IN DEFENCE OF BEAUTY

Exploiting natural variation in thrips resistance in *Chrysanthemum*

Marcella Bovio

Propositions

1. Host plant resistance against thrips is essential for sustainable thrips control. (this thesis)
2. Thrips survival is the key phenotypic trait to select thrips-resistant *Chrysanthemum* plants. (this thesis)
3. Secondary metabolites stand second to nothing in their biological significance.
4. Prioritizing science education is crucial for effectively addressing societal challenges.
5. Job application procedures are designed for extroverts.
6. The Internet is the unreliable memory of humanity.

Propositions belonging to the thesis, entitled

In defence of beauty: exploiting natural variation in thrips resistance in *Chrysanthemum*

Marcella Bovio
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Chapter 1

General introduction

Thrips, an old but growing problem

The pest status of a plant-feeding insect species depends mainly on the degree of production and economic damage it causes. Insect species characterized by a broad host-plant range, a short generation time and high reproduction capacity are overrepresented among pest species, however, their pest status depends also on climate and agronomic practices. Additionally, human expectations and perception may play a role in defining these species as pests within the region where they are found. A pest can be any undesirable animal, but here by pest we mean a species that causes extensive (economic) damage to an agricultural crop or has a demonstrated potential to do so (Mound et al., 2022). Among the most notorious insect pests we can find beetles, moths, whiteflies, planthoppers, aphids, and thrips. Thrips are tiny insects, a few millimetres long, from the order Thysanoptera, that comprises about 7000 species (Maurastoni et al., 2023). The term Thysanoptera comes from the ancient Greek thysanos, "fringe", and pteron, "wing", and in fact, thrips are characterized by fringed wings. Another unique characteristic of this order is the asymmetry of the mouthparts. Because of their small size, they fly using the clap and fling mechanism (Santhanakrishnan et al., 2014; Weis-Fogh, 1973) and are usually transported by wind gusts from host to host (Mound, 2005). Within this order, two suborders are described, Tubulifera and Terebrantia. The members of the first order all belong to one family (Phlaeothripidae) and the females of this family lack an ovipositor. Females of suborder Terebrantia are characterized by a fully developed saw-like ovipositor, through which the eggs are inserted in the host-plant tissues. Only a few species of the 3800 within the family Phlaeothripidae are considered pests. The majority of pestiferous thrips belong to the Terebrantia and are part of a single family, Thripidae, and subfamily, Thripinae (Morse and Hoddle, 2006). These species are considered pests, as they cause extensive damage to crops, are often polyphagous, and can transmit Tospoviruses (Riley et al., 2011). Moreover, they have a short life cycle, allowing a quick population build-up, and thus, substantial damage (Kirk and Terry, 2003). The Thripidae family comprises 1800 species, but less than 1% is known to be a vector of Tospoviruses. Over the past 50 years in Europe, two species, *Frankliniella occidentalis* and *Thrips tabaci*, have been considered the most important thrips pests. However, in recent years invasive species, such as *Thrips parvispinus*, have been reported and in the future may establish themselves in greenhouses (Thorat et al., 2022).

Frankliniella occidentalis, *Thrips tabaci* and *Thrips parvispinus*

Frankliniella occidentalis (Thysanoptera: Thripidae), also known as Western Flower Thrips (WFT), originates from Western North America (Kirk and Terry, 2003). Adult individuals of this species can vary a lot in colour and size. Females are generally 1.5 mm in length, while males are approximately 1 mm in length. Adult females vary in colour from pale yellow to dark brown; adult males do not show colour variation and are yellow (Ghosh et al., 2021; Mound, 2005). Western Flower Thrips is considered a species complex and two strains, called the 'glasshouse' and the 'lupin' strain respectively, can be found in its native distribution range (Nielsen et al., 2010; Rugman-Jones et al., 2010). Starting from the Netherlands in 1983, it spread all over Europe, favoured by the transport of infested plant material, the development of insecticide resistance in the 1970s (Brødsgaard, 1994), and its biological characteristics, i.e. its short life cycle, high reproduction rate, and wide host range. These characteristics also made *F. occidentalis* the main thrips species found on many greenhouse crops globally (Kirk and Terry, 2003; Reitz, 2020).

Thrips tabaci, or onion thrips, originates from the eastern Mediterranean and is widely distributed in Europe, Africa, Asia, (North) America, and Oceania (Loredo Varela and Fail, 2022). Adult individuals are 1.0-1.3 mm in length and, similarly to *F. occidentalis*, vary in colour and size, with yellow to brown females and pale smaller males (Mound, 2005). It is a cryptic species complex consisting of three species or lineages, one tobacco lineage and two leek lineages, all widespread in Europe. These lineages differ in reproductive mode, geographical distribution, and host plants (Loredo Varela and Fail, 2022).

Thrips parvispinus (Thysanoptera: Thripidae), or pepper thrips, originates from Asia. It affects many crops, both vegetables like pepper and ornamentals like rose and chrysanthemum (The Netherlands Plant Protection Organization, 2019). In the past 20 years, *T. parvispinus* greatly extended its geographical distribution and has been reported in many countries: Ghana, Tanzania, Uganda, US, and Australia (Thorat et al., 2022). *Thrips parvispinus* in India has displaced *Scirtothrips dorsalis* on chilli cultivation, while in Indonesia it has displaced *Thrips palmi* (Sridhar et al., 2021). Recently, it has been reported also in Spain, France, Germany, and the Netherlands (Lacasa et al., 2019; The Netherlands Plant Protection Organization, 2019). *Thrips parvispinus* may also pose a threat to Europe in the coming years (Thorat et al., 2022) and changes in agricultural practices and climate may increase the potential impact of this pest (Schneider et al., 2022; Skendžić et al., 2021).

Life cycle of thrips

The life cycle of thrips *F. occidentalis*, *T. tabaci* and *T. parvispinus* lasts approximately 14 days at 25°C and consists of six stages (Thorat et al., 2022; Van Rijn et al., 1995) (Figure 1). It starts with adult females laying their eggs in plant tissues. As oviposition and feeding substrate, *F. occidentalis* and *T. parvispinus* favour flowers, whereas *T. tabaci* prefers leaves. The first instar (L1) larvae hatch from the eggs after 3 to 5 days depending on temperature. The L1 larvae are 0.2 mm long, white in colour, and develop into second instar (L2) larvae within 1-3 days depending on temperature and host. After the two larval stages, pupation occurs. All these three thrips species have two pupal stages, a prepupa and a pupa that differ in the length of the wing buds and the antennal orientation. Unless disturbed, pupae do not move. The prepupal stage lasts 1-2 days, while the pupal stage lasts 3-4 days. Lastly, winged adults emerge.

Thrips damage

Host plants are extensively damaged by thrips feeding. Larvae and adults are the only feeding stages and therefore the only stages causing damage. They have piercing-sucking mouthparts (Hunter and Ullman, 1992), with which they first pierce the leaves and then empty the epidermal and mesophyll cells (Kindt et al., 2003). This results in what is usually referred to as direct damage, i.e. silvery and discoloured spots (or silver damage), and malformation (or growth damage) on both leaves and flowers (Reitz, 2009) (Figure 2). Additionally, extensive thrips feeding indirectly reduces photosynthesis (Shipp et al., 1998).

