter referred to as 'silver damage' was evaluated by visually scoring all leaves. Silver damage was expressed as damaged leaf area in mm^2.

Comparison of JA seed and foliar treatments

To explore the potential role of JA as an active inducer of systemic resistance, we aimed to determine its capacity to induce the production of defensive response markers in tomato and the consequent resistance against WFT. For this, the systemic nature of JA-induced protection against WFT was evaluated by comparing the efficacy of two application methods namely; foliar spray and seed soaking. Seeds of tomato cultivar Virona (provided by Incotec International BV, Enkhuizen, the Netherlands) were immersed in JA solutions at concentrations ranging from 0.1 to 5 mM JA, under the same conditions as described above. Seeds treated with 0.5% aqueous ethanol were used as controls. For foliar application, treatment concentrations and controls were equal to those used in seed treatments. To avoid direct toxic effects of foliar applied JA, 2 ml aliquots were uniformly sprayed 72 hours prior to thrips infestation. Four weeks old plants were subjected to a non-choice whole plant thrips bioassay (n=10). Furthermore, we explored plant defense responses triggered upon JA treatments by measuring the activity of the defense-related marker protein polyphenol-oxidase (PPO; n=4).

Genotypic variation in JA seed receptivity

To determine responsiveness of tomato seeds to JA induced defenses against WFT, an additional eight different cultivars were evaluated. Tomato cultivars, 'Carousel', '72-323', 'Mahitos untreated', 'Mahitos commercial', 'Brioso', 'Trovanzo' and 'Raymos' were kindly provided by Rijk Zwaan (de Lier, the Netherlands), whereas cv. 'Moneymaker' was obtained from Pieterpikzonen BV (Luinjeberd, the Netherlands). The selected commercial tomato cultivars varied in fruit type, with the majority of cultivars producing classic round tomatoes. Brioso, on the other hand, was characterized as cocktail-

type whereas, Mahitos produces beef tomatoes. Four-week old plants , grown from JA (3 mM) or mock treated seeds, were subjected to a non-choice whole plant thrips bioassay (n=10) or sampled for polyphenol oxidase (PPO) activity analysis (n=7-8).

Differences in water permeability among seeds of different cultivars

To explore whether permeability differences among cultivars contribute to the variability in JA responsiveness, an imbibition test was carried out by monitoring the water uptake in the eight selected cultivars. Hundred seeds, separated into four replicates of 25 seeds each, were used for this test and weighed to the nearest 0.0001 g. Seeds were fully submerged in a freshly prepared solution of 3 mM jasmonic acid and kept at 4 °C. The seeds were blotted and weighed at indicated time intervals for 24 hours. The percentage mass increase (fresh mass basis) for each interval was calculated as % increase in mass = $[(W_i - W_d) / W_d] \times 100$, where W_i = mass of imbibed and W_d = mass of dry seeds (Baskin et al., 2004).

Improvement of JA seed receptivity by scarification

With the aim to explore whether seed scarification could augment JA receptivity at the seed stage, we evaluated the effect of scarification on JA-induced defenses against WFT, using tomato seeds of one of the non-responsive tomato cultivars. Initially, three scarification methods were evaluated in cultivar Virona. For mechanical scarification, seeds were individually scarified by a razor blade. Chemical scarification was performed with 10% sulfuric acid. A non-specified commercial scarification method was carried out by the seed company Incotec International BV (Enkhuizen, the Netherlands). Non scarified seeds served as control. Preliminary experimental results demonstrated that chemical scarification with sulfuric acid, prior to JA soaking, was the most potential method to improve thrips resistance in cv. Virona. In a follow-up experiment, seeds of the unresponsive tomato cultivar Moneymaker and the JA-seed receptive cultivar Carousel were used to study the effect of scarification on JA-inducibility. Tomato seeds were chemically scarified with 10% sulfuric acid (H_2SO_4) for 5 minutes and shaken on a platform shaker at slow to moderate speed. After exposure, seeds were thoroughly washed with distilled water to remove residual sulfuric acid and subjected to JA (3 mM) soaking as previously described. Plants at the 3-4 leaf-stage were evaluated for thrips feeding symptoms seven days after infestation.

Polyphenol oxidase (PPO) activity

Polyphenol oxidase (PPO) activity was determined by sampling one leaflet taken from the third leaf from the bottom, following the protocol described in Stout et al. (1998). In brief, 150 mg of fresh leaf material was flash frozen in liquid nitrogen and stored at –80 °C until analysis for PPO activity. The enzyme was extracted by homogenizing ground plant material in 1.25 ml ice-cold potassium phosphate buffer (0.1 M, pH 6.8) containing 7% polyvinyl polypyrolidine, and 400 µl of 10% Triton X-100. Extracts were vortexed for 2 min and centrifuged at 11,000×g for 10 min at 4 °C. The supernatant was then collected as the crude enzyme extract. PPO activity was measured immediately after the

extract was obtained. Activity was assayed by measuring the oxidation of chlorogenic acid as substrate in a reaction mixture containing 5 µl of the enzyme extract and 1 ml of 2.92 mM chlorogenic acid prepared in 0.1 M potassium phosphate buffer at pH 8.0. The increase in absorbance at 470 nm was measured spectrophotometrically (UV-1800 UV-VIS spectrophotometer, Shimadzu Europe GmbH, Duisburg, Germany) for one minute. PPO activity was expressed as changes in absorbance values per min per gram of fresh weight.

Determination of type VI glandular trichome density

In tomato, foliar application of JA or its volatile derivative methyl jasmonate has been reported to induce the production of type-VI leaf glandular trichomes (Escobar-Bravo et al., 2017; Chen et al., 2018). To investigate whether exogenous JA seed treatment also influenced physical leaf properties, we measured the density of type-VI glandular trichomes following the methodology described by Chen et al. (2018). The second terminal leaflet from the third youngest and fully expanded leaf was sampled in four week old plants grown from mock- and JA-treated seeds. A Leica stereomicroscope (MZ16, Leica Microsystems, Wetzlar Germany) equipped with a Leica DFC420 digital camera was used to take pictures on the adaxial surface at both leaf sides of the main vein in the middle section of the leaflet. Trichomes were counted in an area of 12 mm^2 using Fiji ImageJ software (http://fiji.sc/Fiji) and expressed as the number of type-VI trichomes per centimeter square.

GC-MS analysis of terpenes

Terpene production from type-VI trichome-derived leaf exudates was evaluated using the leaf dip method. This protocol was chosen because the terpenoid profile detected in individually collected type VI glands has been shown to be nearly identical to that observed with the leaf dip procedure (Kang et al. 2010; 2014). Four weeks after initial seed treatment, two leaflets belonging to the same leaf used for trichome density measurement, were sampled and leaf fresh weight was measured before extraction. Leaf exudates were obtained by dipping the leaf tissue in 2 ml of pentane. Following an incubation period of 2 min with gentle shaking, leaflets were discarded, and the extracts were analyzed by gas chromatography-mass spectrometry (Kappers et al., 2011). Two microliter of pentane extracts were splitless analyzed on a Hewlett–Packard GC-MS system consisting of a gas chromatograph (5,890 series II, Hewlett–Packard) equipped with DB-5MS column (30 m × 0.25 mm, 0.25 µm film thickness) and a mass-selective detector (model 5972A, Hewlett–Packard). Compounds were identified by comparing retention times and mass spectra with those of authentic standards and reported in Adams (2007). The area under the curve of selected compounds was calculated to be representative for the concentration.

Statistical analysis

Normal distributions were tested by Shapiro-Wilk tests and homogeneity of variances were determined by Levene's tests. Means were analyzed using one-way ANOVA followed by Fisher's least significance difference (LSD) post hoc test. When assumptions were violated, differences in means

were analyzed using the nonparametric Kruskal-Wallis-test or Welch's ANOVA. Data on number of leaves were analyzed by Kruskal-Wallis followed by Dunn's test with Bonferroni correction for multiple comparisons. The effect of foliar JA application on silver damage and PPO activity were analyzed by Welch Anova followed by Games-Howell post-hoc. Generalized Linear Models (GLM) using linear distribution and identity link functions were used to analyze the effect of cultivar, seed treatment and their interaction on silver damage symptoms, PPO activity, number of trichomes, volatile emission and dry mass. Data on silver damage obtained from the scarification experiment were square root transformed and subsequently analyzed by GLM. Differences among groups were tested by Fisher's LSD post-hoc test. All statistical analyses were conducted with SPSS v. 24 software (IBM; SPSS Inc., Chicago, IL, United States).

Results

Effect of elicitor application on thrips feeding damage

Plant defense activators are usually applied as foliar spray treatment, but can also be applied to seeds (Worrall et al., 2012; Smart et al., 2013; Samota et al., 2017). To explore the potential of JA as an active inducer of systemic resistance at the seed stage, we determined its capacity to induce the production of defensive response markers, i.e. protein polyphenol oxidase, in tomato and the consequent implications on resistance against WFT. Firstly, we evaluated whether tomato cv. Virona was receptive to foliar JA sprays. Foliar application of JA caused a marked reduction in silver damage compared to mock-treated Virona plants (Welch's $F(5, 24.27) = 7.53$, $p < 0.001$). With the exception of 0.1 mM JA, significant reductions in damage were observed, ranging between 53% and 97% (Figure 1A). The pattern of responses to thrips feeding mirrored the induction of PPO (Figure 1C). Levels of PPO were greatly enhanced in response to foliar application of JA, showing a dose-dependent trend (Welch's $F(5, 7.34) = 44.23$, $p < 0.001$). Next, we compared the efficacy of foliar applied JA in suppressing silver damage with the efficacy of seed applied JA (Figure 1A and 1B, respectively). In contrast, exogenous application of different JA doses at the seed stage did not affect total silver damage ($H(5) = 4.839$, $p = 0.436$). The lack of increased resistance levels to thrips corresponded with the measured PPO levels (Figure 1D). PPO activity was not increased in JA seed treated plants ($F(5,18) = 0.295$, $p = 0.909$). Costs related to yield were investigated following foliar and seed treatment with JA (Table 1). Dry mass ($F(5,54 = 0.186$, $p < 0,001$), total number of leaves ($H(5) = 32.358$, $p < 0.001$) and plant height ($F(5,54 = 28.721$, $p < 0,001$) were significantly affected by foliar JA treatment. However, the impact of seed treatments on plant performance were minimal. Whereas seed exposure to 5 mM JA adversely affected tomato growth, seeds pre-treated with intermediate concentrations of JA (1 and 3 mM) caused a stimulatory effect on plant dry mass.

Exploring JA seed receptivity among tomato cultivars

In the previous experiment, seed applied JA had no effect on silver damage in tomato cultivar Virona. Yet, application of JA as a seed soak treatment in cultivar Carousel was capable of improving

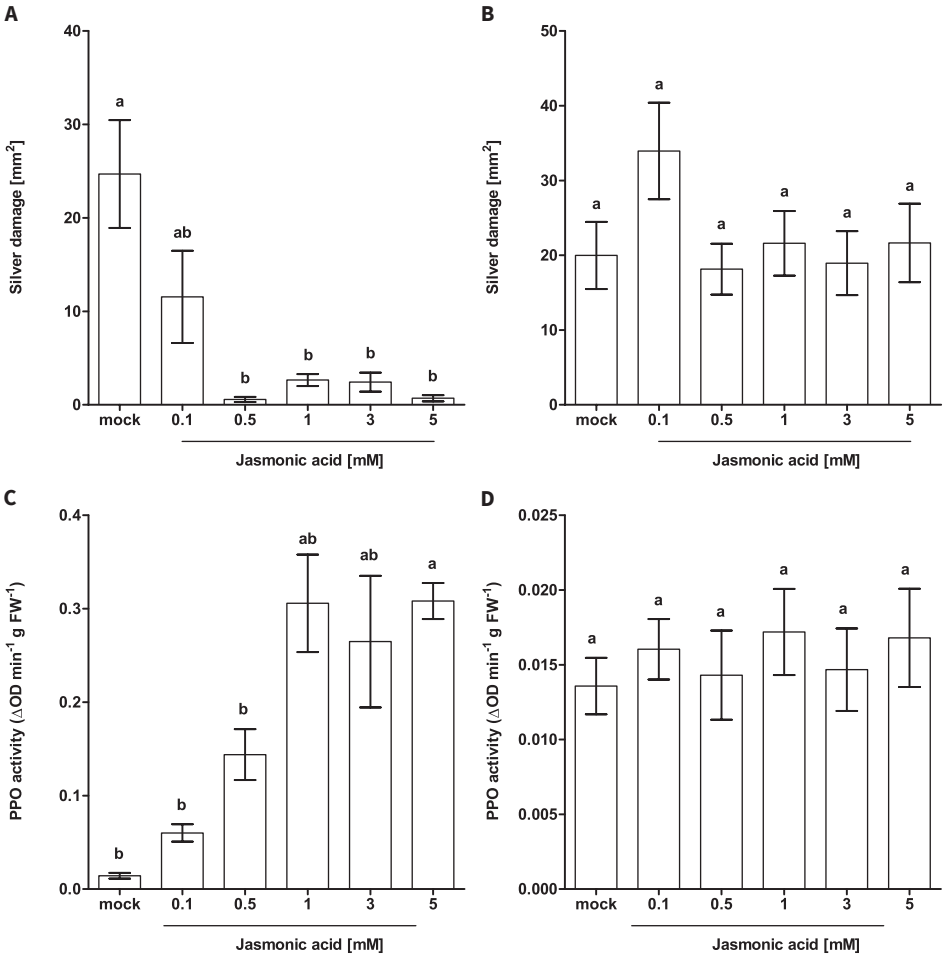

Figure 1. Effect of exogenous JA treatment on tomato resistance against WFT and JA-associated responses. Silver damage symptoms in tomato plants (cv. Virona) following **(A)** foliar application of JA or **(B)** JA seed treatment at different concentrations (n=10). Four-week old plants were infested with WFT for one week or sampled for polyphenol oxidase (PPO) activity. PPO activity (n=4) was measured in the third leaf from the bottom in JA-seed treated plants **(C)** or JA foliar treated plants **(D)**. Data are presented as mean ± SEM. Different letters indicate significant differences among treatments (Welch ANOVA followed by Games–Howell at $p < 0.05$).

resistance against WFT (Supplementary figure S1). Thus, we further examined variation among cultivars in the efficacy of thrips resistance induced by seed application of JA. Concomitantly, we evaluated whether seed applied JA affected the induction of the JA-associated marker polyphenol oxidase. Four week old plants of eight different tomato cultivars, grown from JA or mock treated seeds, were challenged with WFT (Figure 2A). Overall, JA seed treatment had no significant effect on silver damage, but symptoms were affected by plant genotype (GLM: Wald $\chi^2 = 38.94$, $p < 0.001$ for cultivar; Wald $\chi^2 = 0.60$, $p = 0.438$ for treatment and Wald $\chi^2 = 11.87$, $p = 0.105$ for the interaction). However, as in our preliminary experiment with Carousel, a strong reduction in silver damage

Table 1. Effect of jasmonic acid seed soaking and foliar application on plant growth parameters of tomato (cv. Virona). Data are expressed as mean ± SEM (n=10).

	Seed soaking			Foliar spray		
	No. Leaves[1]	**Plant Height[1]**	**Dry mass[1]**	**No. Leaves[2]**	**Plant Height[1]**	**Dry mass[1]**
Mock	6.3±0.15^{a}	11.96±0.64^{abc}	0.37±0.03^{cd}	6.0±0.15^{a}	13.61±0.41^{a}	0.63±0.05^{a}
0.1 mM JA	6.3±0.21^{a}	11.37±0.42^{bcd}	0.39±0.03^{bc}	5.9±0.18^{a}	14.96±0.69^{a}	0.62±0.04^{a}
0.5 mM JA	6.3±0.11^{a}	11.10±0.35^{cd}	0.33±0.01^{cd}	5.8±0.13^{a}	14.44±0.77^{ab}	0.59±0.04^{ab}
1 mM JA	6.4±0.16^{a}	13.29±0.55^{a}	0.50±0.04^{a}	5.5±0.22^{ab}	12.53±0.41^{b}	0.50±0.03^{b}
3 mM JA	6.2±0.20^{a}	12.77±0.53^{ab}	0.47±0.04^{ab}	4.8±0.20^{b}	9.36±0.48^{c}	0.34±0.04^{c}
5 mM JA	6.2±0.13^{a}	10.39±0.65^{d}	0.28±0.03^{c}	4.5±0.17^{b}	7.93±0.26^{c}	0.33±0.04^{c}

Data followed by different letters indicate significant differences among treatment means in a column tested by Fisher's LSD[1] or Dunn's test[2] at $P < 0.05$.

symptoms was observed in JA seed treated Carousel plants. Such a reduction was absent in the remaining other seven cultivars. Silver damage symptoms of plants grown from untreated seeds, revealed that the eight cultivars varied inherently in susceptibility, from cultivar Raymos being moderately resistant with 40.6±7.4 mm^2 silver damage to cultivars Moneymaker and Carousel being highly susceptible with 123.1±23.9 and 123.9±20.1 mm^2 damage, respectively. Furthermore, we observed that the seed treatment was not associated with a reduction in vegetative growth (Supplementary Figure S2). Cultivar, on the other hand, affected dry mass of seed treated plants (GLM: Wald $\chi^2 = 89.44$, $p < 0.001$ for cultivar; Wald $\chi^2 = 0.175$, $p = 0.676$ for treatment and Wald $\chi^2 = 3.86$, $p = 0.795$ for the interaction).

To evaluate whether reductions in thrips feeding damage were associated with the JA signaling pathway, we measured PPO activities in JA-seed elicited plants and control plants in the absence of thrips. PPO activities were not increased in plants grown from JA treated seeds (Figure 2B). The enhanced resistance following JA seed application JA in 'Carousel' plants was not associated with direct induction of plant defenses.

Differences among cultivars in water permeability

To better understand the underlying mechanisms responsible for discrepancies in JA seed induced responses, we examined the eight tomato cultivars in relation to water uptake (Figure 3). The water imbibition pattern of Moneymaker showed a slightly curved pattern, while other cultivars had a more linear pattern. Within 1 hour, water absorption differed strikingly among tomato cultivars ($F(7,16) = 5.376$, $p = 0.03$). Moneymaker absorbed significantly higher amounts of water during this period in comparison to the other cultivars. However, no significant difference in final water uptake (i.e. after24 hours) was observed between tomato cultivars (Welch's F (7, 6,66) = 1.11, $p = 0.452$). Nevertheless, Carousel, together with Raymos seeds hydrated at the slowest rate. Carousel gained only 39% by weight over the initial seed weight after 24 hours soaking. Tomato cultivar Moneymaker, on the other hand, hydrated most rapidly and imbibed 10% more water than Carousel seeds for this time period (49,73±2,35 versus Carousel 39,02±2,15). Moreover, we evaluated whether hydro-priming of

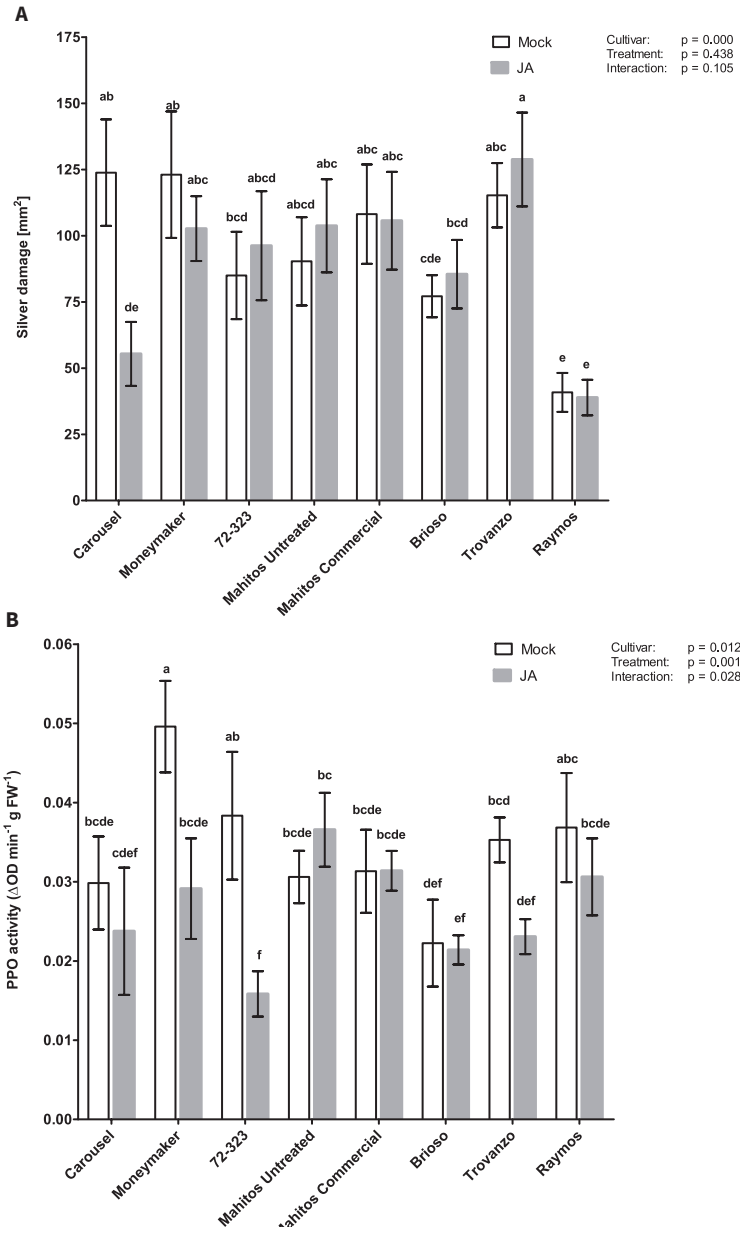

Figure 2. Effect of exogenous JA treatment on tomato resistance against WFT and JA-associated responses. Silver damage symptoms **(A)** and PPO activity **(B)** were measured in 8 tomato cultivars; Moneymaker, Carousel, 72-323, Mahitos untreated, Mahitos commercial, Brioso, Trovanzo and Raymos plants that were subjected to mock or jasmonic acid (3 mM) seed treatment. Four-week old plants were subjected to a non-choice whole plant bioassay and infested with 20 adult thrips (n=10). Silver damage symptoms were visually scored after 7 days of thrips infestation. **(B)** Polyphenol oxidase activity was measured in non-infested plants taking the third leaf from the bottom (n=7-8). Data are presented as mean ± SEM. Different letters denote significant differences among groups as determined by GLM followed by Fisher's LSD test ($p < 0.05$). The overall effects of cultivar, treatment and their interaction are indicated in each graph.