In addition, thrips can also indirectly damage their host by transmitting plant viruses (Reitz, 2009; Riley et al., 2011; Rotenberg et al., 2015). *Thrips parvispinus* is a vector of tobacco streak virus (Klose et al., 1996), whereas *F. occidentalis* and *T. tabaci* are among the most important vectors of tospoviruses (He et al., 2020; Rotenberg et al., 2015). Symptoms of tospoviruses vary from necrotic spots, ringspots, and blotches on leaves to stem necrosis. Tospoviruses are acquired by L1 or early L2 larvae, they replicate in the thrips and migrate from the midgut to the salivary glands (Moritz et al., 2004). Late L2 and adults then transmit the viruses to plants through their saliva during probing (Whitfield et al., 2005). Adults are viruliferous for life but do not transmit the virus to the eggs (Wijkamp et al., 1996a).

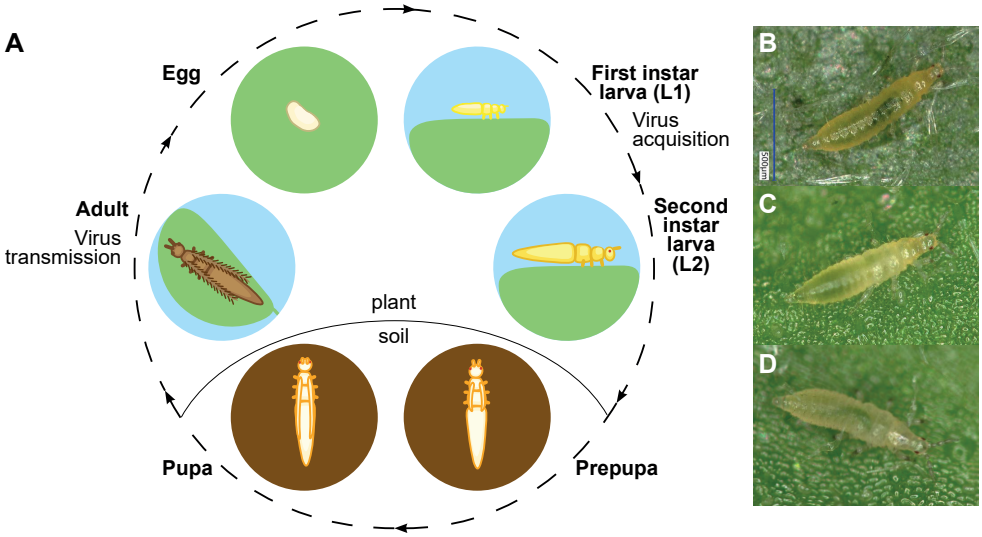

Figure 1. On the left: A) Life cycle of thrips. The adults lay the eggs inside the plant tissue. The first instar larvae hatch and then develop into second instar larvae. Larval stages feed on the plant. The pupal stages are usually found in the soil and do not feed. The adults emerge from the pupae and returns to the plant to feed and reproduce. Adults transmit plant viruses after acquiring them as L1 larvae. Any stage of the life cycle can be affected by plant resistance. On the right: Second instar (L2) larvae of B) *Frankliniella occidentalis*, C) *Thrips parvispinus*, D) *Thrips tabaci*. The vertical blue scale bar in (B) corresponds with 500 µm and is applicable to (C) and (D).

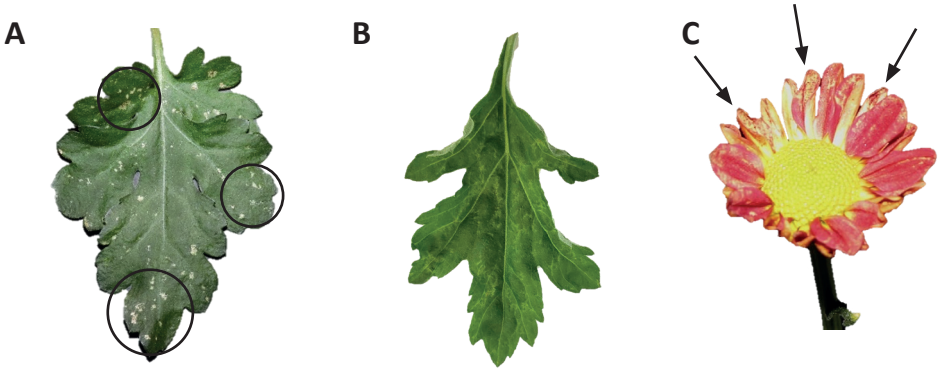

Figure 2. Thrips damage on chrysanthemum leaves: A) silver spots, and B) scars and malformation. C) Malformation and discoloration resulting from thrips feeding on a red chrysanthemum flower. Black circles and arrows indicate the feeding damage.

Control of thrips

Because of the damage that thrips can cause, *F. occidentalis* is a quarantine pest included in the A2 list of the European and Mediterranean Plant Protection Organization (EPPO), thus its presence on plant material in international trade is prohibited (EPPO, 1989). Also, quarantine is required for material infested by *T. parvispinus* in the US (Seal et al., 2023). Therefore, the management of thrips is of extreme importance. Thrips control measures have been developed and applied over the years to try to keep infestations and economic damage to a minimum. These measures include chemical control, biological control, and agronomical practices (Rodríguez and Coy-Barrera, 2023). Nowadays, thrips control still heavily relies on the use of insecticides of various chemical classes such as organophosphates, carbamates, pyrethroids, macrocyclic lactones, and spinosyns (Kivett et al., 2015). However, thrips rapidly develop resistance to different classes of insecticides (namely organophosphates, carbamates and pyrethroids) (Bielza, 2008), leading to an increased use and demand for new active compounds. Moreover, insecticide use raises more and more concerns, as they are hazardous to the environment and mankind. The use of insecticides has negative effects on biocontrol agents and other beneficial insects (Bielza, 2008). For these reasons, the European Union has banned the use of neonicotinoids (European Union, 2018) and strives to reduce the use of chemical pesticides by 50% in 2030. Moreover, thrips' cryptic behaviour, i.e. running away from light, preferring the abaxial side of leaves, and pupation in the soil make them an especially difficult target for contact insecticides, rendering these less effective. Another control strategy is biological control or the use of natural enemies of thrips. Biocontrol agents have optimal conditions under which they effectively control thrips numbers. The most used natural enemies are *Orius* spp. predators, true bugs from the family Anthocoridae, that feed on the thrips by piercing and sucking the haemolymph (Rodríguez and Coy-Barrera, 2023). Also neuropterans from the family Chrysopidae are used as thrips predators in greenhouse cultivation. In addition, foliage predators, soil-dwelling predators, entomopathogenic nematodes and fungi can be used to target pupae in the soil (Manners et al., 2013; Rodríguez and Coy-Barrera, 2023).

In an Integrated Pest Management (IPM) approach, biocontrol agents can be used in combination with host plant resistance (HPR) to control thrips (Mouden and Leiss, 2021). Host plant resistance can reduce pest pressure and maintain thrips population levels within the range in which biocontrol agents can be effective. Moreover, host plant resistance may lengthen the life cycle of thrips, thus reducing the number of generations and the chances for the pest to develop (new) insecticide resistance (Jensen, 2000). Using varieties with host plant resistance, a lower amount

of insecticides will be needed, not only because the population of pests will be smaller but also because these pests will have a decreased vigour caused by feeding on thrips-resistant plants (Smith, 2021). In addition, by reducing thrips number, host plant resistance may also indirectly reduce the transmission of viruses.

Chrysanthemum: economics and the battle against pathogens and pests

Chrysanthemum (*Chrysanthemum x morifolium*) is one of the economically most important ornamental plants, sold as cut flower and potted plant. The annual revenue of the cut flower auctioned in the Netherlands was 369 M€ in 2022, second to rose, and potted chrysanthemums are seventh among pot plants (Royal Flora Holland, 2022). Cut flower chrysanthemums can be categorized into disbudded (one big flower per stem), spray (some big flowers per stem), or Santini (many flowers of maximum 4 cm diameter per stem) (Figure 3). Chrysanthemums intended for sale are, like roses, vegetatively propagated through cuttings because of their genetic complexity. Cultivated chrysanthemums are hexaploid and outcrossing, which results in a very high genetic diversity if propagated through seeds (Drewlow et al., 1973; Van Geest et al., 2017).

Figure 3. Types of cultivated chrysanthemum flowers. From the left, disbudded, spray and pot chrysanthemum.

Due to its ornamental purpose, tolerance for visual damage and insect presence in this crop is very low, but chrysanthemum is, unfortunately, heavily affected by various pathogens and pests. Some of the most relevant pathogens causing diseases are fungi, such as white rust *Puccinia horiana* and powdery mildew *Oidium chrysanthemi*, oomycetes, such as *Pythium* spp. and *Phytophthora* spp., viroids, such as Chrysanthemum Stunt Viroid (CSVd) (Reddy, 2016), and viruses, such as the thrips-transmitted Chrysanthemum stem necrosis virus (CSNV), Tomato spotted wilt orthotospovirus (TSWV) and Impatiens necrotic spot virus (INSV) (Kondo et al.,

2011; Matsuura et al., 2002; Nagata et al., 2004; Okuda et al., 2013; Wijkamp et al., 1996b, 1995). Major pests of *Chrysanthemum* are aphids, such as *Macrosiphoniella sanborni*, two-spotted spider mites *Tetranychus urticae*, and thrips *F. occidentalis* (CABI, 2021; Reddy, 2016). In addition to *F. occidentalis*, other polyphagous thrips species have been reported on chrysanthemum flowers, such as *T. tabaci* and *T. parvispinus* (EPPO, 2022; Loredo Varela and Fail, 2022). In *Chrysanthemum*, thrips feeding causes leaf damage, reduced plant growth (Van Dijken et al., 1994), and damage to flowers (Rhainds and Shipp, 2003). Therefore, the interest of plant scientists and breeders lies not only in improving the appearance and ornamental value of chrysanthemums but also in improving crop resistance to pathogens and pests, like thrips. Attempts to breed for thrips-resistant chrysanthemum cultivars have not yet been successful. The identification of strong resistance sources followed by the study of the underlying genetics and mechanism would contribute to more successful breeding programs for thrips-resistant chrysanthemums.

Crop wild relatives as sources of resistances

Crop domestication is the process by which humans selected plants with characteristics that suit their purpose and that resulted in crops different from their wild ancestors (Dempewolf et al., 2017). Different traits, considered favourable to humans, are selected over time depending on the crop, such as higher nutritious or esthetical value, and yield. However, crop domestication ultimately resulted in a reduction of genetic variation (allelic diversity) (Dempewolf et al., 2017). As a result, many traits that were not selected for in the past because they were not of interest or relevant are not present anymore in the domesticated crops. For instance, until recently in our agricultural system, chemical crop protection was preferred over pest resistance, which was consequently not the focus of selection. Fortunately, these traits can still be found in crop wild relatives (Chen et al., 2015; Whitehead et al., 2017). Four hundred million years of coevolution of plants and insect herbivores led to plants developing diverse defence strategies and insects developing counter-adaptations to overcome these defences (Fürstenberg-Hägg et al., 2013; Kant et al., 2015; War et al., 2012). Crop wild relatives are expected not only to have higher genetic diversity than the cultivated crops, but also to act as pools of resistance traits (Zhang et al. 2017; Warschefsky et al. 2014; Dempewolf et al. 2017). For this reason, crop wild relatives have been extensively used as sources of pest and disease resistance to improve crops such as tomato, wheat, rice, and potato (Hajjar and Hodgkin, 2007). Crop wild relatives are still being screened for new resistance traits. For example, wild relatives of potato show resistance to

Colorado potato beetle (Pelletier et al., 2001; Tai et al., 2014; Wolters et al., 2023), the aphids *Myzus persicae* (Ali et al., 2022; Alvarez et al., 2006) and *Macrosiphum euphorbiae* (Cho et al. 2017). Wild relatives of tomato are resistant to two-spotted spider mite *T. urticae* (Alba et al., 2009), whitefly *Bemisia tabaci* (Firdaus et al., 2012) and *Trialeurodes vaporariorum* (McDaniel et al., 2016), thrips *F. occidentalis* and *M. persicae* (Vosman et al., 2018).

The genus *Chrysanthemum*, in the family Asteraceae, is native to Eurasia and wild relatives can be found not only in China, where the primary gene center is located, but also in Japan, Korea and Alaska (US) (Liu et al., 2012). Within and among the wild species, ploidy ranges from diploid to decaploid. Interspecific crosses among wild *Chrysanthemum* species can be successful, depending on the parental genotypes. Moreover, intergeneric hybrids with *Ajania* spp. are found in nature and are made in breeding programs (Deng et al., 2011; Zhao et al., 2009) and crosses with other related genera, such as *Artemisia* and *Tanacetum*, have also been reported (Deng et al., 2012, 2010; Tang et al., 2011).

Phenotyping methods for thrips resistance

Thrips resistance in plants has been studied across various crops and their relatives, employing a range of assays to evaluate different parameters. These assays can be categorized into two types based on the insect's freedom of choice: choice assays and no-choice assays. In choice assays, thrips are given the freedom to move between plants or plant parts, and antixenosis or non-preference resistance is assessed by counting thrips that choose a plant (part) for feeding or oviposition. On the other hand, no-choice assays restrict the thrips' choice of feeding and oviposition sites to selected plants or plant parts, using cages, Petri dishes, or sleeves. In these assays, antibiosis type of resistance is usually assessed, i.e. the impact of a single feeding substrate or host genotype on survival, oviposition, and development of the thrips. Both types of assays can involve the use of whole plants (whole plant assay), or plant parts, such as individual leaves (detached leaf assay or clip-cage assay), flowers (detached flower assay), or part of these organs (leaf disc assay). Screening for thrips resistance often relied on evaluating feeding damage, so-called silver damage or growth damage seen e.g. as leaf malformation, which can be assessed in both choice and no-choice assays. Thrips resistance in cultivated *C. x morifolium* has been investigated using various assays, and by evaluating different parameters. No-choice whole plant assays have been used to identify resistant plants that show reduced silver damage (Chen et al., 2020b; Leiss et al., 2009b) or reduced thrips population build-up (De Jager et al., 1993); no-choice leaf assays have been used to

assess larval development (Ohta, 2002), larval survival (De Jager et al., 1993), silver damage (Rogge and Meyhöfer, 2021) and reproduction and adult survival (De Kogel et al., 1997a). Whole plant choice assays were performed to assess growth and silver damage resulting from thrips infestation, but also thrips population build-up in flowers (De Jager et al., 1995a; Kos et al., 2014).

Defence mechanisms in *Chrysanthemum*

Plants evolved a vast range of physical and chemical defences against insect herbivores. These defence mechanisms can be constitutively expressed or be induced upon attack by the herbivores (Fürstenberg-Hägg et al., 2013; War et al., 2012). The impact of these plant defences on an herbivore depends on its feeding strategy. When insects start feeding, they first encounter a physical plant barrier that may consist of leaf surface wax, cuticula, thorns, cell walls and trichomes. A dense trichome layer can affect landing, feeding and movement of pests, especially when it consists of glandular trichomes that produce and accumulate specialized defence metabolites (Cho et al., 2017; Stavrinides and Skirvin, 2003; Tian et al., 2012).