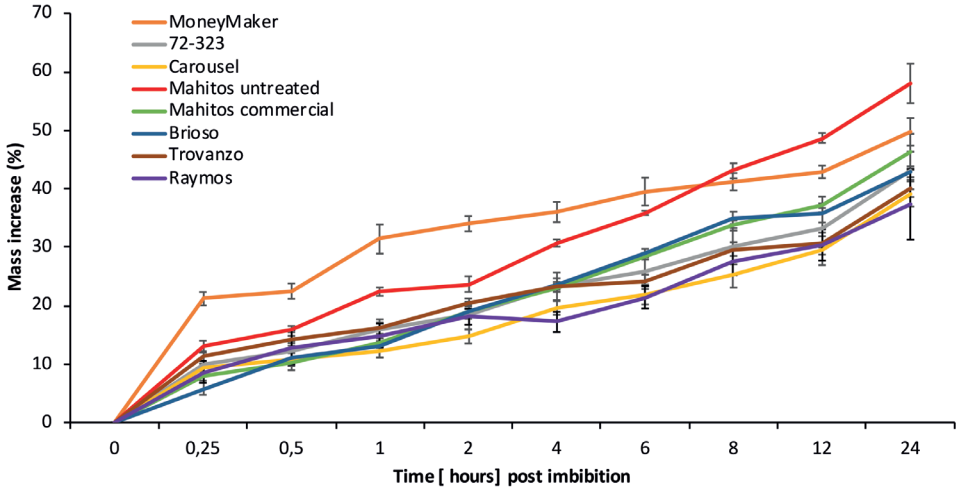

Figure 3. Imbibition curves of eight different tomato cultivars. The percentage of mass increase in weight was used as a measure of water imbibition. Different line colors correspond to different cultivars. Red, orange, green, blue, grey, brown yellow and purple correspond to the following cultivars; 'Mahitos untreated', 'Moneymaker', 'Mahitos commercial', 'Brioso', '72-323', 'Trovanzo', 'Carousel', and 'Raymos', respectively.

commercial Mahitos seeds influenced subsequent water permeability. Seed priming is a commercially used pre-sowing technique which involves the uptake of water by the seed followed by drying to trigger pre-germinative events but do not allow radical protrusion through the seed coat. The achieved physiological state enables seeds to germinate more efficiently (Taylor et al., 1992). Hydro-priming of seeds had no significant effect on water imbibition percentage (46.30 ± 3.08 commercial versus untreated i.e. unprimed Mahitos 58.02 ± 3.37).

Phenotypic characterization of seed receptivity

Based on the above results, Moneymaker and Carousel were chosen as 'non-responsive' and 'highly responsive' in regard to JA seed soaking. With the aim to explore underlying differences in JA seed receptivity, vegetative plants resulting from JA soaked seeds from both cultivars were phenotypically characterized based on morphological and chemical criteria (Figure 4). Type-VI trichome density was significantly affected by cultivar (GLM; Wald $\chi^2 = 22.260$, $p < 0.001$) but not by treatment (GLM; Wald $\chi^2 = 2.576$, $p = 0.108$). The cultivar by treatment interaction was not significant (GLM: Wald $\chi^2 = 1.990$, $p = 0.158$). The number of glandular trichomes in Carousel plants was 2.2 fold lower than Moneymaker (Figure 4A). However, on the contrary, mock seed treated carousel plants displayed significantly higher terpene content (Figure 4B; GLM; Wald $\chi^2 = 9.682$, $p = 0.002$ for cultivar, Wald $\chi^2 = 0.547$, $p = 0.460$ for treatment and Wald $\chi^2 = 3.778$, $p = 0.052$ for interaction). Analysis of volatiles in leaf exudates revealed that cultivars differed in relative abundance of monoterpenes whereas no significant effect of treatment was observed (Figure 4B-D). The results of the interactions suggested that seed treatment affected the emissions of α-terpinolene and β-phellandrene (GLM; Wald $\chi^2 = 7.677$, $p = 0.006$ and

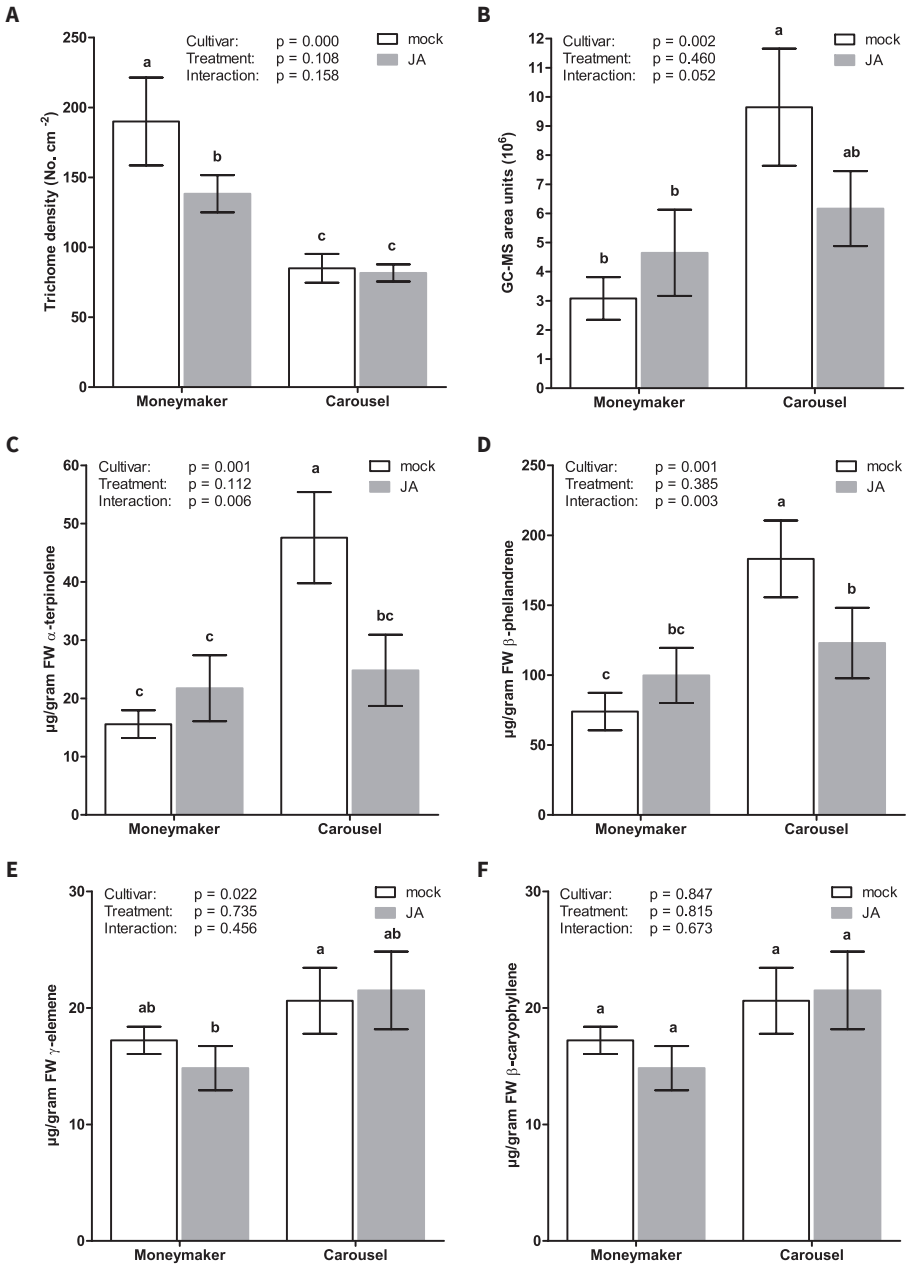

Figure 4. Effect of exogenous JA seed treatment on type-VI trichome density and trichome derived volatiles in uninfested tomato plants. Seeds of cultivar Moneymaker and Carousel were soaked in mock or 3 mM JA for 24 hours. Four weeks after initial treatments, **(A)** type VI glandular trichome density (n=10) was evaluated in the adaxial leafside of leaflets from the third youngest leaf. **(B)** total terpene content and content of monoterpenes **(C)** α-terpinolene, **(D)** β-phellandrene and sesquiterpenes **(E)** γ-elemene and **(F)** β-caryophyllene derived from leaf exudates of mock and JA-seed treated tomato plants (n = 5). Bars represent mean ± SEM. Different letters denote significant differences among groups as determined by GLM followed by Fisher's LSD test ($P < 0.05$). The overall effects of cultivar, treatment and their interaction are indicated in each panel.

Wald $\chi^2 = 4.711$, $p = 0.003$ for interaction, respectively). Whilst the monoterpenes α-terpinolene and β-phellandrene significantly decreased in Carousel, no significant effect was observed for JA seed treated Moneymaker plants (Figure 4C-D). Furthermore, no differences were observed for the sesquiterpenes γ-elemene and β-caryophyllene (Figure 4E-F).

Improvement of JA seed receptivity by scarification

Systemic compounds must be able to permeate the seed coat and diffuse to the embryo in order to exert efficacy. To further demonstrate the importance of the seed coat several scarification methods were explored, prior to JA seed soaking, to determine whether embryonic responsiveness to JA could be augmented. Preliminary experimental results in one of the non-responsive tomato cultivars (Virona) demonstrated that chemical scarification with 10% sulfuric acid was the most potential method to improve JA seed receptivity with no adverse effects on the embryo and subsequent growth (Supplementary Figure S3). Therefore, acid scarification was applied to increase seed coat permeability of the non-responsive cultivar Moneymaker. The effect was compared with the highly responsive cultivar Carousel (Figure 5). Interestingly, acid scarification prior to JA seed treatment augmented seed receptivity in Moneymaker plants. Following JA seed soaking, whole plant silver damage was significantly reduced by 60% as compared to the acid-scarified mock treated control (Figure 5A). The response to JA elicitation, however, varied depending on cultivar and scarification treatment (GLM; Wald $\chi^2 = 3.896$, $p = 0{,}048$ for three-way interaction). Seed applied JA reduced silver

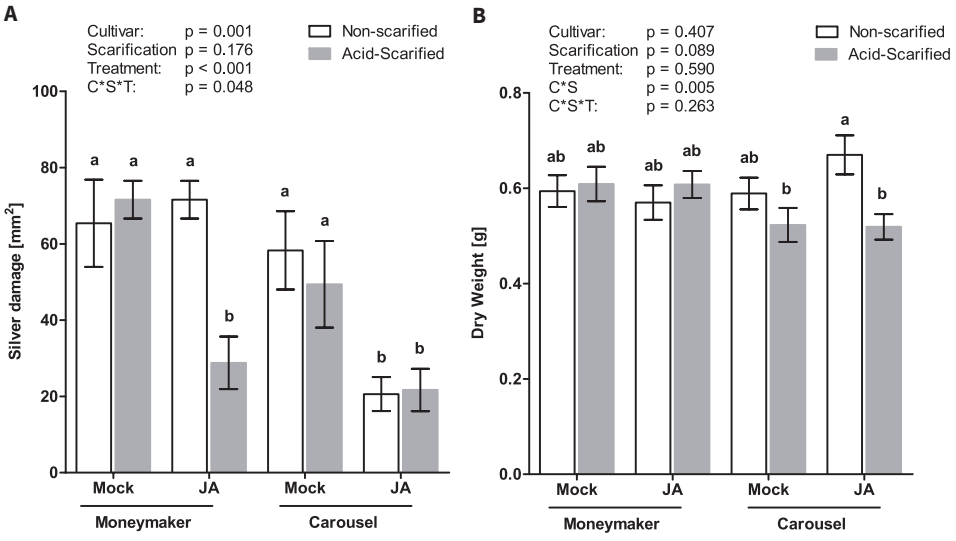

Figure 5. The effect of acid scarification on JA seed receptivity in Moneymaker and Carousel was measured by evaluating whole plant silver damage **(A)** and dry mass **(B)**. Seeds of the cultivar Moneymaker and Carousel were scarified with 10% sulfuric acid prior to seed treatments. Acid scarified seeds were soaked in 3 mM JA or 0.5% ethanol mock for 24 hours. Bars represent mean ± SEM of ten individual plants. Different letters denote significant differences among groups as determined by GLM followed by Fisher's LSD test ($P < 0.05$). The overall effects of cultivar, treatment and the three way interaction are indicated in the graph. Two-way interactions were not significant except the interacting effect of cultivar*scarification on dry mass.

damage symptoms in Carousel plants, independent of acid scarification. Whilst no negative effects of seed pre-treatments on dry mass were observed in Moneymaker, acid scarification significantly reduced dry mass in JA-seed treated Carousel plants (Figure 5B).

Discussion

In the past decades, elicitor-mediated induced resistance has become one of the most challenging research areas in plant herbivory. Recent studies have shown that seeds are receptive to direct application of exogenous phytohormones without being constrained by the inherent costs of defense (Rajjou et al., 2006; Worrall et al., 2012; Haas et al., 2018). However, the beneficial effects of JA seed treatments are not consistent among tomato cultivars (Smart et al., 2013). To further explore the potential of JA as an active inducer of systemic resistance, it was of interest to investigate whether its' application at the seed stage could confer resistance against the economically important pest insect species WFT.

Here we show that in the tomato cultivar Carousel, JA seed treatment induced defenses against WFT. This result is in line with previous studies on other arthropod herbivores (Worrall et al., 2012; Smart et al., 2013). In addition, across eight tomato cultivars we demonstrated that seed receptivity of Carousel seeds appeared to be an exception, rather than the rule. Preliminary experiments with Moneymaker demonstrated that seeds of this cultivar were not receptive to JA-mediated defenses (Supplementary Figure S4). This is line with earlier findings obtained by Smart et al. (2013), who reported that Moneymaker plants lacked systemic induction of defense against the spider mite *Tetranychus urticae* in response to JA seed treatment. These observations prompted us to investigate the differences underlying seed receptivity of different tomato cultivars. To this end, we first studied the influence of JA on elicitation of defense responses in tomato (cv. Virona) by comparing the efficacy of JA seed treatment with foliar application. Thrips resistance was differently affected by treatments. In contrast to foliar application of JA, which significantly reduced silver damage and caused a dose-dependent induction of the defensive enzyme polyphenol oxidase (PPO), JA seed treated plants failed to enhance resistance against thrips consistent with its inability to induce PPO activity. Exogenous foliar application at concentrations above 1 mM, however, imposed significant costs on tomato plant growth, a phenomenon that is well known to be associated with JA-induced defenses (Redman et al., 2001).

Interestingly, when we evaluated the tomato cultivar Carousel, JA seed treatment led to durable resistance against thrips (Supplementary Figure S1). The question naturally arises as to 'what features contribute to Carousels' uniqueness?' A number of hypotheses have been put forth to account for differences in seed receptivity. Among these, genetic variations may play a major role in how plants respond to elicitor induced resistance (Sharma et al., 2010; Walters et al., 2013; Bruce et al., 2017; Goodwin et al., 2017, 2018). So far, however, no studies have explored the underlying mechanisms accountable for variable JA induced responses at the seed stage. Subsequently, we decided to expand our range of cultivars to study response variations to JA seed applied induced defenses. We observed

that cultivar was a major factor in determining the efficacy of JA (Figure 2). Among the eight tomato cultivars, only Carousel seed-treated plants showed a significant decrease in silver damage compared to mock control, without concurrent impact on vegetative growth. Silver damage symptoms in mock-seed treated Carousel plants were, however, for an unknown reason twice as high as in the first experiment (Supplementary Figure S1 vs Figure 2). Nonetheless, the percentage of symptom reduction was in both experiments approximately 50%. Increased resistance to WFT in JA seed-treated Carousel plants, however, was not associated with induction of PPO activities. In contrast, readily measurable PPO activities were found in foliar treated Carousel plants as JA was applied 3 days before thrips challenge (Supplementary Figure S1). One possible explanation for the lack of PPO induction is the time gap following JA seed treatment and PPO measurements. Following induction treatment, there is usually a set period of time required for the establishment of induced resistance. PPO activities may have increased after this lag period with a slow decrease thereafter. Furthermore, induced plant responses often decline with age and, in addition, are markedly influenced by leaf developmental stage (Cipollini and Redman, 1999; Chen et al., 2018).

Perhaps, 4 weeks post treatment, when PPO activity was measured, induction of defense related proteins, in the absence of thrips, might thus not be measurable anymore. Surprisingly, however, Paudel et al. (2014) demonstrated that the induction of plant defense against *H. zea*, following MeJA seed treatment of tomato cultivar Micro-Tom, was correlated with increased PPO activity. Moreover, the effect of seed treatment was found to be considerably long lasting as 25- and 50-day old plants displayed significant increases in PPO levels. Rather than direct induction of defenses, it is plausible that JA seed treatments primed for enhanced defense reactions. So far, it remains inconclusive whether priming of JA responses functions as control mechanism underlying induced resistance in seed-treated Carousel plants. Spatiotemporal distributions of PPO expression levels following JA seed soaking could provide helpful information here.

Seed coat permeability has also been postulated to contribute to discrepancies in JA-induced host responses (Smart et al., 2013). To better understand the underlying mechanisms responsible for variable JA seed induced responses, we measured imbibition rates as a means to characterize seed coat permeability. In contrast to our expectations, water absorption characteristics did not strikingly differ among cultivars (Figure 3). Intriguingly, Carousel seeds were shown to hydrate at the slowest rate and imbibed among the least amount of water after a soaking period of 24 hours. In contrast, the non-responsive cultivar Moneymaker hydrated most rapidly. Although not significant, Moneymaker imbibed more water than Carousel seeds hence, suggesting that discrepancies in JA-induced responses at the seed stage are not directly explained by differences in seed coat permeability. The results of this experiment, however, must be interpreted in the context of a number of potential limitations. The possible diagnostic value of seed coat permeability was assessed by comparing the water uptake which, by itself, does necessarily reflect uptake characteristics and subsequent bioavailability of JA. Jasmonic acid uptake by seeds is pivotal and insufficient penetration into the seed would fail to prime or induce defenses. Dissection and separation of seed tissues, followed by chemical extractions and quantification are a prerequisite to adequately evaluate

whether induced host response variations arose from differences in JA uptake.

It is generally known that differences in seed coat anatomy play an important role in the process of water imbibition and that these differences relate to permeability (Koizumi et al., 2008). Physical properties of seeds such as surface area, sphericity and seed size, however, were not evaluated in the present study. In addition to structural differences, chemical compositions of seed covering layers may also provide a biochemical rationale for variations in uptake and bioavailability (Beresniewicz et al, 1995a; Vu et al., 2014). Comparison of soy bean varieties, for example, revealed that the outer cuticle of non-permeable varieties contained disproportionally more hydroxylated fatty acids than permeable varieties (Shao et al., 2007; Qutob et al., 2008). Moreover, seed coat compositions may differ from that of the embryo and, thus, can affect both permeability and affinity for a compound. Retention by the seed coat could result in suboptimal concentrations reaching the embryo which, as a consequence, is unable to activate defense-related processes. Differential embryonic responses to the plant hormone abscisic acid, for example, has been reported to contribute to variation in dormancy among wheat cultivars (Walker-Simmons, 1990). Therefore, it is important to consider the response of the embryo. Scanning electron microscopy and histochemical analysis could greatly improve our understanding whether certain seed structures attenuate the uptake and how structures correlate with seed treatment efficacy.