Plants constitutively produce many different classes of metabolites and many metabolites associated with thrips resistance have been identified. These belong to different classes of compounds, such as phenols, flavonoids, terpenes, alkaloids and glycosides (Steenbergen et al., 2018). Acyl sugars have been linked to thrips resistance in tomato (Mirnezhad et al., 2010; Vosman et al., 2018). Pyrrolizidine alkaloids (jacobine and jaconine) and a flavonoid (kaempferol glucoside) have been identified in resistant *Senecio* (Leiss et al., 2009a). Luteolin, sinapic acid, and ß-alanine were found in resistant carrots (Leiss et al., 2013). Two acyclic diterpene glycosides and a flavonoid conjugate were found in resistant pepper (Macel et al., 2019; Maharijaya et al., 2019).

Plant defences can also be induced by insect feeding. The response depends on both the insect and the plant. In tomato, a thrips infestation induces both physical and chemical defences: it increases the number of glandular trichomes as well as the concentration of terpenes produced therein (Escobar-Bravo et al., 2017). In *Arabidopsis thaliana*, the synthesis of jasmonic acid (JA) and the downstream JA-pathway are activated after thrips infestation (Abe et al., 2008). Moreover, plants often show increased thrips resistance levels after JA application (Abe et al., 2009). More classes of metabolites can be induced by thrips feeding. For example, in tomato, eggplant, and pepper, thrips infestation induced the production of phenols,

which were associated with thrips resistance (Leiss et al., 2009b; Liu et al., 2022; Maharijaya et al., 2012; Papadaki et al., 2008; War et al., 2012).

In previous research in *C. x morifolium*, thrips resistance did not seem to depend on physical characteristics. Leaf toughness was not associated with thrips resistance (De Jager et al., 1995b). Although T-shaped trichome density seems to play a role in aphid resistance in chrysanthemum (Cheng et al., 2011), leaf trichome density did not correlate with resistance to thrips (Chen et al., 2020b). Chemical defences were also explored, and specialized metabolites are likely to be involved also in thrips resistance in chrysanthemum. Isobutylamide appeared to repel thrips (Tsao et al., 2005), and chlorogenic acid (caffeoyl quinic acid and feruloyl quinic acid) was more abundant in a group of resistant cultivars compared to a group of susceptible cultivars (Leiss et al., 2009b). However, more than one metabolite, or a mix of them, may be responsible for thrips resistance as well.

More than one resistance mechanism against thrips may be present in the *Chrysanthemum* genus. Wild *Chrysanthemum* species are known to produce a vast range of metabolites, among which some classes are associated with plant defence (Hao et al., 2022; Steenbergen et al., 2018). Numerous monoterpenoids and sesquiterpenoids have been identified in *Chrysanthemum indicum* and *Chrysanthemum makinoi* (Jiang et al., 2021). These compounds are also produced in the related Artemisia genus, and studies have been done on the essential oils of *Artemisia feddei*, *Artemisia keiskeana*, *Artemisia vulgaris* and their toxicity towards insects (Deng et al., 2010; Kim, 1997; Suleimen et al., 2019). The wild relative *Tanacetum cinerariifolium* was found to produce and accumulate insecticidal pyrethrins in its glandular trichomes on disc ray florets (Lybrand et al., 2020; T. Yang et al., 2012; Zito et al., 1983). The role of terpenoids in thrips resistance is still to be investigated further, wild *Chrysanthemum* accessions are promising sources for these compounds.

Scope of this thesis

The main goal of the work presented in this thesis was to identify thrips resistance in cultivated and wild *Chrysanthemum* and to unravel the resistance mechanism to develop new thrips-resistant chrysanthemum varieties. Thrips resistance occurring within commercial varieties is sparse and targeted breeding is still not possible because of the lack of genetic information on thrips resistance in this crop and its wild relatives. The first step in efficiently breeding for resistance is identifying reliable sources of resistance. In this thesis a large set of cultivated and wild *Chrysanthemum* accessions was screened, broad-spectrum resistance was found and metabolites correlating with resistance were identified.

In **Chapter 2**, we screened 70 accessions of cultivated chrysanthemum for thrips resistance with two phenotyping methods focusing on insect parameters and not on plant damage: whole plant assays for thrips population build-up, and detached leaf assays for larval development. Then, a subset of cultivars was further characterized with two other phenotyping methods: leaf disc assays for larval survival and development, and leaf disc assays for reproduction. From this set, the resistant cultivar Penny Lane was selected for crosses with the susceptible cultivar Super Pink Pompon.

Because we hypothesised that in wild accessions stronger or novel resistance could be found, in **Chapter 3**, we screened 47 wild relatives of *Chrysanthemum* for thrips-resistance, including cultivar Penny Lane and cultivar Super Pink Pompon as reference. Wild species from the *Chrysanthemum* genus and the related genera *Artemisia* and *Tanacetum* were included in the screening because of their cross-compatibility with cultivated chrysanthemum. Whole plant assays for thrips population build-up and leaf disc assays for larval development, larval survival, and reproduction were performed.

Populations of pest insects can differ in their responses to resistant plants, which can significantly impact the durability of plant resistance. In **Chapter 4**, we studied broad-spectrum resistance of the accessions identified in Chapter 3, by screening with different thrips populations and species, collected in greenhouses in the Netherlands. Additionally, we characterised the genetic diversity of the *F. occidentalis* populations. We further examined the presence of broad-spectrum thrips resistance in *Chrysanthemum* accessions by assessing their resistance against *T. tabaci* and the potential invasive species *T. parvispinus*. Larval development of thrips from five *F. occidentalis* populations, one *T. tabaci*, and one *T. parvispinus* population was assessed on resistant and susceptible wild *Chrysanthemum* accessions, previously selected with one of the *F. occidentalis* populations.

In **Chapter 5**, we aimed to identify metabolites associated with constitutive and induced resistance to thrips. Therefore, we first studied the effect of prior thrips infestation on the resistance level of thrips-resistant (Penny Lane) and thrips-susceptible (Super Pink Pompon) *Chrysanthemum* cultivars resistance levels, and induced metabolites. Next, an F1 population from Super Pink Pompon x Penny Lane cross was phenotyped for *F. occidentalis* larval development, and leaf metabolites were extracted.

Finally, in **Chapter 6**, the main results of this thesis are discussed and their implications for thrips resistance breeding and future research are addressed.

Chapter 2

Identifying resistance to thrips *Frankliniella occidentalis* in cultivated chrysanthemum (*Chrysanthemum x morifolium*) using an array of phenotyping methods

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Abstract

Chrysanthemum (Asteraceae) is an important ornamental crop native to China, Korea, and Japan, but it is now cultivated worldwide. Chrysanthemum cultivation is heavily affected by thrips, especially *Frankliniella occidentalis* or western flower thrips (WFT), that cause damage to chrysanthemum of all developmental stages, from young cuttings to flowers. Host-plant resistance against thrips could play a key role in controlling thrips via an Integrated pest management (IPM) approach. Thrips resistance in *Chrysanthemum* has been investigated but no resistant cultivar is yet available on the market. In this study, we screened 70 *Chrysanthemum* accessions with two phenotyping methods: whole plant assays for thrips population build-up, and detached leaf assays for larval development. We found large variation for both population build-up and larval development among the 70 *Chrysanthemum x morifolium* accessions. Next, a subset of cultivars was re-evaluated in a whole plant assay to validate the population build-up, and further characterized with two other phenotyping methods: leaf disc assays for larval survival and development, and leaf disc assays for adult reproduction. We validated the reduced population build-up of nine resistant accessions. Accessions with a reduced population build-up also showed a reduced larval survival, as well as a slower and reduced development from L1 to L2 stage, which resulted in a lower number of thrips developing into the adult stage, indicating that resistance to thrips affects mostly the earlier larval stages. The identified resistant accessions may be used in follow-up studies on the genetics of thrips resistance in cultivated chrysanthemum, and as a source of resistance in breeding programs for the introgression of this trait.

Introduction

Chrysanthemum is an important ornamental crop from the genus *Chrysanthemum* (Asteraceae) that is native to China, Korea, and Japan (Zeven and Zhukovskiĭ, 1975). Cultivated chrysanthemums are sold as cut flowers and as potted plants. Cut flower chrysanthemums come in three types: spray (big flowers on a stem), disbudded (one big flower per stem), or Santini (when the flowers are maximum 4 cm diameter). Potted chrysanthemums are commonly referred to as pot chrysanthemums or garden mums. They all belong to the species *Chrysanthemum x morifolium*. Most cultivated chrysanthemums are hexasomic and self-incompatible (Drewlow et al., 1973; Van Geest et al., 2017). This results in a highly heterozygous genome. Therefore, chrysanthemums are vegetatively propagated through cuttings.

Chrysanthemum plants suffer damage from many pests, among which the thrips species *Frankliniella occidentalis* is the most important, affecting *Chrysanthemum* cultivation worldwide. *Frankliniella occidentalis* (Thysanoptera: Thripidae), also known as western flower thrips (WFT in the following), originates from Western North America. It appeared for the first time in the Netherlands in 1983 and since then it spread all over Europe due to its wide range of host plants and short life cycle which allows a quick population build-up (Kirk and Terry, 2003). As its name suggests, WFT is attracted by flowers in which it finds hiding places and food in the form of pollen. Larvae and adult thrips feed on plant epidermal cell content through piercing-sucking mouthparts, whereas the pupal stages do not feed (Hunter and Ullman, 1992). Thrips feeding causes direct as well as indirect damage. Direct damage is evident as silvery spots on leaves, discoloration on flowers and malformation of developing organs. Indirect damage is caused by viruses, mainly tospoviruses, which are acquired by the larvae and then transmitted by the adults (Reitz, 2009). Thrips-transmitted tospoviruses that affect chrysanthemums are Chrysanthemum stem necrosis virus (CSNV), Tomato spotted wilt orthotospovirus (TSWV) and Impatiens necrotic spot virus (INSV) (Kondo et al., 2011; Matsuura et al., 2002; Nagata et al., 2004; Okuda et al., 2013; Wijkamp et al., 1996b, 1995).

Despite being particularly attracted by chrysanthemum flowers, WFT causes damage even before the plants start developing flower buds. In fact, also young cuttings are damaged by thrips and especially susceptible to thrips-induced growth malformation. Moreover, young cuttings are shipped worldwide, and presence of thrips makes them unmarketable for export. Therefore, it is necessary to reduce thrips numbers as much as possible on all chrysanthemum plants used for cultivation and trade. One control method is the use of insecticides; however, these are not always effective because thrips prefer protected spaces on the plant and

are therefore not reached. Moreover, thrips have developed resistance to different classes of insecticides (organophosphates, carbamates and pyrethroids) (Bielza, 2008; Herron and James, 2005; Jensen, 2000). In addition to this, concerns about the safety and sustainability of many insecticides are currently leading to a ban in an increasing number of cases (European Union, 2018). Therefore, alternative methods to control thrips are needed.

An alternative method for thrips control is Integrated pest management (IPM), which includes the use of natural enemies. Predators patrolling foliage are more effective in reducing the number of thrips than soil-dwelling predators because these latter prey only on pupal stages (Manners et al., 2013). Nevertheless, this control method does not reduce the number of thrips in chrysanthemum cultivation sufficiently, thus growers still mainly rely on insecticides. Biological control agents combined with thrips-resistant chrysanthemum cultivars, which show a reduced survival and/or development of thrips, may suppress pest populations in greenhouses significantly and could reduce or completely prevent the use of insecticides.

Thrips resistance in *Chrysanthemum* has been investigated but its occurrence within commercial varieties is sparse and targeted breeding is still not possible because of the lack of genetic information on thrips-resistance in this crop. The first step in efficiently breeding for resistance is identifying reliable sources of resistance. In previous research, variation in resistance to thrips was found in cultivated material (De Jager et al., 1995a). The resistance was not associated with leaf hairiness or toughness, but with certain specialized metabolites, specifically chlorogenic acid and feruloyl quinic acid (De Jager, Butôt, et al., 1995; Leiss et al., 2009). Thrips resistance in chrysanthemum was shown to be influenced by many factors, for example, it varied depending on the season (De Kogel et al., 1997a). It was also affected by the ontogenetic stage of the plant. Non-flowering plants were found to be more resistant than flowering plants, and resistant non-flowering plants were not always resistant once flowering (Van Dijken, 1992). Previous studies mainly relied on assessing resistance by observing and quantifying plant damage caused by thrips. However, for the reduction of direct damage and virus transmission, reproduction and population build-up of thrips are more important variables. The objective of this study was to screen a panel of 70 accessions for resistance against *F. occidentalis*, using thrips life cycle parameters. Therefore, evaluations were carried out using four types of assays: 1) whole plant assays for population build-up, 2) detached leaf assays for larval development, 3) leaf disc assays for larval survival and development, and 4) leaf disc assays for reproduction.

Materials and Methods

Plant material

Seventy accessions of *C. x morifolium* were provided by seven breeding companies (Table S1). The accessions were vegetatively propagated without applying insecticides: the growing tips were cut and rooted twice over a six-week period by the companies to reduce the putative effect of insecticides that were routinely used in maintaining the motherplants. After transportation to Wageningen, the cuttings were transplanted in 11 cm square pots with standard potting soil (Lentse potgrond, Lent, The Netherlands). After the first round of evaluations, we selected 13 accessions to be tested together in three validation experiments. To reduce possible effects of company-specific non-genetic factors, after transportation, the growing tips of the cuttings were cut and rooted for two weeks, and then transplanted in 11 cm pots.

All plants were maintained at 25/21°C, 16:8 h light:dark period, 70% RH in a glasshouse at Wageningen University and Research, Wageningen, the Netherlands, without the application of insecticides.

Insect population

The *F. occidentalis* population was maintained in a mass-rearing on yellow-flowering pot chrysanthemums at ~25°C, 16:8 h light:dark period with LED lighting. Twice a week, two new plants with open flowers were added and the two oldest ones were removed from the rearing, maintaining the number of plants at about 16 plants. Female adult thrips were collected directly from the mass-rearing with an aspirator. Synchronized thrips larvae (L1) were obtained by letting females oviposit for 24 h on snack cucumbers placed on paper in glass jars. After removal of the females, the snack cucumbers were incubated in a climate cabinet (25°C, 16-8 h day-night and 70% RH) for four days, after which newly hatched larvae were collected with a fine brush (Mollema et al., 1993).