Seed uptake of organic compounds, however, remains a very complex process, due to the high variability in seed morphology, physiology and the wide range of properties of the chemical compounds. The majority of studies use fluorescent tracers and colored compounds to mimic systemic seed treatments and to visualize movement of compounds through the seed coat. Although these model compounds represent a diverse array of structures, conclusions deducted from these experimental determinations cannot be extrapolated and therefore, might not always give a good estimation of the uptake patterns of a specific compound. Nonetheless, based on this empirical approach tomato seeds were classified as having selectively permeable seed coat characteristics. This semi-permeable layer, composed of suberin, is an important structure for restricting or impeding the penetration of some solutes into embryos during imbibition, while being permeable to water and gases (Beresniewicz et al, 1995b). Non-ionic compounds that are moderately lipophilic in nature should be able to diffuse through the seed coat, whereas ionic compounds are blocked (Taylor and Salanenka, 2012). Consequently, the physicochemical nature of JA in relation to seed coat permeability of different tomato cultivars could shed light on the lack of a systemic induced response against thrips. A molecule's behavior across a pH range profoundly influences the ionization state, and thus the distribution. JA contains an ionisable carboxylic group (-COOH) and, as such, the magnitude of its' electrical charge depends on the pH relative to the charged group (pK_a). At a pH significantly below the pK_a of the ionisable center (pK_a = 4.71; predicted by ChemAxon, Cambridge, MA, USA), the majority of JA occurs in its protonated or unionised form, whereas at a pH above the pK_a, the ionised form predominates. It can be expected that a large proportion of JA, at a concentration of 3 mM, occurs in in a non-dissociated form and thus, theoretically could diffuse through tomato seed coats. Apart from electrical charge, lipophilicity forms another a key determinant for the ability

of a compound to penetrate the seed coat. Lipophilicity, measured by the octanol-water partition coefficient (Log K_{ow} or Log P), describes the affinity of compounds for the lipid phase of plant tissue (e.g. plasma membrane, waxes, cutin, suberin, etc) (Braumann, 1986). Translocation of JA, a derivative of the fatty acid linolenic acid, could potentially be limited by the presence of suberin, which in general is well known to adsorb lipophilic compounds. The lipophilic behavior of JA is most dominant when the carboxylic group is unionised, as it becomes more soluble in the immiscible lipophilic solvent. Recently, Yang and co-workers (2018) studied the uptake and distribution of a series of non-ionic fluorescent piperonyl amides in relation to their chemical lipophilicity (Log K_{ow}) in permeable soy bean and semi-permeable corn seeds. Contrasting trends in uptake between seed tissues were observed for soybean. In tomato, like corn, the overall uptake pattern for seed covering layers was similar to that of the internal tissues (Yang et al., 2017). The maximum uptake for non-ionic compounds to diffuse to the embryo of tomato ranged between Log K_{ow} 3 to 4. Interestingly, at optimal Log K_{ow} less than 5% of the applied compound was measured in the embryo of tomato seeds. The predicted log P or Log K_{ow} for JA is 2.41 (ChemAxon). This value lies in the lower range of permeability, and as such, the uptake efficiency in the embryo is expected to be even lower than 5%. Nonetheless, uptake efficiencies clearly varied among the three evaluated tomato varieties (Yang et al., 2017). Seed applied products with a high potency could, therefore, potentially compensate for their low permeability.

We further investigated whether the enhancement of thrips resistance in the responsive cultivar Carousel could be explained by induction in physical and chemical leaf properties. Type-VI glandular trichomes are controlled by the JA-pathway (Boughton et al., 2005; Maes and Goossens, 2010) and provide physical and chemical barriers which are known to produce and secrete diverse compounds affecting survival (Frelichowski Jr et al., 2001), growth (Kang et al., 2010) and fecundity (Bleeker et al., 2012) of many herbivorous arthropods. In tomato, artificial application of JA or its volatile derivative methyl jasmonate (MeJA) has been reported to induce the production of type-VI leaf glandular trichomes and volatile emissions (Escobar-Bravo et al., 2017; Chen et al., 2018). Carousel plants contained significantly less type-VI glandular trichomes than Moneymaker however, we observed no significant effect of seed treatment on trichome densities. Intriguingly, total terpene content was significantly higher in mock seed treated Carousel plants, suggesting a relative decrease in terpene production per trichome in JA-seed treated plants. Furthermore, JA seed treatments markedly decreased the emission of the monoterpenes α-terpinolene and β-phellandrene in Carousel whereas, no significant effects were observed in JA seed treated Moneymaker plants. These findings are in agreement with Strapasson et al. (2014), who reported that MeJA seed treatment of cultivar Micro-Tom decreased volatile emissions, including that of β-phellandrene, in uninfested tomato plants. Consequently, manipulation of plant volatile emissions could contribute to the control of insect pests as herbivores rely on volatile cues for host location. Reduced levels of emitted volatile compounds involved in volatile-mediated foraging may lead to a decrease in host finding efficiency and, ultimately a lower number of offspring (Bruce et al., 2005; Proffit et al., 2011). By contrast, in the absence of herbivory, Smart et al. (2013) reported an increase in methyl salicylate and (*E,E*)-4,8,12-

trimethyl-1,3,7,11-tridecatetraene (TMTT) upon JA seed incubation, resulting in an increased attractiveness of Carousel plants to predatory mites.

Interestingly, foliar applications of JA or MeJA differentially affect volatile emissions. On the contrary, significant increases in the emission of terpinolene and β-phellandrene were reported in cultivated (cv. Moneymaker) and wild-type (Castlemart) tomato plants and could potentially reinforce defenses as a result of their direct toxic or repellent potency (Bleeker et al., 2009; Escobar-Bravo et al., 2017; Chen et al., 2018). Moreover, enhancement of volatile emissions may also prove to be of value in the context of biological control in mediating natural enemy recruitment (Kappers et al., 2005, 2011). These intriguing findings suggests that indirect defenses can have opposite ontogenic trends and thus highlight the importance of considering ontogeny in studies of tritrophic interactions.

The phenotypical and bio-chemical analyses discussed above did not provide a causal explanation for the underlying characteristic low receptivity of tomato cultivar Moneymaker. Acid scarification prior to JA seed treatment was, therefore, evaluated to further demonstrate the importance of the seed coat permeability. Our results show that pre-treatment with 10% sulfuric acid significantly augmented receptivity in JA-seed treated Moneymaker plants and, hence suggest that the lack of defense induction was likely caused by seed coat impermeability. The observed enhancement in resistance is encouraging; it demonstrates the possibility to manipulate seed coat characteristics of non-responsive cultivars without the need of difficult and time-consuming breeding techniques. Further evidence that the lack of induced resistance against thrips was caused by impermeability comes from extended seed treatment experiments. Extended soaking of germinating tomato seeds (cv. Moneymaker) in JA solutions bypasses the limitations of impermeability of the tomato seed coat and increased the effectiveness of induced resistance. However, this approach is not without risk as prolonged soaking can cause serious negative effects on seed viability (Chen et al., unpublished). Luna et al. (2016) reported similar findings in Moneymaker using 1 mM β-aminbutyric acid (BABA) for durable induced resistance to *Botrytis cinera*. After a 7 day incubation period of seeds in BABA, lesion size was significantly reduced in 4-week old tomato plants.

Concluding remarks

Seed applied JA reduced feeding damage by western flower thrips but, cultivars differed inherently in their response to exogenous JA seed application. Among eight evaluated tomato cultivars, silver damage symptoms were only reduced in JA-seed treated plants of the cultivar Carousel. Seed coat impermeability is a major factor contributing to the low receptivity of cultivars, as acid scarification significantly augmented responsiveness to seed applied JA in Moneymaker. Evaluating genotypes with greater levels of genetic diversity might help to determine the potential range of responses to seed applied JA. This outcome creates opportunities for tomato breeding programs to select for genetic traits that affect seed permeability as well as for private seed companies to pre-treat non-responsive cultivars.

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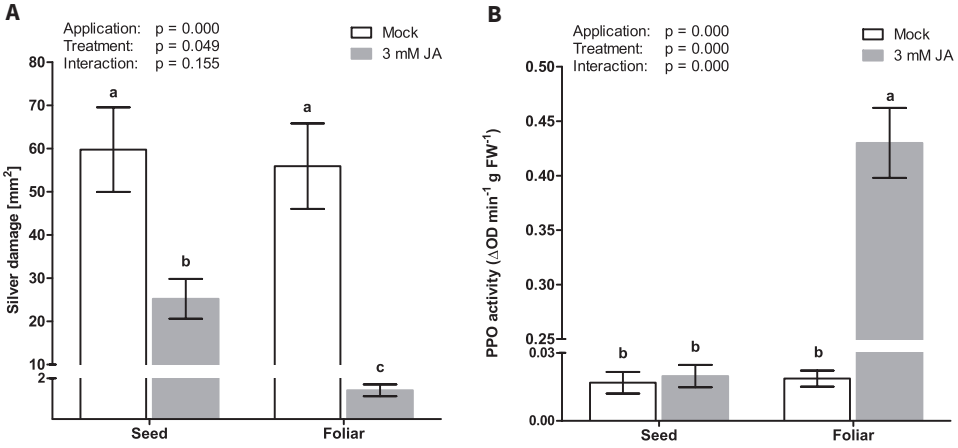
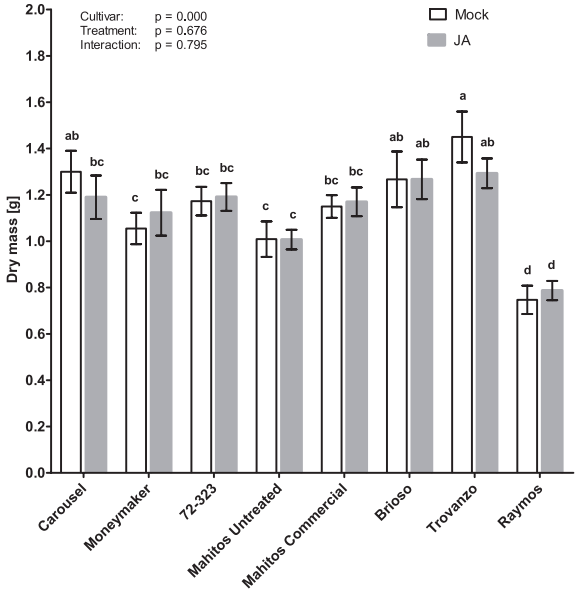
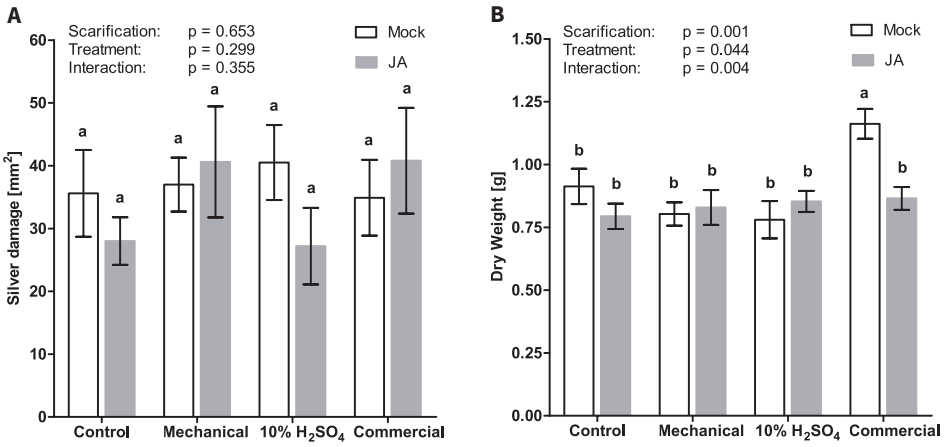
Supplementary figures



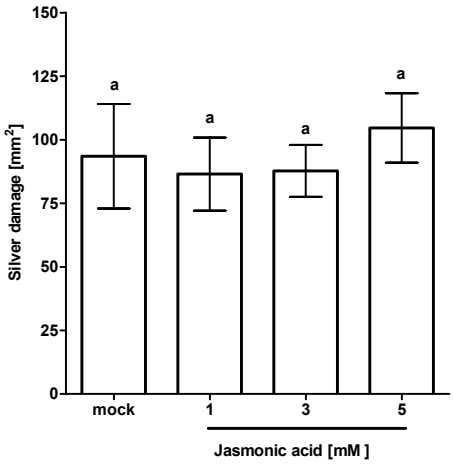
Supplementary Figure S1. Effect of exogenous JA treatment on tomato resistance against WFT and JA-associated responses. **(A)** Silver damage symptoms in tomato plants (n=12) following JA or mock treatment applied at the seed stage or as foliar spray 3 days prior to thrips infestation. Four-week old tomato plants, variety Carousel, were subjected to a non-choice whole plant bioassay and infested with 20 adult thrips. Silver damage symptoms were visually scored after 7 days of infestation. **(B)** Polyphenol oxidase activity was measured in non-infested plants taking the third leaf from the bottom (n=6-8). Data are presented as mean ± SEM. Different letters denote significant differences among groups as determined by GLM followed by Fisher's LSD test ($P < 0.05$). The overall effects of application, treatment and their interaction are indicated in each graph.



Supplementary Figure S2. Effect of seed applied JA on dry mass of tomato plants. Dry mass was measured for 8 tomato cultivars; 'Moneymaker', Carousel', '72-323', 'Mahitos untreated', Mahitos Commercial, Brioso, Trovanzo and 'Raymos' plants that were subjected to mock or jasmonic acid (3 mM) seed treatment. Data are means (±SEM) of ten individual plants and different letters indicate significant differences among groups as determined by GLM followed by Fisher's LSD test ($P < 0.05$). The overall effects of cultivar, treatment and their interaction are indicated in the graph.



Supplementary Figure S3. Effect of different pre-treatments on JA-induced defenses against WFT on silver damage and plant growth. Seeds were mechanically (razor blade), chemically (10% sulfuric acid) or commercially scarified prior to JA or mock seed treatment. Control seeds were non-scarified. **(A)** Total silver damage was evaluated in four-week old tomato plants (cv. Virona) after 7 days of thrips infestation. **(B)** Dry mass was assessed as a measure of plant performance. Data are presented as mean ± SEM, n=12. Different letters indicate significant differences among groups as determined by GLM followed by Fisher's LSD test ($P < 0.05$). The overall effects of scarification method, treatment and their interaction are indicated in the graph.



Supplementary Figure S4. Effect of seed applied JA on tomato resistance against Wester Flower Thrips (*Frankliniella occidentalis*). Four week after seed treatment 'Moneymaker' plants were subjected to a non-choice whole plant bioassay and infested with 20 adult thrips. Silver damage symptoms were visually scored after 7 days of infestation. Data are presented as mean ± SEM, n=8. There was no statistically significant effect among treatments on silver damage symptoms (Welch's F (3, 15.19) = 0.36, p = 0.781).

CHAPTER SIX

WATER DIPPING OF INDOLE-3-BUTYRIC ACID COATED CHRYSANTHEMUM CUTTINGS CONFERS PROTECTION TO INSECT HERBIVORES

Abstract

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Chrysanthemum is a major ornamental plant species. Among the most important production constraints are biotic stresses, in particular thrips and leaf miner infestations form a prominent hazard during its vegetative state. Auxins are commonly used for commercial propagation of chrysanthemums by stem cuttings. However, recent studies suggest that these root-promoting hormones may also affect plant defense responses. The underlying motive of this study stems from the serendipitous observation that water dipping of auxin-coated cuttings beneficially affected thrips herbivory. Therefore, the primary objective of this investigation was to explore the role of indole-3-butyric acid (IBA) in relation to western flower thrips susceptibility in chrysanthemum. We observed contrasting findings concerning the physical presence of IBA and its' role in promoting susceptibility of cuttings to thrips. Nonetheless, we repeatedly demonstrated considerable protection, in some experiments up to 37%, against thrips as well as leaf miner upon water dipping of IBA-coated cuttings. Assessment of polyphenol oxidase activity (PPO), 14 days after dipping treatment, suggests that neither direct induction nor priming of plant defenses are involved. Overall, however, results were highly variable and thus, do not allow us to deduce conclusive statements concerning the involvement of auxin-mediated defense responses. An explanation for the variation may be the large phenotypical variation of cuttings generated from mother stock plants. Future experiments aiming at understanding the early signaling events, including hormonal signaling networks, from a more holistic perspective, may help to explain the physiological basis involved in conferring protection against herbivores.

Keywords: auxin, western flower thrips, *Liriomyza trifolii*, resistance, polyphenol oxidase

Introduction

Chrysanthemum morifolium (Ramat) is a semi hardy herbaceous, perennial flowering plant and belongs to the family of Asteraceae (formerly known as Compositeae). Chrysanthemums are among the most important commercially grown greenhouse ornamentals worldwide and are extensively used as cut flowers and as pot plants (Fletcher, 1992; Machin, 1996; Xia et al., 2006). Classical breeding programs have mainly focused on improving various characteristics to enhance ornamental values, including flower colour, size and shape. Driven by such consumer needs, breeders are often under pressure to supply novel varieties within a restricted timeframe and with very specific choices, leaving few options for altering other agronomic traits. In addition to the limited gene pool, chrysanthemums are hexaploids and genetically highly heterozygous, thus complicating the development of resistant varieties and plant novelties at the same time (Teixeira da Silva et al., 2013). Consequently, many commercial varieties often lack resistance traits to biotic and abiotic stresses.

Cultivars grown under greenhouse conditions are constantly challenged by a number of arthropod infestations among which susceptibility to the western flower thrips (WFT), *Frankliniella occidentalis* (Pergande), is one of the main constraints in the year round production of quality chrysanthemums (Guldemond et al., 1994; Mouden et al., 2017a). Thrips feed by piercing plant tissues with their needle-shaped mouthparts, causing two types of feeding damage (de Jager et al., 1993). Growth damage is caused by feeding on actively growing tissue, whereas feeding on older, expanded tissue causes cells to become filled with air, which imparts a silvery appearance. The latter, known as silver damage, significantly affects product appearance and, hence reduces marketability. Insecticides have been a predominant management strategy, especially for high-value aesthetic ornamentals like chrysanthemum that require nearly zero level damage thresholds. However, once established in a greenhouse, control of thrips may be difficult to obtain due to their thigmotactic behavior and the limited number of active substances available (Mouden et al., 2017a). Management of pests early in the production cycle is, therefore, extremely important to prevent populations from building up to economically damaging levels.

A second important pest species in ornamental greenhouses is *Liriomyza trifolii* (Burgess) (van Dijk et al., 1992). Adult flies of the American serpentine leaf miner puncture leaves for feeding and oviposition. Feeding of larvae on the mesophyll leads to the formation of serpentine mines. When mature, they exit the leaf to pupate in the soil. (de Jong and van de Vrie, 1987). Both larval and adult stages can cause a decrease in the photosynthetic area, facilitate entry of plant pathogens, and adversely affect product appearance and yield.

Cultivars of commercially grown chrysanthemum are predominantly asexually propagated through vegetative terminal cuttings in order to generate genetically identical clones of the mother stock plants. Successful clonal propagation starts with adventitious root (AR) formation in stem cuttings and involves a large set of endogenous and exogenous factors (Druege et al., 2016; Druege et al., 2019). Auxins, a class of plant growth regulating hormones, affect many crucial plant physiological processes and are key determinants of rooting propensity of cuttings (Bellini et al., 2014; Pacurar et

al.,2014). Excision-induced formation of ARs is generally marked by accumulation of the endogenous auxin indole-3-acetic acid (IAA). Although IAA is able to stimulate ARs, indole-3-butyric acid (IBA) promotes rooting more efficiently, a feature partially due to its greater stability to light compared to IAA. Therefore, external application of IBA as talc or water diluted formulations is preferred in clonal propagation of commercial species.

In search of effective dipping treatments to protect cuttings during their vegetative stage, we serendipitously observed a reduction in thrips-associated feeding damage following a dipping treatment of the auxin-coated cuttings in water. Indeed, in spite of the fact that the phytohormone auxin has classically been implicated in developmental processes, recent studies demonstrate that auxin also affects a multitude of plant defense responses through complex interactions among multiple hormone pathways (Kazan and Manners, 2009; Erb et al., 2012; da Costa et al., 2013). Extensive cross talk between auxin and JA exists in both directions as these two hormones share many commonalities in their molecular mechanisms for hormone perception and signal transduction (Pérez and Goossens, 2013). However, only a few reports are available regarding the exogenous effects of auxin on JA synthesis, which in addition, appear to be inconclusive. Auxin acts either synergistically or antagonistically with other hormones to trigger cascades of events leading to AR initiation and development (Lakehal and Bellini, 2019). Several lines of evidence indicate that auxins antagonize JA biosynthesis and signaling. External application of auxin suppressed herbivory-induced accumulation of jasmonates (Baldwin et al., 1997). Likewise, a number of wound induced responses, including the expression of jasmonate-dependent proteinase inhibitor genes and vegetative storage proteins were negatively controlled by the level of auxin (Kernan and Thornburg 1989; DeWald et al., 1994). Down regulation of JA-responsive genes was also observed in auxin treated *Arabidopsis* plants (Rojo et al., 1998; Liu and Wang, 2006). Furthermore, auxin has been shown to induce expression of the JA signaling repressor JAZ1 (Grunewald et al., 2009). Although all these experiments suggest an inhibitory effect of auxin on JA biosynthesis, contradictory results have been reported recently with auxins inducing the expression of JA biosynthetic genes (Tiryaki and Staswick, 2002; Fattorroni et al., 2017). Auxin also mediated increases in JA concentration whereas, mutants with no functional auxin receptors showed a reduction in JA amounts, suggesting a synergistic function of auxin and JA (Grossman et al., 2004; Nagpal et al., 2005).

These results illustrate the complexity of auxin-induced changes and strongly suggest their importance in plant stress responses, particularly toward biotic stresses. Numerous studies, mainly from the analysis of mutant phenotypes, have indeed demonstrated the involvement of auxins in disease development (Kazan and Manners, 2009; Robert-Seilaniantz et al., 2011; Čarná et al., 2014). The changes incurred in plants as a result of these plant growth regulators may, therefore, likely affect plant insect-relationships. However, these effects have been poorly explored (Erb et al., 2012). Given their importance in commercial plant propagation, the impact of auxin on insect herbivory deserves more attention if we are to understand their role as defense regulators. In the present study, we investigated host plant resistance against WFT in auxin-coated chrysanthemum cuttings. In this context, we hypothesized that chrysanthemum susceptibility against thrips herbivory could be

attributed to attenuation of induced defenses by exogenous auxins, possibly through its antagonistic effect on JA. Alternatively, the observed reduction of silver damage symptoms in chrysanthemum cuttings may equally well result from a synergistic mode of action of auxin with JA by improving the uptake of externally applied auxin upon water dipping. Therefore, we aimed to investigate the effect of IBA and water dipping primarily in relation to thrips resistance. Moreover, we explored plant defense responses triggered upon treatments by measuring the expression of the defense-related marker protein polyphenol-oxidase (PPO) and several defense- and growth- related phytohormones.