Phenotyping methods

We screened the 70 *Chrysanthemum* accessions with different types of assays (Table 1). First, the 70 accessions were evaluated in sets of 10-20 genotypes in five whole plant assays and five detached-leaf assays. Based on the results of the first set, two cultivars were selected as references and included in the following assays: a moderately resistant one (Penny Lane) and a susceptible one (Super Pink Pompon). These cultivars respectively exhibited low and high thrips population build-up.

Second, we selected two of the most susceptible accessions and 11 of the most resistant ones and we compared them in three validation experiments, in which we evaluated population build-up, larval development and reproduction rate.

Whole plant assay

In the whole plant assays, we estimated the thrips population build-up on non-flowering plants over three weeks. We randomized five three-week-old plants per accession in a completely randomized design in a greenhouse compartment. Each plant was covered by a thrips-proof sleeve and a water seal was used to assure the isolation of the pot and containment of the thrips. Thrips were collected from the mass-rearing and sedated with CO^2. Fifteen female thrips were placed in a glass vial and then transferred onto each plant. The climate conditions were set at 25/21°C, 16:8 h light:dark period, 70% RH. Three weeks after infestation, sleeves were removed and each plant was shaken so that thrips fell onto a white paper sheet, and the number of thrips larvae and adults were counted. A three-week period was chosen based on the life cycle of the thrips: at the set temperature, it takes two weeks from egg to adult, so in three weeks at least one life-cycle is expected to be completed (Li et al., 2015). The pupal stages and their numbers were not assessed because pupation occurs in the flowers (not present) or in the soil (not examined).

Table 1. Overview of experiments, phenotyping methods, number of accessions screened, measured variables and time of experiments.

Experiment	Phenotyping method	Accessions	Measured parameter	Time of experiment
Initial screening	Whole plant assay	70	Population build-up	Dec 2019 – Aug 2020
Initial screening	Detached leaf assay	65	Fraction of larvae from L1 to L2	Dec 2019 – Aug 2020
Validation experiment	Whole plant assay	13	Population build-up	Oct 2020
Validation experiment	Leaf disc assay	11	Larval survival, larval development	Apr – May 2021
Validation experiment	Reproduction assay	13	Reproduction rate	Apr – May 2021

Detached leaf assay

We observed the fraction of L1 larvae developing into L2 larvae over four days in a detached leaf assays based on (Maharijaya et al., 2012; Van Haperen et al., 2019). We poured ~1 ml of 1.5% water agar on the side of Falcon Tight-Fit Lid Petri dishes. A 1 cm diameter hole was laser cut in the lids of these Petri dishes and covered with

70-micron fine mesh to allow ventilation but prevent thrips escape. From three five-week-old plants per accession, we collected two young leaves and two old leaves, respectively the 1st and 2nd, and the 9th and 10th fully expanded leaves from the top of the plant. Each leaf was placed with its petiole in the agar and was cut to a size that would fit in the Petri dish. Five newly hatched L1 larvae (less than 2 h old) were placed on each detached leaf and the Petri dishes were incubated in a climate cabinet at 25 °C, 16:8 h light:dark period, and 70% RH. After four days, we observed the larvae with a stereomicroscope and determined the number of alive and dead larvae, as well as distinguishing between L1 and L2 larvae.

Leaf disc assay

For the larval survival and development experiment, we collected the 1st and 2nd leaves from three five-week-old cuttings, punched 1.5 cm diameter leaf discs and placed these discs with the abaxial side upward on 1.5% water agar while still soft. There was an average of 17 replicates per accession, depending on how much leaf material was available (Table S2). We placed one newly hatched L1 larva per leaf disc and observed its survival and/or development to a later stage daily during nine days. The Petri dishes were incubated in a climate cabinet at 25 °C, 16:8 h light:dark period, and 70% RH. The larvae were transferred onto fresh leaf discs when the leaf discs showed signs of desiccation. We stopped the observations when the thrips was found dead, reached the adult stage, or was not found for two consecutive days.

Reproduction assay

For the reproduction assay, the first two fully expanded leaves of five-week-old plants were collected. Leaf discs of 1.5 cm diameter were punched and placed in Petri dishes on 1.5% water agar. Three female *F. occidentalis* were placed on a single leaf disc for 48 h in a climate cabinet at 25 °C, 16:8 h light:dark period. There were six replicate discs for each accession; for three leaf discs we counted the number of hatched larvae, for the other three leaf discs we counted the number of eggs. Eggs were counted using a light microscope after staining with acid fuchsin immediately after the females were removed, giving the 0.2 mm kidney-shaped eggs a red appearance (Backus et al., 1988; Martin and Workman, 2006; Simonet and Pienkowski, 1977). The other Petri dishes were incubated in a climate cabinet at 25 °C, 16:8 h light:dark period, and 70% RH for five days. We counted the hatched larvae with a stereo-microscope at seven days post infestation (dpi), because eggs need on average four days to hatch at 25 °C (McDonald et al., 1998).

Statistical analysis

Thrips population build-up, whole plant assays

To estimate thrips population build-up, the total number of thrips per plant was calculated by adding up the number of larvae and the number of adults. The total number was transformed to y=sqrt(x) to stabilise variances. Differences among accessions were analysed with ANOVA, and Tukey's HSD or Dunnett's post-hoc test when cv. Penny Lane was available as reference in the experiment. In the validation whole plant assay, differences among accessions were analysed with ANOVA and Tukey's HSD post hoc procedure.

Larval development, detached leaf assays

For each Petri dish, we assessed larval survival, calculated as (number of living larvae)/(total number of larvae), and larval development, calculated as (number of L2)/(number of L1+L2). Larval survival and larval development data were transformed to y=arcsin(sqrt(x)) and analysed with ANOVA, and Dunnett's post-hoc test was used for comparing all accession means with cultivar Penny Lane. The detached leaf assay results were compared to the whole plant assay results with Pearson's correlations.

Larval survival and development, leaf disc assay

Daily observations of thrips survival on 12 accessions in the validation experiment were analysed with Kaplan-Meier survival analysis (Kaplan and Meier, 1958). The time from the start of the experiment (infestation, day 0) to the event (death) or censoring (lost larva) was calculated in days. The event occurrence was recorded as 1 and the censoring with 0. The data were analysed using the "survival" package in R, specifically the functions "survfit()" and "survdiff()" to test for differences in survival over time per accession (Chi-square test) (R Core Team, 2022; Therneau, 2022). Additionally, we used the "survminer" package and its functions "ggsurvplot()" and "pairwise_survdiff()" to plot the survival curves and calculate the pairwise comparisons between accessions using the Log-Rank test with Benjamini and Hochberg (BH) corrections for multiple testing (Kassambara et al., 2021). The duration of the L1 stage in days was calculated per thrips individual. Differences among accessions were tested using the Kruskal-Wallis Rank Sum test and pairwise comparisons were computed with Mann-Whitney U test with Benjamini-Hochberg p-value adjustment method.

Reproduction analysis

The reproduction rate, which was based on emerged larvae or on eggs, was calculated as (number of offspring)/(number of living females + number of dead females/2). The effects of accession and of the counted parameter (larvae or eggs) were analysed with a two-way ANOVA. Correlation among the reproduction rates based on the two variables was analysed with Spearman's rank correlation test.