Materials and Methods

Plant material and growth conditions

Chrysanthemum plants (*Chrysanthemum morifolium* Ramat) of the cultivar Baltica, which is susceptible to WFT, were used in all experiments and were kindly provided by Deliflor Chrysanten B.V. (Maasdijk, the Netherlands). Unrooted cold-stored chrysanthemum cuttings were imported from different production facilities located in Ethiopia (E) or Uganda (U). As a standard procedure for the production of commercial cuttings, the basal cut ends of freshly pinched cuttings were pre-coated with rooting powder by greenhouse workers, in order to stimulate root growth. Rooting hormone powder consisted of 0.4% indole-3 butyric acid in talc (Chryzoteck beige 0.4%) and was applied as a dry powder formulation. Unrooted chrysanthemum cuttings were approximately 5 cm long with three to four nodes. Commercial pre-coated cuttings were individually planted in plastic trays (4×4×6 cm) containing a 3:1 mixture of potting soil (Horticoop, Lentse Potgrond, the Netherlands) and vermiculite, pre-moistened to saturation using tap water. During the initiation phase of rooting, cuttings were covered with a transparent polyethylene plastic bag fitted over a plastic sliding tray (60×40×55 cm; Beekenkamp Verpakkingen B.V., Maasdijk, the Netherlands) to maintain humidity and prevent desiccation of cuttings. After a rooting period of 11 days, the cover was removed and cuttings were transplanted to plastic pots (Ø 11 cm; Pöppelmann, Germany) containing potting soil, 10 g/L vermiculite and 1.5 g/L osmocote slow release fertilizer (Scott, Scotts Miracle-Gro, Marysville, Ohio, USA: 15:9:11 NPK). Cuttings were grown in a climate room provided with 113.6 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR) and a light/dark cycle of 16/8 h at 20°C and 70% relative humidity (RH) in a completely randomized design. Watering was applied every two days. At 14 days after planting, plants were randomly subjected to a non-choice whole plant thrips bio-bioassay or sampled for chemical analyses as described below.

Non-choice whole plant thrips bioassay

A non-choice whole plant bioassay was used to evaluate resistance against WFT (Leiss et al., 2009). Two-week old cuttings were individually placed in thrips-proof cages consisting of a plexiglass cylinder (50 cm height, 20 cm diameter) closed at one end with a displaceable ring of nylon gauze of 120 µm mesh size. Plants were randomly placed in a climate-controlled growth chamber at a constant temperature of 25 °C, a photoperiod of 16L:8D and 70% RH. For infestation, adult thrips were collected

in glass jars using a mouth-operated aspirator. Western flower thrips were obtained from a continuous mass-rearing on flowering plants of the susceptible chrysanthemum variety Euro Sunny and were maintained in a climate room at 25 °C and 70% RH. Twenty adult thrips, consisting of eighteen females and two males, were released inside the cage, simulating high-density infestations. Seven or 14 days after infestation, cuttings were visually inspected for thrips feeding damage, hereafter referred to as 'silver damage'. Silver damage, expressed as damaged leaf area in mm^2, was evaluated in all leaves. Whole plant cumulative silver damage of plants is presented in the graphs.

Non-choice whole plant leaf miner bioassay

Leaf miners, *Liriomyza trifolii* (Burgess), were obtained from a continuous mass rearing on a susceptible cultivar of chrysanthemum (Ultra Light) in a climate room provided with L16:D8 and 60% RH at 23 °C. In order to obtain pupae, infested plants containing third instar larvae were placed horizontally. One-day old flies were shortly anesthetized by CO_2 and sorted by sex using a fine sable paintbrush prior to release onto the plants. Two unmated males and females each, were placed into a small cage with chrysanthemum cuttings. The adult leaf miners were allowed to deposit eggs on the plants for 24 hours, after which they were removed from the cage. All plants, free of adult leaf miners, were then moved to another clean climate room (L16:D8, 70% RH, at 25 °C) and placed randomly. The total number of mines on the plants were counted 3 days after leaf miner release to prevent overlap in formation of mines and thus, allow for more accurate counts of individual mines. Plants were harvested by cutting them at the crown level and transferring them into individual Ziploc bags. Subsequently, these bags were placed in a climate chamber at 20 °C for pupae to develop. The number of pupae was scored one week after harvesting using a dissecting microscope (25x). Plants were carefully checked for remaining pupae in leaf tissue, which were indicated as non-emerged pupae.

Dipping treatments

Bio-insecticidal dipping of unrooted cuttings

The use of natural compounds with low risk profiles offer a simple and a cost-effective opportunity for sustainable pest management. Among them, β-alanine has been reported confer resistance against WFT (Leiss et al., 2013). Therefore, bio-insecticidal dips were evaluated as a pre-treatment of unrooted chrysanthemum cuttings to enhance resistance against thrips. Treatments were based on the results of preliminary trials in the laboratory. The basal cut end (~1 cm) of unrooted cuttings were individually dipped in an aqueous solution of beta-alanine at a final concentration of 100 mg/ml. Water dipping treatment was considered as control whereas, a non-dipped group of unrooted cuttings was included as a negative control group. Furthermore, the effect of exogenously applied jasmonic acid was included as a positive control. Based on preliminary experimental results, a dipping time of 60 minutes was selected as an effective duration for JA treatment. The JA stock solution (1.19 M) was prepared by dissolving 2.5 mg in 1 ml absolute ethanol which, prior to use, was

diluted to a final working concentration of 5 mM. Following dipping treatments, cuttings were planted in a mixture of pre-moistened soil and vermiculite. Two weeks after rooting, chrysanthemum plants were subjected to a non-choice whole plant bio-assay as previously described. Various dipping durations were evaluated (e.g. 30, 45 and 60 min) but pooled silver damage data were used for analysis.

Water dipping of IBA pre-coated cuttings in relation to thrips resistance

To investigate the role of auxins in chrysanthemum susceptibility and to explore the potential of water dipping treatments for resistance improvement, commercial cuttings pre-coated with IBA (Chryzoteck beige 0.4%) and non-coated control cuttings free of IBA were used for this experiment. The basal end (~1 cm) of the cuttings were individually dipped in water for 30, 45 or 60 minutes, whereas non-dipped cuttings served as negative control (indicated as t=0). The cuttings were immediately planted and randomly grown under conditions described above. Two weeks after rooting, chrysanthemum plants were subjected to a non-choice whole plant bio-assay or sampled for dry weight measurements, with ten replications per treatment.

Water dipping of IBA pre-coated cuttings in relation to leaf miner resistance

Furthermore, we evaluated the involvement of auxins in relation to leaf miner resistance. To examine the role of IBA in leaf miner susceptibility, unrooted Baltica cuttings with and without IBA coating were grown for 2 weeks after which they were infested with four adult leaf miners for a period of 24 hours (n=15). In a second experiment, the effect of water dipping was investigated in the presence of IBA coating. Commercial cuttings pre-coated with IBA (Chryzoteck beige 0.4%) were dipped in water for various durations (30, 45, 60 minutes), whereas non-dipped cuttings served as negative control (indicated as t=0). Additionally, a positive control was included by dipping the basal cut ends for 60 minutes in a solution of 5 mM jasmonic acid. After treatment, all cuttings were directly planted in soil. After the rooting period, cuttings were subjected to a leaf miner bioassay (n=13) or sampled for polyphenol oxidase measurements as described elsewhere (n=8 to 10). After scoring pupae emergence the plants were transferred to paper bags and dried in an oven at 50 °C for at least 3 days for measurements of plant dry mass .

Standardization of IBA applied powder formulation

The objective of the present experiment was to standardize hormone powder application in order to potentially reduce IBA-induced response variations among cuttings. For powder formulations the main variables were those factors determining the amount of powder adhering to the cutting base and epidermis, including pre-dipping in water, retaining the powder through careful handling and standardizing the diameter of the cutting insertion (5 mm) at the soil surface to prevent loss of hormones. Rooted Baltica cuttings, planted in small compost blocks of 64 cm^3, were manually pinched to obtain stem cuttings of 5 cm in length. The full factorial experiment contained two factors, each with four levels with main treatments being auxin application (no dip, 0.4 and 0.8% IBA and

talc) and water dipping time (0, 30, 45 and 60 minutes). Indole-3-butyric acid was applied as a dustable talc-based powder formulation to basal cut ends. Talc powder, primarily consisted of magnesium silicate ($Mg_3H_2(SiO_3)_4$, Sigma-Aldrich with particle diameters measuring between 1.9 and 2.3 µm) and was used as an inert carrier for dry powder formulations and was homogeneously mixed with IBA at a final concentration of 0.4 and 0.8% (w/w). To satisfactorily adhere to the cut ends and to provide a medium of contact through which the hormone could enter plant tissues, the basal cut ends were pre-wetted with water. Subsequently, cut bases were dipped in 0.4 % IBA, 0.8% IBA or talcum powder. In order to ensure precise application the amount of powder that was applied to the cut base of the stem was standardized to approximately 150 mg. Excess powder was brushed off. Other sets of cuttings were untreated and served as negative control. To mimic the proposed commercial application and to keep cuttings turgid, they were stored in sealed plastic bags under cold (4 °C) and dark conditions for a week prior to water dipping treatments. For water dipping treatments, unrooted cuttings were vertically placed in glass vials filled with a layer of water at room temperature, covering 1 cm of their basal cut end. Cuttings were individually dipped in water for 30, 45 or 60 minutes, whereas control cuttings were not dipped (t = 0 min). All cuttings were planted on the same day, after their respective basal water dip treatments, in a 3:1 potting mix consisting of potting soil with vermiculite. Fifteen cuttings were treated as a sampling group of which ten cuttings from each treatment group were subjected to a non-choice whole-plant thrips bioassay and five cuttings per treatment were used for plant hormone analysis as described below. For statistical analysis, data for silver damage, hormone concentrations and dry mass were pooled (30-45 and 60 min) for each coating treatment

Comparing dry-dip rooting powder with water-based rooting solution

The efficacy of two different application methods for IBA was investigated to avoid potential confounding effects exerted by talc-powder. The basal cut end of commercially provided unrooted Baltica cuttings, pre-coated with IBA, was dipped in water for 30 minutes. The commercial rooting powder Chryzotek beige 0.4% contains 4000 parts of indole-3-butyric acid to a million parts of talc and thus, basal liquid dips were performed using concentrations equimolar to powder applied IBA. To this end, eight water soluble IBA tablets of 50 mg each (Rhizopon AA) were dissolved in 100 ml MilliQ water at RT to obtain a final concentration of 4000 ppm (Rhizopon, Hazerswoude-Rijndijk, the Netherlands). Cuttings, free of hormone powder were inserted in a floating mat of which approximate 1 cm of the basal cut end was dipped in the IBA suspension for 30 minutes. Water soluble IBA solution was constantly stirred to avoid precipitation. After dipping, cuttings were grown as previously described. Each treatment consisted of 15 replicates. Two weeks after rooting cuttings were subjected to a non-choice whole-plant thrips bioassay .

Effect of dipping on different forms of induced resistance in chrysanthemum

To evaluate whether reductions in thrips associated feeding damage operate in a JA-dependent manner, polyphenol oxidase activities were measured. Forty IBA-coated cuttings were dipped in

water for 30 minutes. Control cuttings, pre-coated with IBA, were directly inserted in a pre-mixture of soil and vermiculite (n=40). Two weeks post treatment cuttings were randomly divided in two groups of 20 each and were either infested with thrips or sampled for PPO measurements (i.e. direct induction). To demonstrate whether the underlying mechanisms govern direct induction of defenses or prime for a potentiated response, cuttings were also sampled for PPO assessment after thrips infestation.

In parallel to this experiment, an additional 280 cuttings were subjected to the same treatments as above to determine whether defense responses were time-dependent. For this time course experiment, chrysanthemum cuttings pre-coated with IBA were subjected to control (no dipping) or water dipping (30 min) treatment. At 14 days after the start of the dipping treatments, all leaves were sprayed with 2 mL of 7.5 mM MeJA (Sigma-Aldrich) or treated with the corresponding mock solution consisting of 0.8% aqueous ethanol. JA-associated defenses were artificially induced using methyl jasmonate (MeJA) because inductive effects on PPO bioactivities were observed to be more consistent than herbivore induction. Ten cuttings from a given treatment were periodically sampled for PPO measurement at 0 (i.e. before hormone treatment), 12, 24, 36, 48, 72 and 168 hours (7 days) after hormone application.

Polyphenol oxidase (PPO) activity

Polyphenol oxidase (PPO) activity was measured following the method of Stout et al. (1998) with slight modifications. Two weeks after treatment (i.e. before infestation) or one week post infestation, the third leaf from the bottom was sampled. Fresh leaf material was ground using a tissue lyser (Qiagen, Hilden, Germany) and stored at –80 °C until analysis for PPO activity. Hundred fifty milligrams of fine powder was extracted with 1.25 ml ice-cold potassium phosphate buffer (0.1 M, pH 6.8) containing 7% (w:v) polyvinyl polypyrolidine. To this homogenate, 0.4 ml of a 10% solution of Triton X-100 was added. Plant extracts were vortexed for 2 min and centrifuged at 11,000 g for 10 min at 4 °C. The resulting supernatant was used directly as an enzyme source using chlorogenic acid as a substrate. The reaction mixture consisted of 5 µl enzyme extract and 1 ml of 2.92 mM chlorogenic acid dissolved in 0.1 M potassium phosphate buffer at pH 8.0. The rate in change of absorbance of this mixture was spectrophotometrically measured at 470 nm for one minute (UV-1800 UV-VIS spectrophotometer, Shimadzu Europe GmbH, Duisburg, Germany). PPO activities were calculated from the linear slope and were reported as changes in absorbance values per min per gram of fresh weight.

Hormone analysis

To investigate the signaling pathways involved in auxin-mediated induction of defenses against WFT, we determined how coating and water dipping treatments influenced plant defense- and growth-related hormone levels. Two weeks after initial dipping treatment, prior to thrips infestation, the basal third leaf was sampled for hormone analysis (n=5). Analysis of jasmonic acid (JA), its biosynthetic precursor 12-oxo-phytodienoic acid (OPDA), jasmonic acid-isoleucine (JA-Ile), salicylic acid (SA),

abscisic acid (ABA) and auxin (indole-3-acetic acid, IAA) were performed following the procedures described by Machado et al. (2013) and Schäfer et al. (2016), with some modifications. Leaves were flash frozen in liquid nitrogen and stored at –80 °C until freeze-drying. Approximately 100 mg of frozen and homogenized leaf material was aliquoted in 2 ml Eppendorf tubes and extracted with 1 ml of methanol containing 40 ng of the phytohormone standards D_6-ABA (Olchemin), D_6-JA (HPC), D_6-JA-Ile (HPC), D_6-SA (Olchemin), and D_5-IAA (Olchemin). Samples were vortexed for 10 min and centrifuged at 14,000 rpm for 10 min at 4 °C. Subsequently, the supernatants were transferred to new Eppendorf tubes and evaporated to dryness in a vacuum-concentrator at room temperature. The residue was dissolved in 20 µl 70% aqueous methanol (v/v) for 5 min using an ultrasonic bath, and centrifuged 5 min at 14,000 rpm. The supernatants were transferred to glass vials and then analyzed by LC-MS/MS (EVOQ, Bruker Daltonics). One µl of each sample was injected onto a C_{18} Zorbax-Eclipse column (50 × 4.6 mm, 1.8 µm, Thermofisher). The mobile phase was comprised of LCMS-grade water (solvent A) and acetonitrile (solvent B), both containing 0.05% (v/v) formic acid. The program had a constant flow rate of 400 µl min^{-1} and consisted of 0–0.5 min 95% solvent A; 0.5–2.5 min 50% solvent A, and 50% solvent B; 2.5–3.5 100% solvent B; 3.5–4.5 min 95% solvent A. The column temperature was set at 42 °C. The cone, probe, and nebulizer gas were set at the following flow conditions (arbitrary units/temperature): 35/350 °C, 60/475 °C and 60 (arbitrary unit), respectively. The phytohormones were quantified using the signal of their corresponding internal standard and were expressed on the basis of fresh leaf weight.

Statistical analyses

Normal distributions were confirmed by Shapiro-Wilk tests and homogeneity of variances were determined by Levene's tests. Means were compared, where appropriate, using an unpaired Student's t-test or one-way ANOVA followed by Fisher's least significance difference (LSD) post hoc test. When assumptions were violated, data transformations were performed or differences in means were analyzed using the nonparametric Mann–Whitney tests or Welch's ANOVA.

To evaluate the effect of water, beta-alanine and jasmonic acid dipping treatments after one week of thrips infestation silver damage data were compared to non-dipped control cuttings using an unpaired Student's t-test. Likewise, differences among same treatment groups following an infestation period of two weeks were analyzed by a Student's t-test but, were square root transformed prior to analysis to meet normality assumptions. The test for normality, examining standardized skewness and the Shapiro-Wilks test, indicated that the data for mine and pupae counts were statistically non-normal. Therefore, differences in the total number of mines and total number of pupae between IBA-coated and non-coated control cuttings were analyzed by Mann–Whitney tests with a 5 % level of significance. An unpaired Student's t-tests was used to assess significant differences in number of leaves between both groups.

To investigate the effect of dipping treatments on leaf miner resistance, one-way ANOVA was used to analyze differences in total number of mines among groups. However, for pupae counts, the Levene's F test revealed that the homogeneity of variance assumption was not met ($p = 0.046$).

Therefore, a Welch's ANOVA followed by Games-Howell's post hoc was used to assess significant differences in total number of pupae among dipping treatments. Differences in emergence of pupae (emerged and non-emerged) were analyzed by a Welch's t-test. Data transformations were performed to significantly reduce heteroscedasticity and normalize residuals of PPO levels, but failed to meet the assumption of normality. Subsequently, reciprocally transformed PPO data were analyzed by Mann-Whitney test.

To study the underlying mechanisms of resistance (i.e. induction or priming), silver damage data between control and water dipped cuttings were analyzed using the Student's t-tests. Likewise, for the time-course experiment, differences in PPO levels between control and water dipped plants sprayed with MeJA or mock at each time point were tested using Student's t-tests whereas, PPO activities, before and after thrips infestation, were analyzed by Generalized Linear Models (GLM)) using linear distribution and identity link functions. Moreover, GLM were used to analyze the effect of dipping treatment, coating treatment and their interaction on silver damage symptoms, PPO activity, hormone concentrations and dry weight. Differences among groups were tested by Fisher's LSD post-hoc test. Differences in all analyses were considered significant at $p < 0.05$. As an indication which plant hormone variables would best predict variation in silver damage among treatments, multiple backward linear regression analysis was performed according to the Akaike information criterion (AIC). Variables were removed from the full model when the variance explained did not significantly improve the model (α=0.05). Among the five generated models, the most significant model, retaining a set of two strong predictors of silver damage, were further analyzed using GLM. For comparison of dry-powder application and liquid dips with IBA, square root transformed silver damage data were analyzed by Kruskal-Wallis followed by Dunn's test with Bonferroni correction for multiple comparisons. All statistical analyses were conducted with SPSS v. 24 software (IBM; SPSS Inc., Chicago, IL, United States).

Results

Effect of bio-insecticidal pre-pretreatments on thrips resistance

In preliminary experiments we observed that chrysanthemum cuttings were more resistant to thrips, i.e. displayed less silver damage, than untreated cuttings when the basal portion of cuttings were dipped in a solution with β-alanine (Supplementary figure S1; ($t(7) = 2.149$, $p = 0.069$). Therefore, we further investigated the potential of basal liquid dipping treatments, using aqueous solutions of β-alanine, as a possible strategy to enhance thrips resistance of chrysanthemum cuttings during their early vegetative stage. In comparison to non-dipped control cuttings, water dipping, but not β-alanine significantly reduced silver damage when cuttings were infested with thrips for one week (Figure 1A; $t(23) = 2.137$, $p = 0.043$). Furthermore, upon dipping in jasmonic acid, thrips-associated feeding damage was markedly reduced by 65% relative to the control ($t(18) = 4.680$, $p < 0.001$). Following an infestation period of two weeks, the effect of β-alanine dipping treatment was highly significant (Figure 1B; $t(46) = 3.623$, $p = 0.001$) however, did not differ significantly from water dipping

with respect to reduction in silver damage (Figure 1B). JA dip reduced silver damage by two-fold as compared to the control ($t(28) = 5.439$, $p < 0.001$). Taken together, these observations indicate that water dipping of IBA-coated cuttings is involved in enhancing resistance against thrips. Moreover, dipping treatments had no negative effect the total dry mass of cuttings ($F(7, 79) = 1.31$, $p = 0.257$).

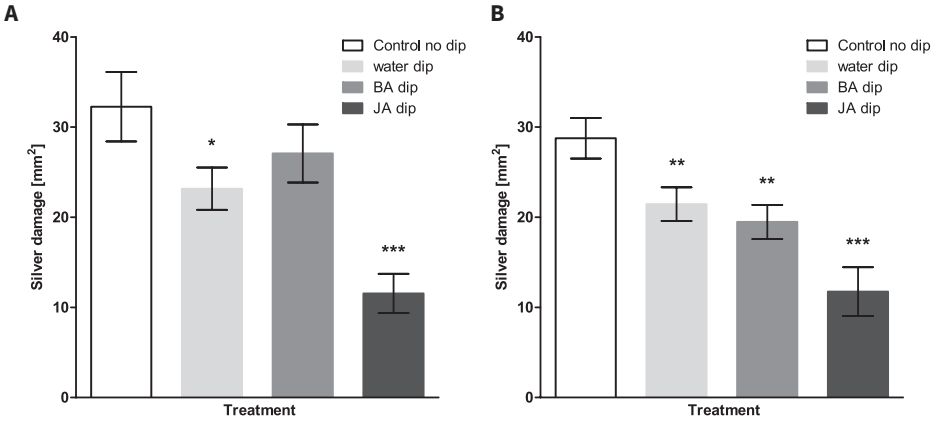

Figure 1. Effect of basal liquid dips on chrysanthemum resistance against western flower thrips (WFT). The basal portion of stem cuttings were dipped in 100 mg/ml of β-alanine (BA), water or 5 mM JA. Untreated, non-dipped, cuttings served as control. All commercially provided cuttings were pre-coated with indole-3-butyric acid IBA (Chryzotek beige 0.4 %). Two weeks post treatment, cuttings were infested with 20 adult thrips for a period of **(A)** 7 days or **(B)** 14 days. Silver damage symptoms were visually scored and expressed as damaged leaf area in mm². Means for water dipping and β-alanine represent pooled cumulative silver damage of three dipping timepoints (30, 45 and 60 minutes). Data are presented as mean ± SEM. Asterisks indicate significant differences in comparison to non-dipped control as determined by an unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Water dipping of IBA-coated cuttings confers protection against thrips

The observations of reduced silver damage upon water dipping gave rise to the hypothesis that auxins may exert an antagonistic effect on the jasmonic acid signaling pathway, and consequently, increase WFT susceptibility of cuttings commercially pre-coated with auxins. In order to evaluate the influence of auxin on thrips susceptibility, unrooted cuttings with and without IBA were infested with adult thrips after a rooting period of two weeks. Furthermore, we evaluated the effect of water dipping at three different time points namely; 0 – 30 – 45 and 60 minutes (Figure 2). Significant differences among treatments were observed, both for dipping time and auxin coating (GLM: Wald $\chi^2 = 9.92$, $p = 0.019$ and Wald $\chi^2 = 4.55$, $p = 0.033$, respectively). The time by coating interaction was not significant (GLM: Wald $\chi^2 = 3.02$, $p = 0.388$). Auxin coating of cuttings significantly affected silver damage symptoms however, mean comparisons within dipping time revealed there were no significant differences between IBA-coated and non-coated cuttings under control conditions (i.e. non-dipped cuttings at t=0). Additionally, at 45 and 60 minutes no significant differences were observed between cuttings pre-treated with IBA and untreated cuttings. Interestingly, however, water dipping of IBA treated cuttings significantly increased thrips resistance. The response to water dipping was independent of the time. After 30 and 60 minutes plants displayed significantly less silver damage.

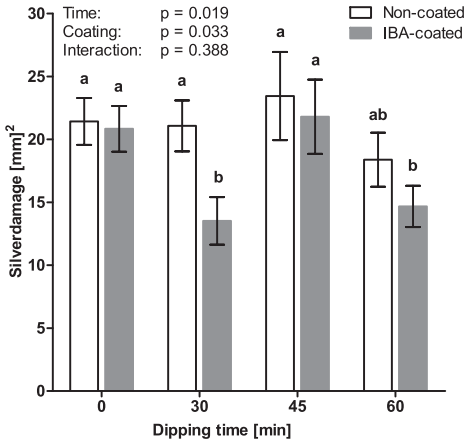

Figure 2. Effect of IBA coating and water dipping on chrysanthemum susceptibility against thrips. Basal cut ends (cv. Baltica), in the presence or absence of the powder formulated rooting hormone IBA, were dipped in water at various durations. Two weeks post treatment, cuttings were infested with 20 adult thrips for a period of one week. Cumulative silver damage data are presented as mean ± SEM (n=10). Different letters denote significant differences among groups as determined by GLM followed by Fisher's LSD test ($P < 0.05$). The overall effects of dipping time, auxin coating and their interaction are indicated in the graph.

Silver damage symptoms were reduced up to 35 % compared to plants grown from non-dipped IBA-coated cuttings. In contrast, in the absence of exogenous auxin coating, cuttings displayed no significant differences following water dipping.

Water dipping of IBA-treated cuttings reduced pupae emergence

The unexpected benefit of water dipping triggered our curiosity to further evaluate whether such an approach could improve resistance against other herbivores. Therefore, we evaluated the effect of water dipping treatments on celery leaf miner resistance, a second important pest insect species of chrysanthemum. Two separate experiments were performed to explore the role of IBA in leaf miner resistance. Firstly, we investigated the physical effect of the rooting hormone IBA on leaf miner resistance by comparing IBA-coated and non-coated cuttings (Supplementary Figure 2). We observed that the mere physical presence of IBA does not influence susceptibility of chrysanthemums to leaf miners. Total number of mines ($U = 74.5$, $p = 0.114$) as well as the number of pupae ($U = 91$, $p = 0.372$) were not affected by exogenous application of IBA to cut ends (Supplementary figs. 2A and 2B, respectively). The growth of cuttings, on the contrary, was significantly influenced by IBA. After two weeks of rooting, the number of leaves in cuttings pre-treated with the rooting hormone IBA (9.60 ± 0.25) was significantly higher than the untreated control group (8.72 ± 0.23) ($t(28) = -2.536$, $p = 0.017$). Additionally, in a second experiment we further assessed the effect of water dipping in the presence of the rooting hormone IBA on leaf miner (Figure 3). Group means of the total number of mines did not differ significantly ($F(4, 59) = 1.08$, $p = 0.374$). In contrast, the number of pupae was significantly affected by dipping ($F(4, 28.52) = 10.59$, $p < 0.001$). The amount of time cuttings were dipped in water had no effect on pupation. Post hoc comparisons revealed that non-dipped control cuttings and

water dipped cuttings were similar to each other, but were significantly different from the jasmonic acid treated group (Figure 3B). It is noteworthy, however, that the effect of water dipping becomes more evident when the ratio between emerged and non-emerged pupae is examined. Leaf miner larvae pupate in hibernacula within mines and emerge before or after leaf abscission. Interestingly, upon further evaluation a significantly lower rate of emergence was observed for groups receiving a dipping treatment whereas, in comparison to the non-dipped control group, no differences for non-emerged pupae were observed (Figure 4A). The number of pupae successfully emerging from chrysanthemum leaves was significantly lower after 30 and 45 minutes of dipping ($t(15,27) = 2.55$, $p = 0.022$ and $t(16,69) = 2.18$, $p = 0.044$, respectively). For cuttings that were dipped in water for 60 minutes we also observed a reduction although not statistically significant ($t(20,64) = 1.65$, $p = 0.115$). Treatment of cut ends with JA significantly reduced emergence rate by 97% as compared to non-dipped control cuttings ($t(12,08) = 3.93$, $p = 0.002$).

To investigate whether reduction in pupae emergence rates were related to induction of defenses, we assayed the activity of the defense-related protein polyphenol oxidase (PPO) by sampling leaf material prior to infestation (Figure 4B). An irregular pattern of PPO activity was observed in water and JA treatment groups. Activities of PPO were not significantly stimulated following dipping treatments suggesting that enhanced resistances were not explained by direct induction. Nonetheless, upon a dipping duration of 30 minutes in water, PPO levels were only marginally statistically enhanced in comparison to the non-dipped control ($U = 19.0$, $p = 0.068$).

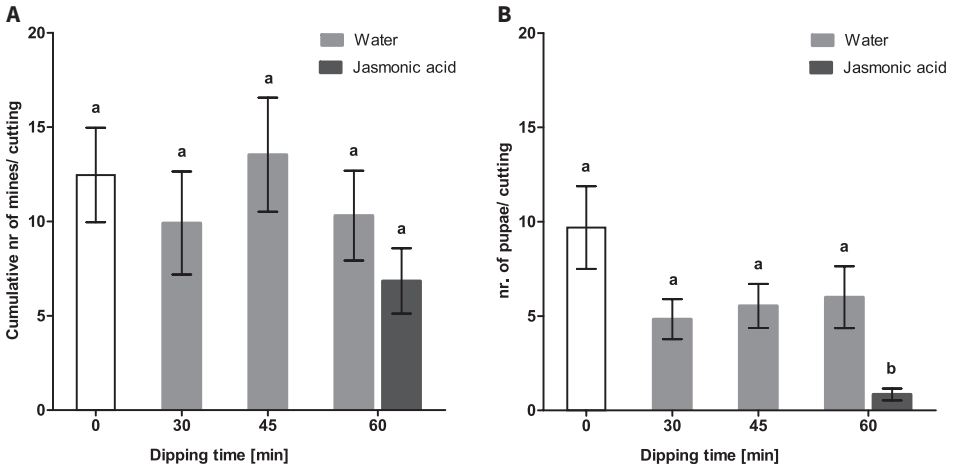

Figure 3 Effect of water dipping and jasmonic acid on leaf miner resistance (*Liriomyza trifolii*). Unrooted cuttings, pre-coated with indole-3-butyric acid (IBA), were dipped in water for various durations or in a solution of 5 mM jasmonic acid for 60 min. Two weeks post treatment, cuttings were individually caged and infested with four flies for 24 hours. **(A)** Total number of mines were scored after 3 days, whereas the number of pupae were counted after one week **(B)**. Data are represented as mean ± SEM, n=13. Different letters indicate significant differences among treatments as determined by **(A)** Fisher's least significant difference (LSD) test or **(B)** Games–Howell at $p < 0.05$.

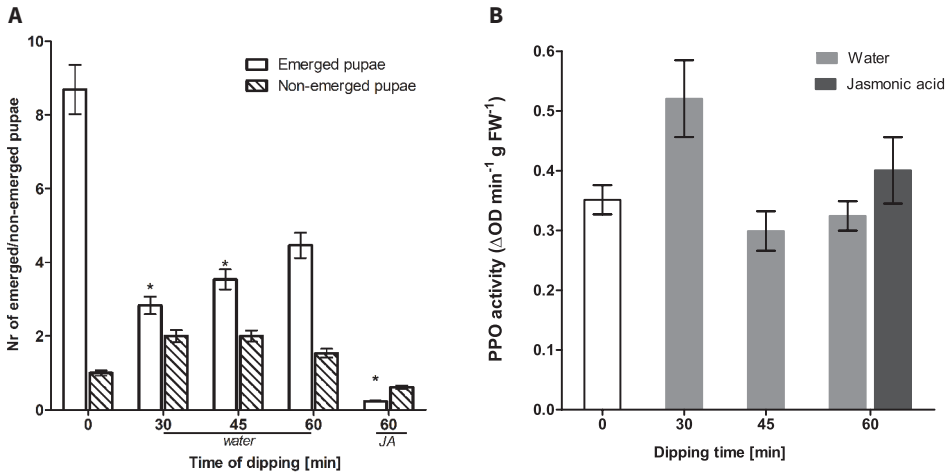

Figure 4. Effect of dipping on the leaf miner pupation and induction of polyphenol oxidase (PPO) activity. Cut ends of chrysanthemum, pre-treated with 0.4% IBA, were dipped in water for various durations and in a solution of 5 mM jasmonic acid for 60 min. Non-dipped cuttings, indicated at t=0 in the graph, serve as control. **(A)** Number of pupae emerging from chrysanthemum leaves and non-emerged pupae were scored after one week (n=13). Asterisks indicate statistical significance in comparison to non-dipped control cuttings determined by Welch's t–test (*p <0.05). **(B)** PPO (n=8-10) was measured in the third leaf from the bottom prior to leaf miner infestation. Reciprocally transformed PPO data were analyzed by Mann-Whitney test. Data shown are representative of means ± SEM.

Water dipping does not induce nor prime for enhanced defenses

After evaluating the effect of auxin and dipping treatments on resistance to thrips and leaf miner, the most effective treatment was selected to further study the dynamics of induced resistance. We observed that efficacy of water dipping was most evident at 30 minutes. To demonstrate whether defense responses were induced directly or primed for potentiated expression upon thrips infestation, the activity of PPO was assayed by sampling leaf material before and after thrips infestation, respectively. Firstly, we investigated how water dipping of IBA-coated cuttings affected the induction of the JA-associated marker PPO and, concomitantly, the effect on resistance against WFT (Figure 5). In contrast to our previous observations, however, we found only a minor reduction in silver damage by water dipping and this was not significant ($t(38) = 1.45$, $p = 0.156$). The lack of enhanced resistance corresponds with the measured PPO levels. PPO activities were not stimulated upon water dipping treatment of cuttings (GLM, Wald $\chi^2 = 1.05$, $p = 0.307$). To further address whether water dipping primed plant defense, we assessed the induction of PPO activity under thrips infestation in control and water-dipped cuttings. Thrips infestation had no effect on the level of PPO (GLM, Wald $\chi^2 = 0.09$, $p = 0.763$).

Plants respond to exogenous methyl jasmonate (MeJA) with a myriad of inducible defense responses, including the production of anti-digestive proteins such as PPO (Constabel and Ryan, 1998). The similarities to defense responses triggered by herbivores allows to closely mimic wound-induced responses by artificial elicitation and hence, provides potential means to explore how dipping treatments of chrysanthemum cuttings contribute to enhanced thrips resistance. Therefore, in a

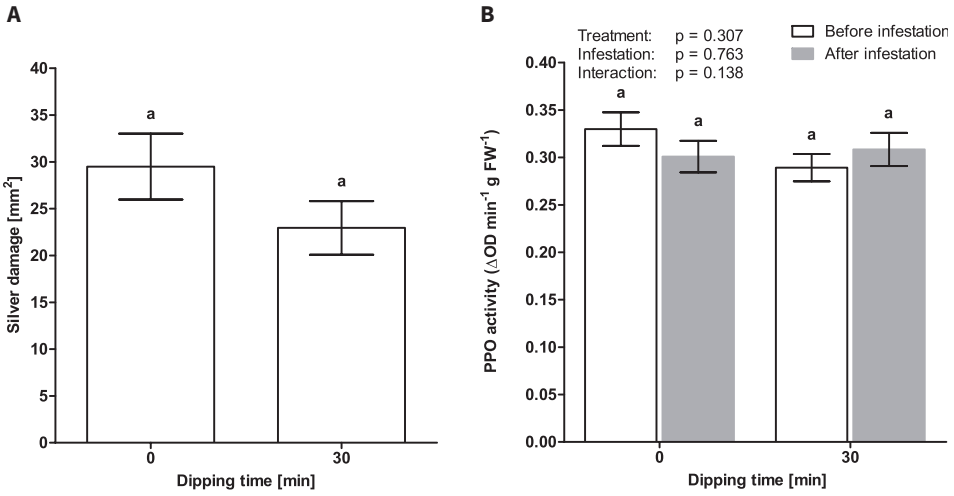

Figure 5. Effect of water dipping treatment on thrips-associated silver damage **(A)** and polyphenol oxidase (PPO) activity **(B)**. Cut ends of chrysanthemum cultivar Baltica, pre-coated with the powder formulated rooting hormone IBA (Chryzotek beige 0.4%), were dipped in water for 30 min. Two weeks post treatment, cuttings were infested with 20 adult thrips or sampled for PPO measurements before infestation. One week after thrips infestation, silver damage symptoms were visually scored and expressed as damaged leaf area in mm^2 and subsequently sampled for PPO activity after infestation. Data in the graphs represent mean ± SEM of 20 individual cuttings. The main effects of dipping time, infestation and their interaction are indicated in the graph.

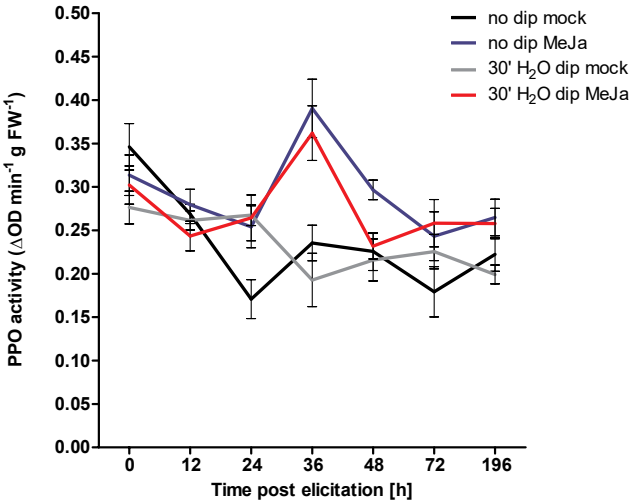

Figure 6. Effect of water dipping treatment on polyphenol oxidase activity (PPO). Comparative graph of PPO activity on a time course after MeJA elicitiation in water dipped cuttings and non-dipped control cuttings. Basal ends of chrysanthemum cuttings (cv. Baltica), coated with the powder formulated rooting hormone IBA (Chryzotek beige 0.4%), were dipped in water at for 30 minutes or left untreated. Two weeks after rooting, cuttings were directly harvested for PPO analysis (t = 0 min) or foliarly sprayed with 7,5 mM MeJA to mimick thrips infestation. Mock treated plants served as control. PPO activity was measured in the third leaf from the bottom. Values represent the mean of 10 replicates with SEM. Asterisks indicate significant difference at α=0.05 as determined by an unpaired Student's t-test.

further attempt to elucidate the role of auxin and water dipping treatments in establishing enhanced herbivore resistance, a time-course experiment was performed using MeJA for artificial induction of JA-associated defenses (Figure 6). In mock-treated plants PPO activities initially decreased and were then stabilized over time. PPO activities reached a peak level 36 h after methyl jasmonate (MeJA) application and then gradually declined at the end of the experiment. Exogenously applied MeJA significantly enhanced PPO activities by two-fold in water-dipped plants as compared to their corresponding mock-treated control (30 min H_2O dip-mock; t (18)=3.85, p = 0.001). Interestingly, the rate of PPO synthesis changed almost coordinately with non- water dipped plants and a similar trend was observed upon hormone treatment. PPO activity was directly responsive to exogenous MeJA treatment. Likewise, 36 h hours post elicitation, PPO levels were significantly amplified relative to the corresponding mock-control (t(18)=3.95, p < 0.001).

Standardization of IBA applied powder

Dipping treatments of chrysanthemum cuttings generally suffer from inconsistency. Many reasons can account for this situation among which a lack of standardized IBA formulation can be hypothesized. Indeed, within each batch of commercially provided unrooted cuttings, we observed considerable variation in the amount of IBA applied rooting powder at the basal cut ends (Supplementary Figure S3). In view of this, the current experiment aimed to reduce response variations by regulating factors that may potentially influence absorption of IBA through the cut base. To this end, the loading amount of powder applied IBA at the cut base and duration of water dipping were systematically studied (Figure 7A). Silver damage did not significantly differ among various dipping treatment (GLM: Wald χ^2 =1.41, p = 0.236). Similarly, exogenous powder application neither affected silver damage symptoms (GLM: Wald χ^2 = 6.73, p = 0.081). However, a significant interaction was observed between auxin treatment and dipping time (GLM: Wald χ^2 = 13.91, p = 0.003). The least amount of silver damage was observed in non-coated cuttings under control conditions (i.e. control cuttings at t= 0 min). In the absence of a water dipping treatment, powder formulated hormonal application at the basal cut ends, irrespective of the type and concentration, significantly increased silver damage whereas, with increased duration of water dipping these negative effects were not observed.

These results contrast our previous observations, where no significant differences in silver damage were observed between IBA-coated and non-coated control cuttings (Figure 2). Upon water dipping, silver damage was significantly increased in control cuttings free of powder applied hormones while an opposite trend was observed for powder-coated cuttings. Notably, upon water dipping, IBA-coated cuttings, at a concentration of 0.4%, displayed considerably lower silver damage symptoms in comparison to non-dipped IBA cuttings. Silver damage symptoms were significantly reduced by approximately 33%. By contrast, the beneficial effect of water dipping was far less evident for cuttings treated with 0.8% IBA where, water dipping only marginally reduced silver damage at 45 minutes. Furthermore, powder coating, but not water dipping, significantly influenced dry mass of chrysanthemum cuttings (GLM: Wald χ^2 = 52.81, p < 0.001 for coating; Wald χ^2 = 0.40, p = 0.527 for powder coating and Wald χ^2 = 2.16, p = 0.539 for the interaction). Among hormone concentrations, application of 0.8% IBA to the cut base of the stem adversely affected dry mass (Figure 7B).