Results

Initial screening of 70 accessions for population build-up

The thrips population build-up on 70 accessions was tested in five whole plant assays (Table S1), where plants were infested with 15 females. After three weeks, the average number of thrips per plant varied from 2.2 ± 0.4 on PAR001 to 69.3 ± 5.2 on Super Pink Pompon. In the first assay, the population build-up differed among accessions (ANOVA; $p < 0.01$), with Penny Lane and Fenix showing significantly lower population development than Super Pink Pompon and Vibe (Tukey's HSD; $p < 0.05$). In subsequent experiments we included Super Pink Pompon and Penny Lane as, respectively, susceptible and resistant reference cultivars. In all but the third whole plant assay we found significant differences among accessions for thrips population development (ANOVA; $p < 0.05$). We compared thrips number on Penny Lane with that on other accessions to identify material that was as resistant as Penny Lane or more resistant. Only MG163 showed a significantly lower population development (Dunnett's test; $p < 0.05$). Ten accessions that showed a thrips population build-up similar to Penny Lane were selected to be tested in the validation experiments.

Initial screening of 65 accessions for larval development

Sixty-five accessions that were evaluated for thrips population build-up were also evaluated for larval development in five sets using detached leaf assays. We recorded the number of larvae that developed from L1 to L2 stage after four days. Of the five larvae the assay started with, an average of 2.6 larvae developed to L2 on Penny Lane, while on Super Pink Pompon leaves, an average of 4.0 larvae developed to L2. In the first and fifth detached leaf assay the accession effect on larval development was not significant (ANOVA; $p > 0.05$) (Table S1). In the second, third, and fourth detached leaf assay, we found significant differences among accessions (ANOVA; $p < 0.01$), however, none exhibited significantly lower larval development than Penny Lane.

Correlation between larval development and population build up

Pearson's correlation was calculated to compare the thrips population build-up and the larval development in the initial screenings. The detached leaf assay for larval development and the whole plant assay for population build-up showed a weak but significant positive correlation (R = 0.27, p < 0.05) (Figure 1, S1, S2).

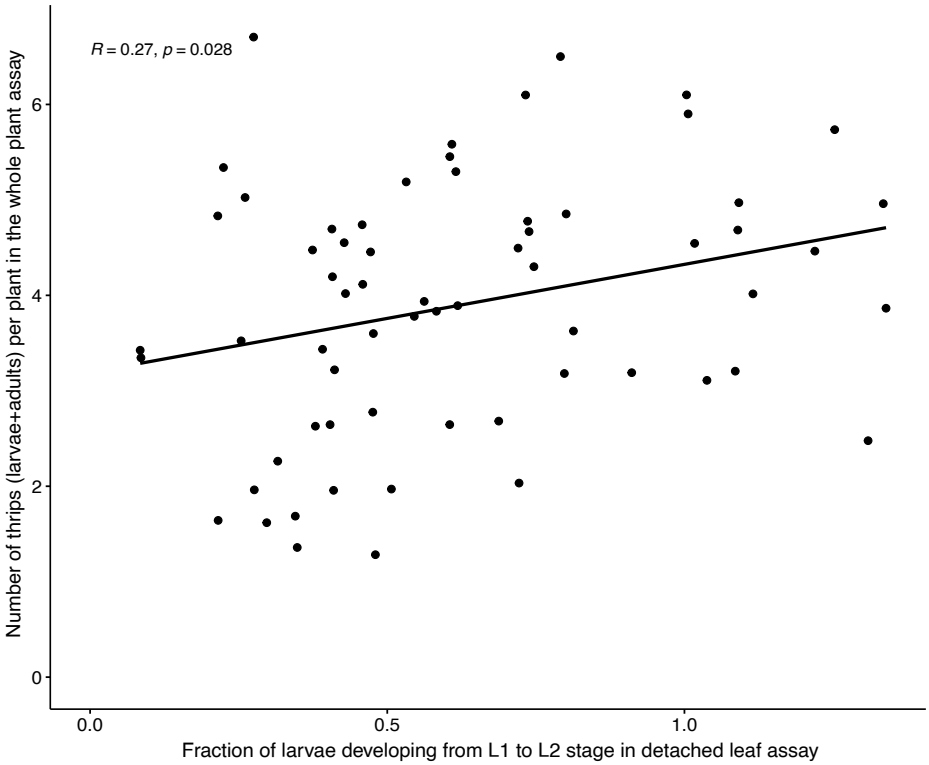

Figure 1. Correlation plot between number of thrips (larvae + adults) per plant in whole plant assay and fraction of larvae developing from L1 to L2 stage in detached leaf assay from the initial screening. Prior to the Pearson's correlation test (R = 0.27, p < 0.05), individuals data points for number of thrips per plant and larval development in the leaf disc assay were respectively transformed as y=sqrt(x) and y=arcsin(sqrt(x)).

Validation of population build-up of 13 accessions

A whole plant assay with 13 of the 70 accessions was carried out to validate the differences in thrips population build-up previously observed. Super Pink Pompon and S5 were included as susceptible accessions. Penny Lane was included as resistant reference. The other ten accessions were selected as candidate resistant material because they had a similar thrips population build-up as Penny Lane in the initial screenings. At 21 dpi we found more than 30 thrips on average per plant in the two pre-selected susceptible accessions, and 12.6 thrips on average on Penny Lane. The

number of thrips found per plant at 21 dpi significantly differed among accessions (ANOVA; $p < 0.0001$) (Table 2). Four of the accessions supported a high population build-up, of these two were pre-selected as susceptible (Super Pink Pompon and S5) and two as resistant (Grassly and T3). A reduced population build-up was confirmed for nine of the accessions selected as resistant.

Table 2. Thrips population build-up per plant assessed in the validation experiment on 13 accessions. The average number of larvae, adults and their sum per plant is listed per accession. The number of plants (N) tested in the whole plant assay is also included. Means of total number of thrips per plant per accession sharing the same letter (Tukey's HSD) were not significantly different ($p > 0.05$).

Accession	Pre-selected as resistant (R) or susceptible (S)	Average number of larvae/ plant	Average number of adults/ plant	Average total number of thrips/ plant	N	Validation experiment result	Tukey's HSD
T4	R	0	1	1	1	R	bc
T2	R	1.8	3.5	5.3	4	R	c
PAR001	R	0.4	5.2	5.6	5	R	c
PAR007	R	2.8	2.8	5.6	5	R	c
18.5847.000	R	1.6	4.6	6.2	5	R	c
T6	R	2.4	4.6	7	5	R	c
Mona Lisa	R	2	9.2	11.2	5	R	bc
Penny Lane	R	6.4	6.2	12.6	5	R	bc
09.1021.000	R	5	8.8	13.8	5	R	bc
Grassly	R	7.3	13.3	20.5	4	S	abc
T3	R	11.3	11.3	22.5	4	S	abc
S5	S	12.7	16	28.7	3	S	ab
Super Pink Pompon	S	18.6	16.2	34.8	5	S	a

Leaf disc assay for reproduction of thrips

We assessed the reproduction rate of female WFT on 13 accessions in a leaf disc assay (Figure 2). The reproduction rate of females was assessed with two methods, by counting the eggs at day 2 and by counting the number of hatched larvae at day 7. Overall, the reproduction rate did not significantly differ among accessions and the effect of the stage counted (eggs or larvae) was not significant (ANOVA; accession: $p > 0.05$; stage counted: $p > 0.05$). No correlation was found between the reproduction rate assessed by counting eggs and by counting larvae ($r = -0.14$, $p > 0.05$) (Figure S3). Moreover, reproduction rate counting either eggs or larvae

did not correlate with the population build-up (reproduction rate (larvae) – total number of thrips per plant: r = 0.096, p > 0.05; reproduction rate (eggs) – total number of thrips per plant: r = 0.0068, p > 0.05) (Figure S4, S5).