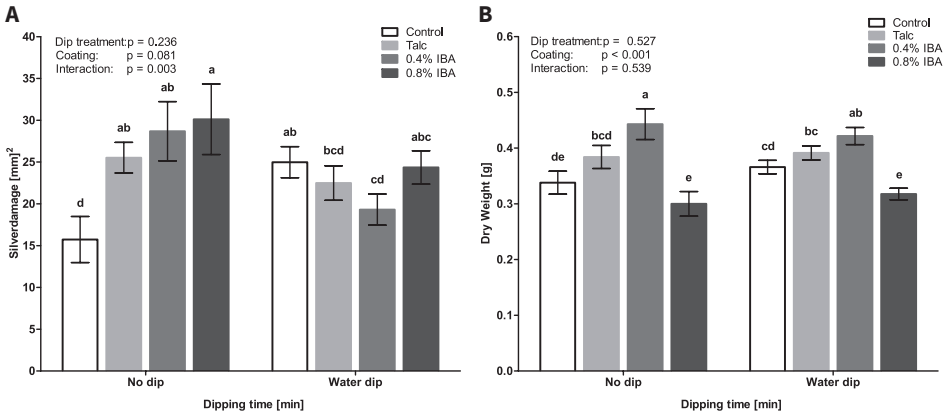

Figure 7. Effect of auxin formulation and duration of dipping on **(A)** thrips-associated feeding damage and **(B)** dry mass. Standardized powder treatments include pre-wetting of cutting base in water prior to coating in talc or 0.4 and 0.8% indole-3 butyric acid (IBA). Chrysanthemum cuttings were individually placed in thrips proof cages and were exposed to adult thrips released at rate of 20 thrips per cutting. One week post infestation, silver damage symptoms were visually scored. Data are expressed as damaged leaf area in mm^2 (means ± SEM). Means for water dipping represent pooled cumulative silver damages of three different dipping durations. Different letters denote significant differences among groups as determined by GLM followed by Fisher's LSD test ($p < 0.05$). The overall effects of dipping treatment, coating and their interaction are indicated in each graph.

Hormonal profiling

In an attempt to further elucidate factors underlying the phenomenon of enhanced resistance upon water dipping of IBA-coated cuttings, six major plant hormones were measured two weeks after dipping treatment (i.e. before thrips infestation). Multiple backward regression analysis was used to reveal the relative importance of plant hormones by eliminating variables with a low level of significance (Supplementary Table S1 and S2). The most significant model revealed that jasmonic acid and its isoleucine conjugate, JA-ile, were significant predictors of silver damage among different treatments. The most significant model revealed that jasmonic acid and its isoleucine conjugate, JA-ile, were significant predictors of silver damage among different treatments ($F(2, 13) = 5.03$, p = 0.024, R2 = 0.436). Subsequently, generalized linear models were applied to assess the main effects of cutting treatment and dipping time, as well as their pairwise interaction on JA and JA-ile (Table 1). The concentration of jasmonic acid varied significantly among treatments (GLM: Wald $\chi^2 = 6.75$, $p = 0.080$ for dipping time; Wald $\chi^2 = 39.50$, $p < 0.001$ for cutting treatment and Wald $\chi^2 = 29.90$, $p < 0.001$ for the interaction). Jasmonic acid-isoleucine concentration, on the other hand, was only influenced by dipping time (GLM: Wald $\chi^2 = 9.45$, $p = 0.024$ for dipping time). Coating of cuttings with talc or IBA only marginally affected the concentration of JA-ile (Wald $\chi^2 = 7.21$, $p = 0.065$ for coating and Wald $\chi^2 = 13.02$, $p = 0.162$ for the interaction). However, reductions in silver damage were not accompanied by marked increases in jasmonic acid nor by upregulation of its isoleucine conjugate and thus, do not support a synergistic nor an antagonistic mode of action of IBA.

Table 1. Hormonal profiling upon cutting treatments. Hormone concentrations are expressed as mean in ng/mg freeze dried leaf material ± SEM (n=5).

	Cutting treatment	Dipping time	Hormone concentrations					
			Ja-Ile			JA		
Treatments	Control	0 min	0.11 ± 0.05	bcde		58.91 ± 8.61	cdef	
		30 min	0.05 ± 0.01	de		50.31 ± 7.07	fg	
		45 min	0.04 ± 0.01	de		84.88 ± 8.30	ab	
		60 min	0.09 ± 0.05	cde		77.28 ± 7.02	abd	
	Talc	0 min	0.33 ± 0.12	ab		82.55 ± 7.00	ab	
		30 min	0.04 ± 0.01	de		75.89 ± 5.26	abc	
		45 min	0.41 ± 0.26	a		92.40 ± 10.12	a	
		60 min	0.06 ± 0.04	cde		75.43 ± 10.15	abc	
	IBA 0.4%	0 min	0.08 ± 0.03	cde		69.83 ± 4.80	bcd	
		30 min	0.21 ± 0.16	abcde		68.74 ± 11.59	bcde	
		45 min	0.20 ± 0.03	abcde		61.11 ± 6.46	cdef	
		60 min	0.02 ± 0.01	e		40.33 ± 5.27	g	
	IBA 0.8%	0 min	0.26 ± 0.09	abcd		53.70 ± 5.79	defg	
		30 min	0.12 ± 0.06	bcde		48.14 ± 5.41	fg	
		45 min	0.29 ± 0.11	abc		51.32 ± 3.40	efg	
		60 min	0.14 ± 0.05	bcde		61.42 ± 7.34	cdef	
GLM	Coating	Wald χ^2	7.21	p	= 0.065	39.50	p	< 0.01
	Dipping time	Wald χ^2	9.48	p	= 0.024	6.75	p	= 0.08
	Interaction	Wald χ^2	13.02	p	= 0.162	29.90	p	< 0.01

Abbreviations: indole-3-butyric acid (IBA); jasmonic acid (JA); JA-isoleucine (JA-Ile). Different letters denote significant differences among treatment groups as determined by Generalized Linear Models (GLM) followed by LSD at $p < 0.05$.

Comparing dry-dip rooting powder with water-based rooting solution

Because of possible confounding effects exerted by talc, we aimed to differentiate between the impact of IBA and that of the carrier chemical talc contributing to the absence of plant defense responses. In this context, basal liquid dips were performed using concentrations equimolar to powder applied IBA. Concomitantly, commercially provided cuttings pre-coated with 0.4% IBA were included in the experiment to compare the relative effectiveness of application and possibly the influence of IBA absorption through the cut base. Figure 8 shows that, in general, non-coated cuttings displayed the least amount of silver damage whereas, strikingly, water dipping of IBA-coated cuttings yielded the highest level of silver damage. The Kruskal-Wallis test showed that there was a statistically significant difference in silver damage symptoms between the different treatments groups, H(4) = 17.138, $p = 0.002$. However, we observed no evidence that auxin supplementation enhanced reduced feeding symptoms. With the exception of water dipped IBA-coated cuttings, symptoms were equal across all groups. Under control conditions, when cuttings did not receive a water dipping, an increase in silver damage was observed in IBA-coated cuttings (32.3 ± 6.2), although this effect was not significantly different from non-coated cuttings (19.1 ± 3.4).

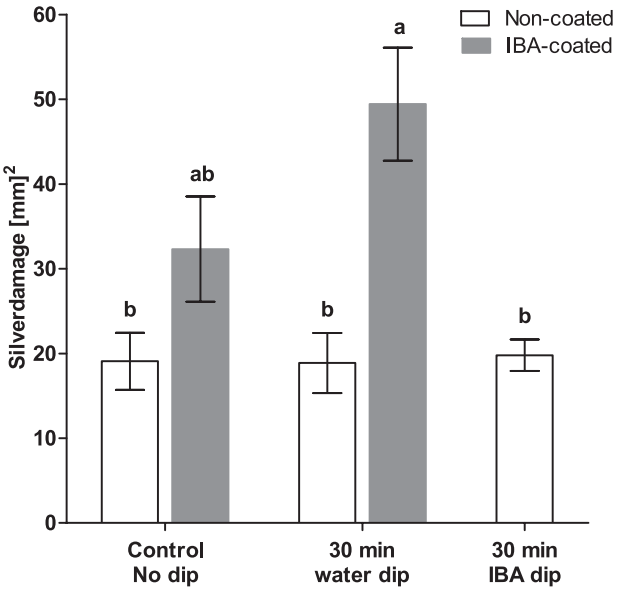

Figure 8. Effect of powder and liquid auxin formulations on silver damage. Unrooted indole-3-butyric acid (IBA)-coated (cv. Baltica) cuttings were dipped in water for 30 minutes. Non-coated cuttings were dipped in an equimolar concentration of 4000 ppm or water. Control cuttings did not receive a water dipping treatment. Bars (mean ± SEM, n=13 -15) represent the cumulative silver damage expressed as damaged leaf area in mm^2 Different letters indicate significant differences among groups as determined by Kruskall-Wallis followed by Games-Howell multiple post hoc comparison ($p < 0.05$).

Discussion

The exploitation of various secondary metabolites as defensive compounds holds great promise as a sustainable strategy for the control of pest insects (Cantrell et al., 2012; Lorsbach et al., 2019). Many secondary metabolites display interesting bioactivities, which either act directly as a result of their insecticidal properties or are mediated through the plant by induction of defenses. In the present study, we evaluated the effect of various basal liquid dipping treatments as a pre-propagation treatment to enhance resistance against thrips, early in the production cycle of chrysanthemums. The majority of experiments exploring the potential of bio-insecticidal dips are often carried out as immersion treatments. These studies mainly evaluate this approach as a postharvest disinfestation strategy of propagative cuttings in order to manage insect populations prior to shipment. Furthermore, such intervention strategies allow to control pest populations prior to entering the production cycle, and could potentially reduce reliance on chemical pesticides (Buitenhuis et al., 2006). In the present study, we explored naturally occurring plant compounds as a preventative means to enhance plant defenses against two of the most economically important pest insects of Chrysanthemum; WFT and leaf miner.

Among the defensive metabolites, β-alanine has frequently been implicated in resistance against herbivores of numerous taxa, including western flower thrips (Leiss et al., 2013). In preliminary

experiments we observed that chrysanthemum cuttings were more resistant to thrips, i.e. displayed less silver damage, than untreated cuttings when the basal portion of cut ends was dipped in an aqueous solution of β-alanine. Silver damage symptoms were reduced by 36% as compared to non-dipped cuttings. While the effect was comparable with that of Abamectin stem dipping, it should be noted that Abamectin acts as a contact insecticide and has a very limited systemic activity. Subsequently, in seeking to optimize the dipping treatments of chrysanthemum cuttings, we surprisingly observed that thrips associated feeding damage was remarkably lower in water dipped cuttings (Figure 1). The scientific practice of including an appropriate control had serendipitously yielded an unexpected benefit. Because the base commercial chrysanthemum cuttings is covered with rooting power containing auxin, this outcome raised two potential hypotheses on the role of auxins in relation to plant-insect defenses suggesting that either (1) antagony or (2) synergy could explain our observations. Firstly, it can be hypothesized that exogenous application of the growth regulating hormone, IBA, attenuated thrips resistance by exerting an antagonistic effect on the JA-signaling pathway. While jasmonates are generally recognized as the prominent hormone in plant defense against herbivores, recent studies revealed that auxins may also have a key role in modulating this process (Zhao, 2018). The assumption of a dual role of auxins is supported by several lines of evidence. A number of studies have demonstrated an inhibitory effect of exogenously applied auxins on JA-biosynthesis. A direct prediction of our first hypothesis is that the physical presence of IBA on stem cuttings promotes thrips susceptibility. We tested this hypothesis by performing water dipping treatments in the presence and absence of the rooting hormone IBA. Silver damage symptoms were significantly different among treatments and were influenced both by period of dipping and coating treatment of cuttings (Figure 2). When cuttings received no water dipping treatment, we observed no significant differences in silver damage between IBA-coated and uncoated cuttings. Likewise, we found no significant differences between coated and non-coated cuttings when they were dipped for 45 and 60 minutes. The present findings therefore, do not support the hypothesis that auxins play a direct role in promoting susceptibility of cuttings to thrips. Intriguingly, water dipping treatments in the presence of IBA-coating were shown to significantly reduce silver damage symptoms while in the absence of exogenous auxins, cuttings displayed no significant differences in thrips damage following water dipping. Consequently, removal of externally applied hormone powder by water dipping treatments could have mitigated the antagonistic effect of auxin. Alternatively, rather than removal of auxins, water dipping treatments could have enhanced the uptake of exogenously applied auxin, and subsequently leading to increased resistance through a synergistic mode of action with JA.

The beneficial effect of water dipping triggered our curiosity to further evaluate whether such an approach could improve resistance against multiple herbivores. As such, we evaluated the effect of dipping treatments on celery leaf miner resistance, a second important pest insect species of chrysanthemum. In agreement with our thrips data, it is clear that the mere physical presence of IBA did not explain susceptibility of chrysanthemum cuttings to *Lyriomyza trifolii*. Furthermore, we observed that water dipping had no direct effect on the number of mines (Figure 3A). Although seemingly lower, no significant differences were detected in total pupation (Figure 3B). However, the

number of pupae successfully emerging from leaves was significantly lower after water dipping (Figure 4A). The most prominent effect was observed in JA treated cuttings. Cut ends dipped in JA significantly reduced the emergence rate of pupae by 97%. In order to determine whether these enhancements were associated with JA-dependent defense traits, we measured the activities of polyphenol oxidase (PPO). Induced resistance may result from a direct activation of defense mechanisms, including increased basal levels of defense-related proteins such as PPO which serve an anti-nutritive role by reducing the digestibility of dietary protein (Felton, 2005; Conrath et al., 2006; Robert-Seilaniantz et al., 2011). Although significant reductions in the number of emerged pupae were observed, none of the dipping treatments stimulated PPO activity. Surprisingly, JA dipping did not induce PPO levels either. This could be explained by the time gap following dipping treatment and PPO measurements. Furthermore, rather than direct induction of defenses, it is plausible that dipping treatments of auxin-coated cuttings primed for enhanced defense reactions. The priming defense processes remain dormant until herbivore infestation and reflects a cost-effective approach by which the plant can avoid expending energy under low pest pressure (Conrath et al., 2006; Huot et al., 2014).

To establish whether enhanced resistance in chrysanthemum was associated with priming of the plant defensive capacity, a comparative study was undertaken. In the first experiment, we compared thrips-mediated induced responses in IBA-coated chrysanthemum cuttings following a 30 minute water dipping treatment. Again we found a reduction of silver damage after water dipping but this time this was not statistically significant. (Figure 5A). In parallel, as a marker of plant defense responses, the activity of PPO was measured two weeks post treatment (i.e. before thrips infestation) and one week post thrips infestation (Figure 5B). We observed no induction of PPO activities upon 30 minutes of water dipping. Furthermore, no significant differences were observed between thrips-infested and non-infested chrysanthemum cuttings . These observations suggest that neither induction nor priming of plant defenses are involved. However, the current experimental set-up as well as the minor effect on silver damage, do not allow us to draw a conclusive picture of underlying mechanisms and indicate the necessity for further study.

Activation of the JA signaling pathway through exogenous application of jasmonates, such as the volatile form of JA methyl jamonate (MEJA), is reported to induce the expression of the defensive protein PPO more efficiently than JA (Farmer and Ryan 1990; Jang et al., 2014; Jiang and Yan et al.,2018). MeJA-mediated induction of PPO was assayed as a means to mimic herbivore-induced responses (Constabel and Ryan, 1998) and hence, explore which role dipping treatments in the presence of auxin play in establishing resistance against thrips. Subsequently, a separate time-course experiment was performed to assess water dipping effects on artificially induced plant defense responses triggered by exogenous application of MeJA. The time course of PPO levels in cuttings treated with MeJA peaked after 36 hours for both the non-dipped and water dipped plants. Taken together, these experiments suggest that water dip treatments in the presence of IBA do not directly induce nor prime JA related defenses and, possibly operates independently from proteinaceous mediators such as PPO.

Besides their well-known defensive role, PPO is an important co-factor and acts as a stimulant of adventitious root formation by catalyzing the condensation between phenolic compounds and IAA

(Nag et al., 2001; Rout et al., 2006). Exogenous auxin treatment increases PPO activity while, decreasing the activity of indoleacetic acid oxidase (IAAO) (Zhang et al., 2017; Yan et al., 2017). peak values of PPO activity induced by exogenous auxin, appear to occur at different time points, depending on the plant species and hormone treatment. For example, in 1-naphthylacetic acid treated cuttings of hybrid aspen, PPO levels significantly increased 6 days after treatment (Yan et al., 2017) whereas, Zhang and co-workers (2017) reported a PPO peak value 45 days after planting IBA treated stem cuttings of *Malus hupehensis*. Future studies investigating the signaling pathways that govern the expression of defenses could contribute further to our understanding of mechanisms underpinning the processes of enhanced herbivore resistance. More specifically, aiming at understanding the early signaling events, by performing time course experiments during rhizogenesis may elucidate whether the defenses activated by water dipping are mediated through changes in PPO levels.

Considering earlier conflicting results concerning defense responses of chrysanthemum cuttings to water dipping, we emphasize the importance of standardizing hormone application. Within batch variations in hormone application may potentially have led to varying defense responses. Despite these extensive efforts, dipping and coating treatments continued to suffer from a high level of irreproducibility in thrips responses. Contrary to our earlier observations, we observed that Baltica cuttings under control conditions, i.e. 0 minutes of water dipping, exhibited significantly lower amounts of silver damage symptoms in the absence of talc-based IBA coating suggesting a potential antagonistic action of IBA. These contrasting results, as compared to our previous experiment, leads us to make two comments. The first concerns a plausible explanation of plant responses in relation to their water status. One of the main constraints in large-scale production of unrooted cuttings is the time delay between excision of cuttings from stock plants and insertion into a rooting environment. Once a stem is cut from its mother plant, its nutrient and water supply is lost. Traditionally, herbivorous insects are thought to exhibit enhanced performance on water-stressed host plants due to induced changes in plant physiology, largely through their effects on nitrogen availability (Huberty and Denno, 2004). One of the first physiological changes to occur in water deficit plants, is the loss of cell water content and turgor, which in turn influences plant resistance and susceptibility to herbivore attack. Whilst earlier we have demonstrated that, in the absence of exogenously applied IBA, water dipping of cut bases did not significantly affect silver damage symptoms ,the results obtained in the standardized experiment show a different trend. Surprisingly, water dipping of control cuttings free of powder applied hormones negatively affected silver damage and, symptoms were markedly increased by 37% relative to non-dipped cuttings(Figure 7). Our observations implicate that a positive cell turgor promotes susceptibility of chrysanthemum cuttings by enhancing thrips performance.

Notwithstanding, in line with our previous results, we observed that water dipping significantly reduced silver damage in cuttings treated with 0.4% IBA as compared to its corresponding non-dipped control (Figure 7). However, there was no clear consensus in the levels of auxin along with the associated improvement in silver damage symptoms. IBA application at basal cut ends did not increase endogenous levels of IAA in comparison to non-coated cuttings (Table 1).

Notably, IBA-coating of cut bases elevated the susceptibility of cuttings to thrips, as manifested by

significant increases in silver damage at t = 0 minutes (Figure 7). Although these results suggest that IBA exerts an antagonistic effect on the JA-signaling pathway, talc might act as a confounder. Talc, a clay mineral composed of hydrated magnesium silicate with the chemical formula $Mg_3H_2(SiO_3)_4$), is a functional carrier in many agricultural products and is extensively used as an inert chemical for active premixed ingredients. Talc is a strongly hydrophobic and an extremely platy mineral. As they are naturally water-repellent, talc particles can form a barrier when they envelop other particles and thus reduce the evaporation and uptake of water by preventing the formation of hydrate bridges, which is used to enable longer storage periods (IARC, 2010). Hence, the level of water stress in cuttings, as a result of talc-based coating, may have negative consequences for herbivores, especially for those that rely on high tissue water content and turgor (i.e. osmotic) pressure.

Multiple backward linear regression analysis revealed that differences in silver damage among chrysanthemum treatments could be explained in a model comprising JA and JA-isoleucine. However, multiple pairwise comparisons yielded no consistent pattern how dipping and coating treatments in relation to hormones were associated with reductions in thrips-associated feeding damage. As such, we have no firm evidence for a causal antagonistic or synergistic relationship in auxin-mediated responses. Importantly, these results should be interpreted in the context of spatial and temporal limitations. Hormonal concentrations were measured prior to thrips infestation and thus, two weeks post treatment, such measurements do not necessarily encompass hormonal changes induced by treatment of cuttings.

In an effort to identify and avoid possible confounding effects of talc-powder, basal liquid dips were performed using concentrations equimolar to powder applied IBA. We detected no evidence that IBA supplementation by liquid dips improved thrips resistance as silver damage, with the exception of water dipped IBA-cuttings, was equal across treatments). Strikingly, upon water dipping, IBA-coated cuttings displayed significantly more silver damage as compared to non-coated cuttings.

The large variation within and between experiments as well as the contrasting results leads to our second remark and concerns the status of the mother stock which is, perhaps, of greater importance than the water status of plant cuttings. An explanation of the apparent disparity of plant responses to thrips-associated feeding patterns may be the phenotypic heterogeneity of plant cuttings. We speculate that the physiological status of stock plants is key to explaining the discrepancy in the variable and inconsistent performances of thrips on generated cuttings. Maternal life history traits may affect the progeny ,i.e. generated stem cuttings, and thus, despite being clonal in nature, cuttings can display different response patterns when facing the same infestation conditions. First and foremost, cuttings originated from Ethiopia or Uganda, and as such, the considerable variation in results is likely to be affected by environmental conditions. Cuttings used for exploring the physical effect of IBA as illustrated in Figure 2, were obtained from a production site located in Ethiopia whereas, in the follow-up experiment exploring standardized IBA-loading, cuttings were obtained from Uganda (Figure 7). Furthermore, discrepancies in experimental results could be linked to collection time of cuttings. Experiments conducted with cuttings from Ethiopia were harvested from mother plants in week 49 (December 2017) whereas, in the latter experiment for IBA standardization,

pruned cuttings from mother plants grown in a greenhouse located in Uganda differed by one week in harvest date (week 27 and week 28; July 2018). In addition to regional or climatic influences, it is important to note that growth conditions among these commercial greenhouses are optimized for each production site and, as such, may for example affect the nutritional status of stock plants (S. Kos, pers. comm.). This lends additional support to the contention that phenotypic heterogeneity involves genotype by environment interactions. Other hypothetical possibilities that influence physiological responses include; but are not limited to, substrate, cutting position along the canopy structure of the stock plant, age and the health of mother plants. Moreover, despite the fact that greenhouses provide a controlled and constant environment suitable for year-round production of chrysanthemum cuttings, variation in pest activities still exists from one season to the next (de Kogel et al., 1997; Hewitt et al., 2014).

Concluding remarks

In summary, water dipping of IBA-coated cuttings repeatedly reduced herbivory, both by thrips as well as by leaf miner. However, results were highly variable and inconsistent. Furthermore, separation of IBA and talc implies a possible confounding effect of the carrier chemical. Assessment of polyphenol oxidase activity indicates that neither direct induction nor priming of JA related plant defenses are involved. Although inroads have been laid, future experiments aiming at understanding the early signaling events, including hormonal signaling networks, from a more holistic perspective may help to explain the physiological basis involved in conferring protection against herbivores.
A possible explanation of the anomalies appears to hinge on the large phenotypical variation in cuttings generated from stock plants and warrants the necessity to improve intra- and inter- variability and reproducibility. Nonetheless, our study provides an interesting starting point to investigate alternative roles of auxins in commercial propagation and may add, in addition to their growth regulating function, a promising defense strategy to the horticultural toolbox of chrysanthemum propagators. In view of these findings, we advise that the putative defensive role of auxins should be exploited and integrated in coherent models of response and function in model plant species prior to translation to commercially important horticultural crops. Future work with auxin formulations is needed to determine whether such an approach is of commercial value over a broader range of herbivores.

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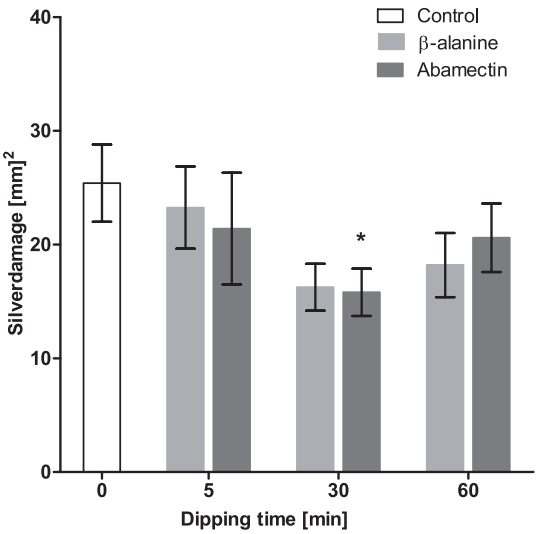
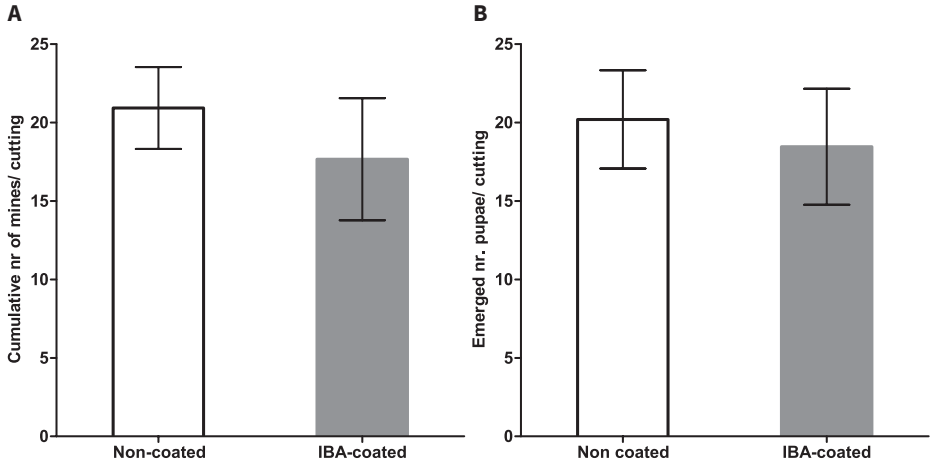
Supplementary Figures



Supplementary Figure S1. Efficacy of bio-insecticidal dips on chrysanthemum resistance against western flower thrips (WFT). The basal cut ends were dipped in 100 mg/ml of β-alanine or 0.3 ml/L abamectin (Avis EC, Syngenta, Syngenta Crop protection Inc., Greensboro, NC, USA) for various time points. Untreated, non-dipped cuttings served as control. Two weeks post treatment, cuttings were infested with 20 adult thrips. One week after thrips infestation, silver damage symptoms were visually scored and expressed as damaged leaf area in mm^2. Data represent cumulative silver damage and are presented as mean ± SEM of 5 replicates per treatment. Asterisks indicate significant difference at α=0.05 as determined by an unpaired Student's t-test.



Supplementary Figure S2. Effect of exogenously applied indole-3-butyric acid (IBA) on celery leaf miner (*Liriomyza trifolii*) resistance. Basal ends of chrysanthemum cuttings (cv. Baltica) were pre-coated with powder formulated rooting hormone (Chryzotek beige 0.4% IBA). Non-coated cuttings served as control. After 14 days of rooting, cuttings were infested with four one-day old leafminers (2 males and 2 females) for 24 hours. **(A)** cumulative number of individual mines and **(B)** the number of emerged pupae. Data represent means ± SEM of 15 replicates. Differences between IBA-coated cuttings and control plants were evaluated by the Mann-Whitney U test (two-tailed) at $p < 0.05$.

Supplementary Figure S3. Substantial variation in the amount of hormone applied rooting powder within a batch of 50 commercially provided Baltica cuttings. Cuttings at the left upper part of the image contain considerably more rooting powder than the lower part whereas, cuttings on the left upper part are loaded with a mediocre amount. Approximately 1 cm of the basal cut end is pre-coated with the rooting powder Chryzoteck beige 0.4% (Rhizopon, Hazerswoude-Rijndijk, the Netherlands) consisting of 0.4% indole-3 butyric acid (IBA) in talc. Baltica cuttings require IBA applied rooting hormones for quick root initiation.

Supplementary Table S1. Backward multiple regression models of independent predictors of variation in silver damage.

Model	Independent variables	B	SE	β	t	*p*
1	OPDA	0.049	0.055	0.212	0.891	0.396
	JA-ile	-22.390	10.812	-0.561	-2.071	0.068
	JA	0.183	0.094	0.583	1.951	0.083
	IAA	-0.054	0.111	-0.149	-0.484	0.640
	ABA	0.163	0.137	0.301	1.190	0.265
	SA	0.065	0.090	0.184	0.720	0.490
2	OPDA	0.045	0.052	0.194	0.860	0.410
	JA-ile	-23.247	10.249	-0.583	-2.268	0.047
	JA	0.158	0.075	0.504	2.099	0.062
	ABA	0.143	0.126	0.264	1.140	0.281
	SA	0.066	0.087	0.186	0.760	0.465
3	OPDA	0.048	0.051	0.210	0.955	0.360
	JA-ile	-19.522	8.827	-0.489	-2.212	0.049
	JA	0.146	0.072	0.465	2.021	0.068
	ABA	0.146	0.123	0.269	1.184	0.261
4	JA-ile	-19.950	8.783	-0.500	-2.271	0.042
	JA	0.159	0.070	0.507	2.253	0.044
	ABA	0.113	0.118	0.208	0.956	0.358
5	JA-ile	-21.308	8.639	-0.534	-2.466	0.028
	JA	0.176	0.068	0.562	2.594	0.022

Note: Independent variables: concentration of 12-oxo-phytodienoic acid (OPDA), jasmonic acid-isoleucine (JA-Ile), jasmonic acid (JA), indole-3-acetic acid (IAA), abscisic acid (ABA) and salicylic acid (SA). Abbreviations: B: unstandardized regression coefficient, β: standardized regression coefficient, SE: standard error Linear regression through the origin at $p < 0.05$, t-value and 2-tailed p-value.

Supplementary Table S2. Model summary of the backward elimination multiple regression analysis.

Model	Adj R^2	df 1	df 2	F	Sig.
1	0.257	6	9	1.865	.192[b]
2	0.314	5	10	2.374	.115[c]
3	0.340	4	11	2.935	.071[d]
4	0.345	3	12	3.636	.045[e]
5	0.350	2	13	5.030	.024[f]

Note: Abbreviations: Adj, adjusted; df, degrees of freedom. *p < 0.01 (two-tailed).
[a] Dependent variable: silver damage
[b] Predictors: (Constant), SA, IAA, OPDA, ABA, JAile, JA
[c] Predictors: (Constant), SA, OPDA, ABA, JAile, JA
[d] Predictors: (Constant), OPDA, ABA, JAile, JA
[e] Predictors: (Constant), ABA, JAile, JA
[f] Predictors: (Constant), JAile, JA

CHAPTER SEVEN

SUMMARY AND DISCUSSION

Summary and General Discussion

In this final chapter I summarize the main findings and discuss the overall contribution of the thesis by presenting a critical opinion about the present work and future research needed to follow up strategies for early thrips management. In doing so, the discussion is considered in light of our main research question: 'Can we employ plant derived compounds as a strategy to boost the plant's defense system?'

Whether modern agriculture without conventional pesticides will be possible or not is a matter of debate. This debate is only meaningful within the context of environmental awareness on one hand, and the availability of sustainable substitutes on the other.

The general introduction, in **chapter one**, discusses the need for changes in the techniques and compounds that, until recently, have been the mainstay for dealing with pest insects. Additionally, it offers a brief overview of the concept sustainable use of pesticides, from a regulatory context with some insight on changes triggered by the implementation of the regulatory framework. The need for increased agricultural production, together with the necessity to reduce insecticide use, has stimulated scientists to study the ability of plants to survive and adapt to insect attacks over aeons of time and, how such innate defense systems can be adopted or manipulated in agricultural settings. Consequently, the importance of phytochemicals and their use as candidates for pest management has increased during recent years. Against this background, subsequent sections in the introduction focus on the methodology by describing the key research aim of this project and the experimental research system. As a contribution to the changing legislation and evolving societal attitudes concerning environmental issues, this project aims to explore the use of plant-derived compounds for early management strategies in tomato (*Solanum lycopersicum* L.) and *Chrysanthemum morifolium* (Ramat) to combat one of the most economically important insect species – western flower thrips (WFT; *Frankliniella occidentalis*). To manipulate and augment chemical basal defenses, known putative defense secondary metabolites were exogenously applied (**chapter four**) whereas, in **chapter five** and **six** external application of plant hormones were explored as a means to trigger innate defense responses against WFT.

Chapter two reviews the concept and the main control tactics of Integrated Pest Management (IPM) in light of WFT. The acronym 'IPM' has gained global recognition and is endorsed as the future paradigm for crop protection, resonating with public and political perceptions. The holistic concept of IPM emphasizes on systems approaches by integrating preventative and therapeutic intervention measures which are briefly introduced. Among the topics considered here, this chapter particularly focusses on two key approaches as areas of major progress for sustainable pest management, being (1) host plant resistance, the selection or development (via traditional breeding or genetic modification) and the use of crop plants that possess defensive traits against herbivores, and (2) biological control, the use of living organisms as natural enemies of crop pests. Knowledge gaps are identified and innovative approaches are emphasized, highlighting the advances in -omics technologies, from genomes to metabolites, which have been a major driver for the adaptation of

systems-based approaches. Such integrative approaches, particularly when put in the context of high throughput screening (HTS), enable a comprehensive view of defense mechanisms and provide a powerful tool to drive innovation in crop protection.

Amongst such innovative approaches, the search for environmentally acceptable substitutes has amplified (Adeyemi, 2010; Isman et al., 2006; Kortbeek et al., 2019; Lorsbach et al., 2019). Biopesticides appear in the horizon as an attractive solution for sustainable plant protection (Villaverde et al., 2013). Accordingly, **chapter three** discusses main strategies for selecting bioactive plant-derived secondary metabolites as sustainable alternative candidates for crop protection agents. This chapter provides a simplified guide, from plants to practice, and comprises three essential elements. The first element describes a robust, reliable and quantitative eco-metabolic approach to screen for bioactive metabolites followed by (2) a rigorous validation process to study and verify the insecticidal activity. Examples are provided to illustrate the respective advantages of using naturally based crop protection agents as regards to pesticides, in meeting sustainability objectives. Ten selected metabolites were surveyed which, beyond their natural function as defensive chemical weapons against insect attack, appear to provide numerous desirable health benefits. Central to the control of pest insects is the question of how these green substituents can be formulated and promulgated. Many of the insecticidal metabolites are crystalline solids with limited water solubility which thus, potentially hamper commercial formulation. As such, the third element describes a strategy for improving the solubility of sparingly soluble compounds. Natural Deep Eutectic Solvents (NADES), a new class of innovative environmentally benign solvents composed of a wide range of primary metabolites, were evaluated as a concept for enhancing solubility and stability of scarcely water soluble secondary metabolites. This chapter is further supported with experimental results demonstrating the solubilizing capacity of six insecticidal metabolites in a variety of NADES. The highest solubilities were achieved using lactic acid: 1,2-propanediol in a molar ratio of 2:1 and 1,2-propanediol: choline chloride: water (molar ratio 1:1:1).

Hence, this investigation was further taken up with the final aim to apply this concept as a pre-sowing seed treatment (e.g., soaking) for enhancement of tomato defenses against WFT (**chapter four**). The effect of eight different NADES on tomato seed germination and plant growth performance was studied prior to exploring their potential as vehicles for insecticidal plant secondary metabolites. Exogenous applications of three representative model compounds (beta-alanine, sinapic acid and chlorogenic acid) were evaluated for their ability to protect tomato plants from their most vulnerable stage onwards. NADES has proven to be an interesting solvent to significantly improve solubility of plant metabolites. However, in exploring its use as a carrier solvent for defensive secondary metabolites, both phenolic compounds as well as beta-alanine did not improve thrips resistance in terms of silver damage symptoms. Furthermore, an inherent drawback of exogenously applied NADES is its' phytotoxic effect on seed germination and viability, particularly when the duration of tomato seed soaking was increased. The work described in this chapter was therefore, a main driver to explore inducible responses as an alternative component of the resistance arsenals.

Modulation of endogenous insecticidal metabolite levels by exogenous application

The production of the impressive arsenal of endogenous insecticidal molecules has been exploited by man throughout history and, still serve either directly or as lead compounds for semi-synthesis of modern agrochemicals (Lorsbach et al., 2019). The search for lead compounds from natural sources however, may be complicated by the co-occurrence of numerous plant metabolites as well as the precense of plant matrices (Inui et al., 2012). Exogenous application with pure phytochemicals may thus, not always be able to fully reveal their insecticidal potency, especially when the *in planta* insecticidal activity is due to synergy or interactions between different constituents present in the plant. Such interactions may occur through different mechanisms including: bioavailability, activation of pro-metabolites or deactivation of active compounds into inactive metabolites, inhibition of binding to target proteins or interference with cellular transport processes (Efferth and Koch, 2011; Jeschke, 2016). Among these mechanistic reasons, the importance of bioavailability has been highlighted throughout the experimental chapters.

Seed treatments are routinely applied worldwide to protect seeds and early developmental stages from attack by insect pests and pathogens (Brandl, 2001) however, they only provide a transient window of systemic protection which often declines as the bioactive compound is degraded or diluted as growth progresses (Quérou et al., 1998). This might very well explain the lack of improvement in trips resistance upon tomato seed soaking with plant secondary metabolites (**chapter four**). Additionally, various other factors, including soil moisture, water solubility, substrate retention, treatment dose and formulation as well as seed coat permeability, all influence the translocation and the final efficacy of an active ingredient applied as seed treatment (Quérou et al., 1998). For many decades, crop plants have typically been bred and cultivated for constitutive defenses whereas, inducible responses are strongly underutilized management tactics (Stout et al., 2002). Alternatively, seed treatments with plant-derived elicitors enable young plants to mount defenses early in the growth cycle by triggering innate defense responses (Song et al., 2017). Being mediated through the plant's complex metabolic pathways where many feedback and trade-off mechanisms operate, induced defenses may represent a more durable form of resistance (Stout et al.,2002; Bruce et al., 2017).

Accordingly, **chapter five** seeks to induce defenses against thrips at the seed stage using the ubiquitous plant hormone jasmonic acid (JA). Elicitor-mediated induction of plant defenses is a widely accepted eco-friendly strategic approach for modern plant protection as it aims to substantially minimize the use of toxic pesticides (Vallad and Goodman, 2004; Steenbergen et al., 2018). Seeds are receptive to direct application of exogenous phytohormones without being constrained by the inherent costs of defense and could therefore, offer broad-spectrum protection at the early stages of plant development (Rajjou et al. 2006; Worrall et al., 2012; Haas et al., 2018). However, previous work has demonstrated that these beneficial effects of JA seed treatments are not consistent among tomato cultivars (Smart et al., 2013). This is in line with the work described in chapter five, where I examined the extent of variation of resistance inducibility in eight commercial tomato cultivars. Among the evaluated cultivars, their response to exogenous JA seed application differed inherently.

Silver damage symptoms were only reduced in JA-seed treated plants of the tomato cultivar Carousel. Enhancement of resistance, however, was not associated with activation of defense-related traits such as polyphenol oxidase activity (PPO), trichomes nor with induction of volatiles. Assessment of these traits early in the growth phase could shed more light on the mediated defense responses. Most promising however, was the fact that sulfuric acid scarification significantly augmented responsiveness to seed applied JA in the non-responsive cultivar Moneymaker. This observation lends added support to the contention that seed coat permeability is undoubtedly an important factor for facilitation of JA absorption (i.e. bioavailability) and thus, increased physiological activity. Additionally, this outcome creates opportunities for tomato breeding programs to select for genetic traits that affect seed permeability as well as for private seed companies to pre-treat non-responsive cultivars.

Commercial utility of elicitors

Nonetheless, the observed variability in host responsiveness between cultivars constitutes a major obstacle, because one of the foremost conditions for successful commercialization of elicitor-mediated induced resistance is consistent efficacy. Intriguingly, in scientific literature, Moneymaker along with tomato cultivars Aisla Craig, Micro-Tom and Castlemart are frequently used to represent tomato as a plant model system. Despite being regarded as a model plant of the Solanaceae family, the fundamental question remains whether or not it is correct to refer to tomato as model species in general whilst, there are significant differences among individual cultivars. The variability in responsiveness in different cultivars clearly demonstrated the necessity to include multiple cultivars as biological test system to establish consistent statements. Moreover, it shows that neglect of this issue at the screening stage for new chemical elicitors unnecessarily contributes to an excess of false-negative findings. To avoid potential lead candidates being incorrectly classified as inactive, it is important to distinguish true-negative results from false-negative elicitors. Alternatively, transgenic plants and cells containing defense gene promotor-reporter gene fusions may provide convenient, sensitive and specific assay systems for screening potential enhancers of natural resistance to herbivores (Doerner et al., 1990; Corrado and Karali, 2009; Halder and Kombrink, 2018). Elicitors can be introduced by treating young seedling or cell suspension cultures. Subsequently, for example, the response can be monitored in a high throughput fashion using a microplate reader imaging system for visualizing reporter degradation (Larrieu et al., 2015).

Despite the inherent potential of elicitors for sustainable agricultural use, an imperative question is why very few have achieved commercial success? Adoption of such approaches have a strong regulatory dimension which may act as a barrier to the development of reliable and efficacious commercially viable products. Moreover, the development of a new active compound is expensive and time consuming. The vast majority of commercial products claiming to induce disease resistance often involve SA-mediated signalling (Gorelick and Bernstein, 2014). On the contrary, not much headway has been made in the commercialisation of products controlling herbivory. Large-scale application of JA can be prohibitively costly but, perhaps of bigger importance is the well-known

growth repressing action of JA, particularly at higher doses (Redman et al., 2001). Screening for or designing structurally related analogues that retain the resistance-inducing capacity of JA (Haider et al., 2000) but, are less active in growth repression may be more promising in this respect. It is also crucial to recognise that the agricultural utility of JA-induced defenses do not produce an immediate knockdown effect and rarely confers complete resistance (Stout et al., 2002). Nonetheless, one of the main advantages of induced resistance responses is that it can be compatible with, or even enhance biocontrol of plant pests by promoting plant attractiveness to natural enemies and therefore, could be considered as a parallel or sequential approach (Smart et al., 2013; Bruce et al.,2017; Pappas et al., 2017). Finally, elicitor induced resistance is differentially effective against different pests and thus, hormonal crosstalk following exogenous JA treatment may leave some plants more vulnerable to pathogens associated with the SA-regulated pathway.

Challenges in irreproducibility

In the context of mechanistic crosstalks, the complex interaction between the root promoting auxin hormone indole-butyric acid (IBA) and JA was explored in **chapter six**. Recent studies demonstrate that auxins also affect a multitude of plant defense responses through complex interactions among multiple hormone pathways, including JA (Kazan and Manners, 2009; Robert-Seilaniantz et al., 2011; da Costa et al., 2013). In search of effective dipping treatments to protect cuttings during their vegetative stage, we serendipitously observed that water dipping of IBA-coated cuttings repeatedly reduced herbivory, both by thrips as well as by leaf miner. Furthermore, separation of IBA and talcum powder implies a possible confounding effect of the carrier chemical. Assessment of polyphenol oxidase activity (PPO), 14 days after dipping treatment, suggests that neither direct induction nor priming of plant defenses are involved. Future experiments aiming at understanding the early signaling events, including hormonal signaling networks, from a more holistic perspective may help to explain the physiological basis involved in conferring protection against herbivores. Overall, however, results were highly variable and thus, do not allow us to deduce conclusive statements concerning the involvement of auxin-mediated defense responses. A possible explanation of the anomalies appears to hinge on the large phenotypical variation in cuttings generated from stock plants and warrants the necessity to improve intra- and inter- variability and reproducibility, which both form a cornerstone in science for critically assessing the correctnes of scientific claims and conclusions drawn (Plesser, 2018). It is therefore, strongly advised that the putative defensive role of auxins should be exploited and integrated in coherent models of response and function in model plant species prior to translation to commercially important horticultural crops. Our study provides an interesting starting point to investigate alternative roles of auxins in commercial propagation and may add, in addition to their growth regulating function, a promising defense strategy to the horticultural toolbox of chrysanthemum propagators. Future work with auxin formulations is needed to determine whether such an approach is of commercial value over a broader range of herbivores.

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Samenvatting en discussie

In dit laatste hoofdstuk worden de belangrijkste bevindingen samengevat door een kritische blik te werpen op het huidige werk beschreven in dit proefschrift. Tevens wordt er vooruitgeblikt op toekomstig onderzoek dat nodig is om succesvolle strategieën, ten behoeve van trips controle, vroeg in de productie cyclus te kunnen realiseren. Hierbij staat de onderzoeksvraag: "Kunnen plant-eigen inhoudsstoffen als duurzame strategie ingezet worden om de plantweerbaarheid te verhogen?" centraal.

Of de toekomstige landbouw zonder chemische middelen mogelijk is of niet, blijft een punt van discussie. De discussie omtrent de vergroening van het middelenpakket is echter alleen zinvol in de context van milieubewustzijn enerzijds en de beschikbaarheid van duurzame alternatieven anderzijds.

De algemene inleiding, in **hoofdstuk één**, bespreekt de noodzaak voor veranderingen in strategieën, die tot voor kort de belangrijkste steunpilaren vormden voor gewasbescherming. Het concept duurzaam pesticiden gebruik (2009/128/EG) wordt geïntroduceerd, met enig inzicht in regelgeving die ten grondslag ligt van de huidige beleidsveranderingen, en de daarmee voorgestelde beperkingen. Het verhogen van gewasopbrengsten ten behoeve van de fors groeiende wereldbevolking, tezamen met de noodzaak het gebruik van chemische middelen te sterk minderen, heeft ertoe geleid dat het vermogen van planten zich te verdedigen tegen insectenvraat, en de mogelijkheid om deze verdedigingsmechanismen te manipuleren, een veel bestudeerd onderwerp is. Ten gevolge hiervan is zowel het belang van plant inhoudsstoffen als het gebruik ervan, ter bevordering van de duurzaamheid, in de afgelopen jaren sterk toegenomen. De opvolgende paragrafen in de inleiding richten zich vervolgens op de methodologie en beschrijven het onderzoeksdoel en het experimentele onderzoeksysteem. Het, in dit proefschrift beschreven onderzoek, beoogt natuurlijke plantenstoffen in te zetten om de weerbaarheid in tomaat en chrysant tegen één van de belangrijkste sleutelplagen namelijk, de Californische trips, te verhogen. Aanleiding hiervoor zijn onder meer veranderingen in het gewasbeschermingsbeleid en de maatschappelijke opinie omtrent milieukwesties. Om de chemische basale afweer te manipuleren dan wel te verhogen, worden secundaire plantmetabolieten extern toegediend (**hoofdstuk vier**), terwijl in **hoofdstuk vijf** en **zes** externe applicatie van planthormonen wordt onderzocht om de weerbaarheid tegen trips te induceren.

Hoofdstuk twee introduceert het concept geïntegreerde gewasbescherming (EN: Integrated Pest Management; IPM) en de daarbij behorende maatregelen in het kader van de Californische Trips. De afkorting 'IPM' heeft inmiddels wereldwijde erkenning gekregen en wordt onderschreven als het toekomstige paradigma voor gewasbescherming, wat duidelijk resoneert met zowel maatschappelijke als politieke kwesties. Het holistische concept van IPM legt de nadruk op systeembenaderingen door preventie- en interventiemaatregelen te integreren. In deze review worden een tweetal strategieën, waarin de afgelopen jaren grote vooruitgang is geboekt, uitgelicht namelijk (1) waardplant resistentie, waarbij rassen veredeld worden (traditioneel of genetisch) op intrinsieke weerbaarheid en afwerende eigenschappen, en (2) biologische bestrijding met behulp van natuurlijke vijanden. Een aantal

kennisgaten en innovatieve oplossingen worden besproken. De nadruk wordt daarbij gelegd op verschillende omics-technologieën die een belangrijke drijfveer vormen in de systeembiologie. Dergelijke systeemgerichte benaderingen dragen bij aan het verkrijgen van nieuwe inzichten over afweermechanismen en ziekte- en plaagresistentie. Het gebruik van screeningsprocedures met grote verwerkingscapaciteit (EN: high-throughput) bieden daarbij een krachtig hulpmiddel om innovatieve ontwikkelingen voor gewasbescherming te stimuleren.

Onder dergelijke innovatieve benaderingen is de zoektocht naar milieuvriendelijke substituten in de afgelopen jaren enorm versterkt (Adeyemi, 2010; Isman et al., 2006; Kortbeek et al., 2019; Lorsbach et al., 2019). Het gebruik van biopesticiden lijkt daarbij een aantrekkelijke oplossing voor duurzame gewasbescherming te zijn (Villaverde et al., 2013). In **hoofdstuk drie**, worden de belangrijkste strategieën besproken voor het selecteren van bioactieve, plant-eigen inhoudsstoffen als duurzame alternatieve kandidaten voor gewasbeschermingsmiddelen. Dit hoofdstuk biedt een vereenvoudigde gids, van plant tot praktijk, en bespreekt drie essentiële elementen. Het eerste element beschrijft een robuuste, betrouwbare en kwantitatieve eco-metabolische benadering om bioactieve metabolieten te screenen, gevolgd door het tweede element namelijk, een rigoureus validatieproces om de insecticide activiteit te bestuderen en te verifiëren. Een tiental geselecteerde metabolieten werden beoordeeld die, naast hun natuurlijke functie als chemische afweerstof tegen insecten, een tal van gewenste gezondheidsvoordelen lijken te bieden. Centraal in de bestrijding van plaaginsecten staat de vraag hoe deze groene substituten kunnen worden geformuleerd. Veel van deze insecticide metabolieten zijn namelijk kristallijne vaste stoffen met een zeer beperkte oplosbaarheid in water die aldus commerciële formulering mogelijk belemmeren. Als zodanig beschrijft het derde element een strategie voor het verbeteren van de oplosbaarheid. Natuurlijke diepe eutectische oplosmiddelen (EN: Natural Deep Eutectc Solvents; NADES), een klasse van innovatieve milieuvriendelijke oplosmiddelen bestaande uit een breed scala veelvoorkomende primaire plant metabolieten, werden geëvalueerd als concept voor het verbeteren van de oplosbaarheid van slecht wateroplosbare metabolieten. Dit hoofdstuk wordt verder ondersteund met experimentele resultaten en toont de oplosbaarheid van een zestal insecticide metabolieten in verschillende NADES aan. De hoogste oplosbaarheid werd bereikt met behulp van melkzuur: 1,2-propaandiol in een molaire verhouding van 2: 1 en met 1,2-propaandiol: choline chloride: water (molaire verhouding 1: 1: 1).

Dit onderzoek werd verder opgepakt met het uiteindelijke doel om dit concept toe te passen als zaadbehandeling (weken van zaden) voor het verbeteren van de afweer van tomatenplanten tegen de Californische trips, hierna trips genoemd (**hoofdstuk vier**). Het effect van acht verschillende NADES op de ontkieming van tomatenzaden en de groeiprestaties van planten werd bestudeerd. Tevens werd het potentieel van NADES als drager voor insecticide plant-secundaire metabolieten verkend. Externe toediening (d.m.v. zaadbehandeling/weken) van drie representatieve modelverbindingen (beta-alanine, sinapinezuur en chlorogeenzuur) werd geëvalueerd op basis van hun vermogen om tomatenplanten, al vanaf hun meest kwetsbare fase, te beschermen tegen trips. Hoewel NADES heeft bewezen een interessante vloeistof te zijn om de oplosbaarheid van plant metabolieten aanzienlijk te verhogen, blijkt het gebruik van NADES als drageroplosmiddel voor

insecticidale secundaire metabolieten de trips-resistentie, in termen van zilverschade symptomen, niet te verbeteren. Bovendien is een inherent nadeel van extern aangebracht NADES het fytotoxisch effect ervan op de zaadkieming en levensvatbaarheid, met name wanneer de duur van het weken van tomatenzaad werd verlengd. Het werk dat in dit hoofdstuk wordt beschreven, was daarom een belangrijke drijfveer om induceerbare afweerreacties te onderzoeken als een alternatief onderdeel van de verdedigings-mechanismen.

Moduleren van endogene insecticide metabolieten door exogene toediening

De productie van het indrukwekkende arsenaal van endogene insecticidemoleculen is door de geschiedenis heen door de mensheid geëxploiteerd en dient vaak nog steeds rechtstreeks of als 'lead' verbindingen voor de semi-synthese van moderne agro-chemicaliën (Lorsbach et al., 2019). De zoektocht naar 'lead' verbindingen uit natuurlijke bronnen kan echter gecompliceerd zijn door het gelijktijdig voorkomen van talloze plant metabolieten (Inui et al., 2012). Hierdoor kan het zijn dat externe toediening met zuivere plantenstoffen niet altijd de volledige insecticide potentie onthult, vooral wanneer de ***in planta*** insecticide activiteit te danken is aan synergie of interacties tussen verschillende bestanddelen in de plant. Dergelijke interacties kunnen plaatsvinden via verschillende mechanismen, waaronder: biologische beschikbaarheid, activering van pro-metabolieten of de-activering van actieve verbindingen in inactieve metabolieten, remming van binding aan doeleiwitten of interferentie met cellulaire transportprocessen (Efferth en Koch, 2011; Jeschke, 2016). Onder deze mechanistische verklaringen wordt het belang van biologische beschikbaarheid van bioactieve stoffen benadrukt in de opvolgende experimentele hoofdstukken.

Zaadbehandelingen worden wereldwijd routinematig toegepast om zaden en vroege ontwikkelingsstadia te beschermen tegen aantasting door insectenplagen ziekteverwekkers (Brandl, 2001). Echter, zaadbehandelingen bieden slechts een tijdelijke systemische bescherming die vaak afneemt als de bioactieve verbinding wordt afgebroken of verdunt naarmate de groei vordert (Quérou et al., 1998). Dit zou mogelijkerwijs een verklaring kunnen zijn voor het gebrek aan verbetering in trips-resistentie in tomatenplanten (**hoofdstuk vier**). Bovendien beïnvloeden verschillende andere factoren, waaronder bodemvocht, oplosbaarheid in water, behandelingsdosis en –formulering, substraatretentie en zaadpermeabiliteit, zowel de translocatie als de uiteindelijke werkzaamheid van een actief ingrediënt (Quérou et al., 1998). Al tientallen jaren worden gewassen voornamelijk veredelt op natuurlijke constitutieve afweer, terwijl induceerbare afweer een sterk onderbenutte strategie lijkt te zijn (Stout et al., 2002). Zaadbehandeling met chemische activators, ook wel elicitors genoemd, kan planten in staat stellen vroeg in de groeifase verdedigingen aan te zetten door aangeboren afweermechanismen te activeren (Song et al., 2017). Geïnduceerde afweermechanismen kunnen daarom een duurzamere vorm van resistentie vormen (Stout et al., 2002; Bruce et al., 2017). Derhalve beoogt **hoofdstuk vijf** de afweer tegen trips, met behulp van het plantenhormoon jasmonzuur (JA), in het zaadstadium te stimuleren. Elicitor-gemedieerde inductie van afweer is een algemeen aanvaarde, milieuvriendelijke benadering voor moderne gewasbescherming, omdat dit het gebruik van giftige pesticiden aanzienlijk kan minimaliseren (Vallad en Goodman, 2004;

Steenbergen et al., 2018). Zaden zijn gevoelig voor externe toediening met fyto-hormonen, zonder daarbij beperkt te worden door de inherente kosten van afweer en kunnen daarom breedspectrum bescherming bieden in de vroege stadia van plantontwikkeling (Rajjou et al. 2006; Worrall et al., 2012; Haas et al. al., 2018). Eerder onderzoek heeft echter aangetoond dat deze gunstige effecten van JA-zaadbehandelingen niet altijd even consistent zijn onder tomatenrassen (Smart et al., 2013). Deze bevinding is in overeenstemming met het werk beschreven in **hoofdstuk vijf**, waarin ik de mate van variatie in induceerbare resistentie in acht commerciële tomatenrassen onderzocht heb. Onder de geëvalueerde cultivars verschilde hun reactie op externe JA-zaadtoepassing inherent. Symptomen van zilverschade werden alleen verminderd in JA-zaad behandelde planten van de tomatencultivar Carrousel. Verhoging van de afweer was echter niet geassocieerd met activering van afweer gerelateerde eigenschappen zoals polyfenoloxidase-activiteit (PPO), trichoom dichtheid noch met de inductie van vluchtige stoffen. Echter, beoordeling van deze eigenschappen eerder in de groeifase kan mogelijkerwijs meer licht werpen op de onderliggende afweermechanismen. Chemische scarificatie met zwavelzuur verbeterde de zaadresponsiviteit op JA aanzienlijk in de niet-responsieve cultivar Moneymaker, hetgeen een belangwekkende en veelbelovende bevinding is. Deze waarneming ondersteunt de bewering dat de permeabiliteit van de zaadhuid ongetwijfeld een belangrijke factor is voor het vergemakkelijken van JA-absorptie (d.w.z bio-beschikbaarheid) en daarmee dus ook de verhoogde fysiologische activiteit. Bovendien biedt deze uitkomst interessante kansen voor veredelingsprogramma's door te selecteren op genetische eigenschappen die de permeabiliteit van zaad beïnvloeden. Tevens biedt het mogelijkheden voor particuliere zaadbedrijven om door middel van voorbehandelingen de fysische barrière van de zaadhuid, en de daaraan gelinkte responsiviteit, af te stemmen.

Commercieel gebruik van elicitors

Desalniettemin vormt de waargenomen variabiliteit in de responsiviteit tussen cultivars een belangrijk obstakel, omdat een van de belangrijkste voorwaarden voor succesvolle commercialisering van van elicitors een consistente werkzaamheid is. In de wetenschappelijke literatuur wordt Moneymaker samen met tomatencultivars Aisla Craig, Micro-Tom en Castlemart vaak gebruikt om 'tomaat' te vertegenwoordigen als een plantmodelsysteem van de ***Solanaceae***-familie. Desondanks blijft de fundamentele vraag of het correct is om tomaat als modelsoort in het algemeen te beschouwen, terwijl er aanzienlijke verschillen bestaan tussen individuele cultivars. De variabele responsiviteit tussen verschillende cultivars heeft duidelijk de noodzaak aangetoond om meerdere cultivars op te nemen als biologisch evaluatie systeem om zodoende consistente uitspraken te kunnen doen. Bovendien laat het zien dat het verwaarlozen van deze kwestie in de screeningfase voor nieuwe chemische elicitors onnodig bijdraagt tot een overmaat aan fout-negatieve bevindingen. Om te voorkomen dat potentiële kandidaten ten onrechte als inactief worden geclassificeerd, is het belangrijk om echt-negatieven te onderscheiden van vals-negatieve elicitors. Als alternatief kunnen bijvoorbeeld transgene planten en cellen die promotor-reporter gen fusies bevatten, bijdragen aan de ontwikkeling van gevoelige en specifieke systemen voor het screenen van potentiële elicitors van

natuurlijke herbivoor resistentie (Doerner et al., 1990; Corrado en Karali, 2009; Halder en Kombrink, 2018). Elicitors kunnen worden geïntroduceerd door jonge zaailingen of cel suspensie culturen te behandelen. Activatie van het afweersysteem kan vervolgens met behulp van een microplaatlezer met hoge verwerkingscapaciteit worden gevolgd door middel van visualisering van de reporter degradatie (Larrieu et al., 2015).

Ondanks het inherente potentieel van elicitors, is een dwingende vraag waarom slechts weinigen commercieel succes hebben behaald? De goedkeuring van duurzame alternatieven heeft een sterke regulerende dimensie die mogelijk een belemmering kan vormen voor de ontwikkeling van doeltreffende commerciële producten. Bovendien is de ontwikkeling van een nieuwe actieve verbinding duur en tijdrovend. De overgrote meerderheid van commerciële producten die claimen ziekteresistentie te induceren, hebben vaak te maken met SA-gemedieerde signalering (Gorelick en Bernstein, 2014). Integendeel, er is niet veel vooruitgang geboekt bij de commercialisering van producten ter bestrijding van herbivoren. Grootschalige toepassing van JA kan onbetaalbaar zijn, maar misschien wel van groter belang is de bekende groei-onderdrukkende werking van JA, met name bij hogere doseringen (Redman et al., 2001). Het screenen op of het ontwerpen van structureel verwante analogen die het resistentie-inducerende vermogen van JA behouden (Haider et al., 2000), maar minder actief zijn in groeionderdrukking, kunnen in dit opzicht veelbelovender zijn. Het is ook cruciaal om te erkennen dat het agrarisch nut van JA-geïnduceerde afweersystemen geen onmiddellijk knock-downeffect heeft en zelden volledige weerstand verleent (Stout et al., 2002). Toch is een van de belangrijkste voordelen van geïnduceerde resistentie dat het compatibel kan zijn met, of zelfs kan bijdragen aan een verbeterde biologische bestrijding van plantenplagen door de aantrekkelijkheid van planten voor natuurlijke vijanden te bevorderen. Geïnduceerde afweer kan daarom als een parallelle of sequentiële aanpak worden beschouwd (Smart et al. , 2013; Bruce et al., 2017; Pappas et al., 2017). Ten slotte is elicitor-geïnduceerde resistentie verschillend effectief tegen plagen en dus kan hormonale cross-talk na exogene JA-behandeling sommige planten juist kwetsbaarder maken voor pathogenen die geassocieerd worden met de SA-gemedieerde afweer route.

Uitdagingen in reproduceerbaarheid

In het kader van mechanistische cross-talk werd in **hoofdstuk zes** de complexe interactie tussen het wortel-bevorderende auxinehormoon indoolboterzuur (IBA) en JA onderzocht. Recente studies tonen aan dat auxines ook invloed hebben op een scala aan afweerreacties in planten, door complexe interacties tussen meerdere hormoonroutes, waaronder JA (Kazan en Manners, 2009; Robert Seilaniantz et al., 2011; da Costa et al., 2013). Op zoek naar effectieve dip behandelingen om stekken tijdens hun vegetatieve fase te beschermen, merkten we onverwacht op dat het dippen van IBA-gecoate stekken in water herhaaldelijk de herbivoor schade, zowel door trips als door de mineervlieg, verminderde. Beoordeling van de polyfenol oxidase activiteit (PPO), 14 dagen na de dip behandeling, suggereert dat er geen sprake is van directe inductie noch van priming van de plantenafweer. Toekomstige experimenten die gericht zijn op het begrijpen van de vroege signaleringsgebeurtenissen,

met inbegrip van hormonale signaalnetwerken, vanuit een meer holistisch perspectief, kunnen helpen om de fysiologische basis te verklaren die betrokken is bij het bieden van bescherming tegen herbivoren. Over het algemeen waren de resultaten echter zeer variabel en staan deze ons dus niet toe om concluderende uitspraken af te leiden over de betrokkenheid van auxine-gemedieerde afweerreacties. Een mogelijke verklaring van deze tegenstrijdigheden lijkt te berusten op de grote fenotypische variatie in stekken die uit moederplanten worden gegenereerd. Dit benadrukt de noodzaak om intra- en inter-variabiliteit en reproduceerbaarheid, die beide een kern vormen in de wetenschap voor het kritisch beoordelen van de juistheid van wetenschappelijke claims en conclusies (Plesser, 2018), te verbeteren. Het suggereert ook dat het succes van een behandeling tijdens een bepaald stadium van het groeiproces sterk kan afhangen van wat er in de vorige stadia gebeurd is. Het is dan ook sterk aanbevolen om de potentieel defensieve rol van auxines verder te onderzoeken en deze te integreren in coherente modellen van modelplantensoorten, alvorens ze te vertalen naar commercieel belangrijke tuinbouwgewassen. Deze studie biedt een interessant uitgangspunt om de alternatieve rol van auxines in de commerciële stek propagatie te onderzoeken en kan, naast hun groei-regulerende functie, een veelbelovende afweerstrategie toevoegen voor chrysantenveredelaars en telers. Toekomstig werk met auxine-formuleringen is nodig om te bepalen of een dergelijke aanpak commercieel waardevol is voor een breder scala van herbivoren.

Curriculum Vitae

List of publications

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