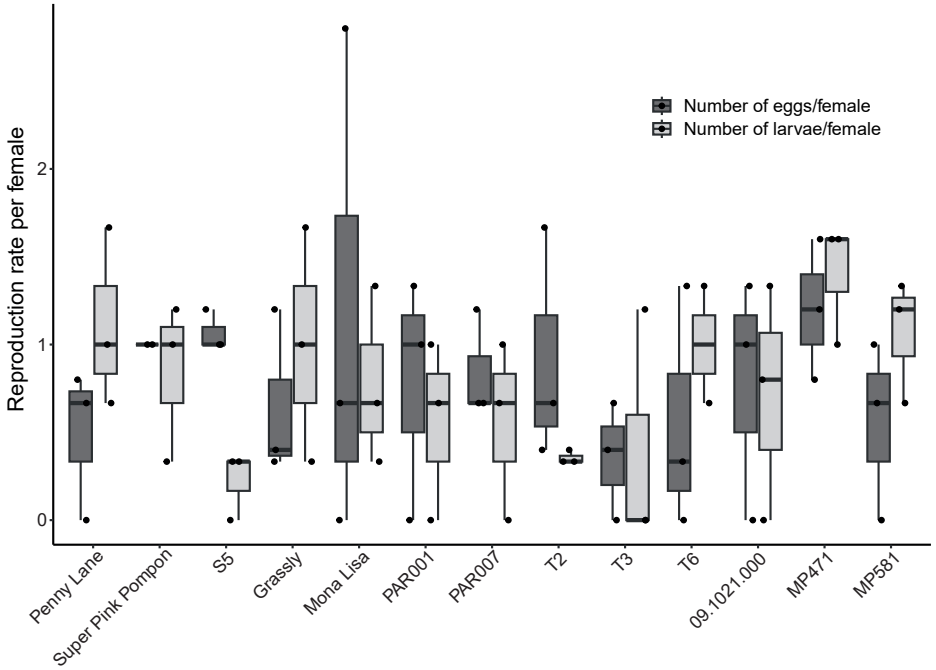

Figure 2. Reproduction rate of *Frankliniella occidentalis* on leaf discs of *Chrysanthemum* accessions. Three female thrips were allowed to oviposit on discs of young chrysanthemum leaves. There were six replicate discs for each accession. Of these, in three discs the eggs were stained with acid fuchsin on day 2 and counted, and in the other three the hatched larvae were counted on day 7. The reproduction rate was calculated as (number of offspring)/(number of living females + number of dead females/2). The individual observations are plotted as black dots. Boxplots show the distribution of the observations: the box represents the interquartile range, the horizontal line inside the box represents the median, and the whiskers indicate the range of data. Differences between accessions, counting either eggs or larvae, were not significant (ANOVA; accession: p > 0.05; counting-method: p > 0.05).

Larval development and survival in the validation experiment

We followed the development of thrips larvae until the adult stage or death, on leaf discs of 11 *Chrysanthemum* accessions. In the same experiment, we also analysed the duration of the different life cycle stages. The majority of the larvae developed from L1 to L2 stage one or two days after infestation. The duration of the L1 stage differed significantly between accessions (Kruskal-Wallis Rank Sum test; Chi-square = 22.04, df = 10, p < 0.05). On accession 18.5847.000, the L1 stage was significantly longer than on 09.1021.000, Grassly, Super Pink Pompon, T2, and T6 (Mann-Whitney U test with BH correction; p < 0.05) (Figure 3, Table S2). Most of the larvae were

either L2 or dead after the first four days. Thrips survival curves significantly differed between accessions (Kaplan-Meier test; Chi-square = 58.2, df = 10, $p < 0.001$). From day 2, larvae on Super Pink Pompon, the most susceptible accession, showed significantly higher survival than larvae on all of the other accessions (Log-Rank test for pairwise comparison with BH correction; $p < 0.001$). On accession PAR007 the chance of survival was lowest, and significantly lower than on T2, T6, PAR001, Mona Lisa and Super Pink Pompon (Log-Rank test for pairwise comparison with BH correction; $p < 0.05$) (Figure 4). The survival of larvae began to decrease from day 3 and by day 9, all larvae had died except those on Super Pink Pompon (8 emerged adults), T6 (2 adults), and Mona Lisa (one adult).

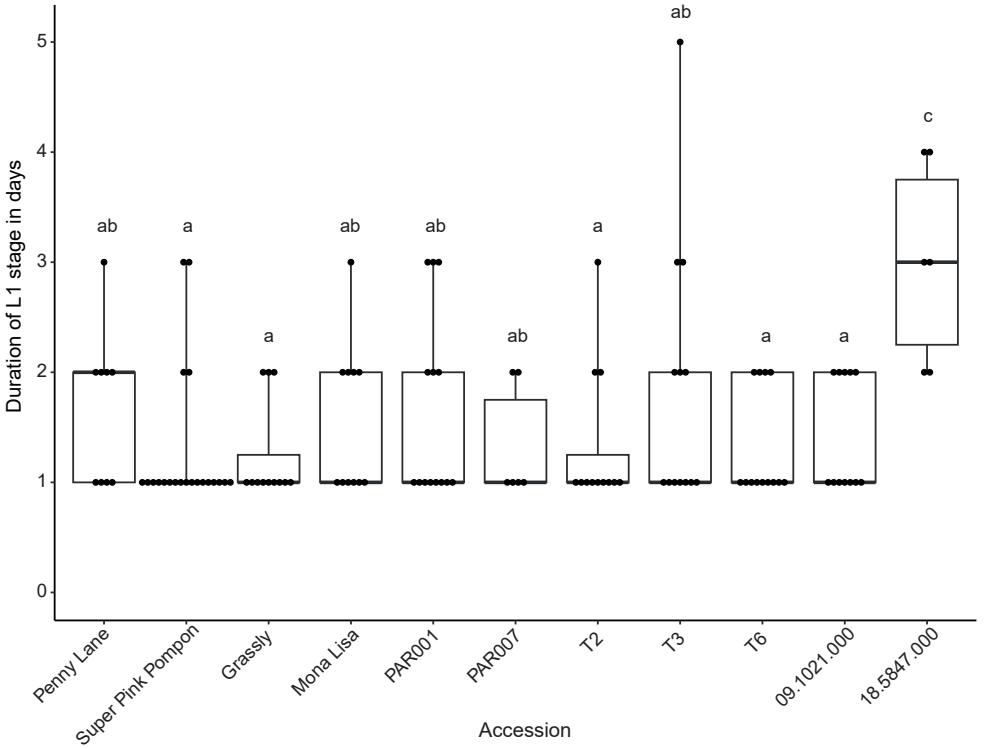

Figure 3. Duration of L1 stage of thrips *Frankliniella occidentalis* on 11 *Chrysanthemum* accessions. The graph shows the duration of the L1 stage in days (Y axis) per accession (X axis) for surviving larvae. The experiment started with on average 17 newly emerged L1 larvae per accession, one larvae per leaf disc. Observations started on day 1. Black dots indicate individual observations of the duration of the L1 stage of individual thrips that survived this stage, i.e. the larvae that developed into L2; data of larvae that died during this L1 stage were not included in this plot. Boxplots show the distribution of the observations: the box represents the interquartile range, the horizonal line inside the box represents the median, and the whiskers indicate the range of data. The duration of the L1 stage differed significantly between accessions (Kruskal-Wallis Rank Sum test; $p < 0.05$); means of duration of L1 stage per accession sharing the same letter were not significantly different (Mann-Whitney U test with BH correction; $p < 0.05$).

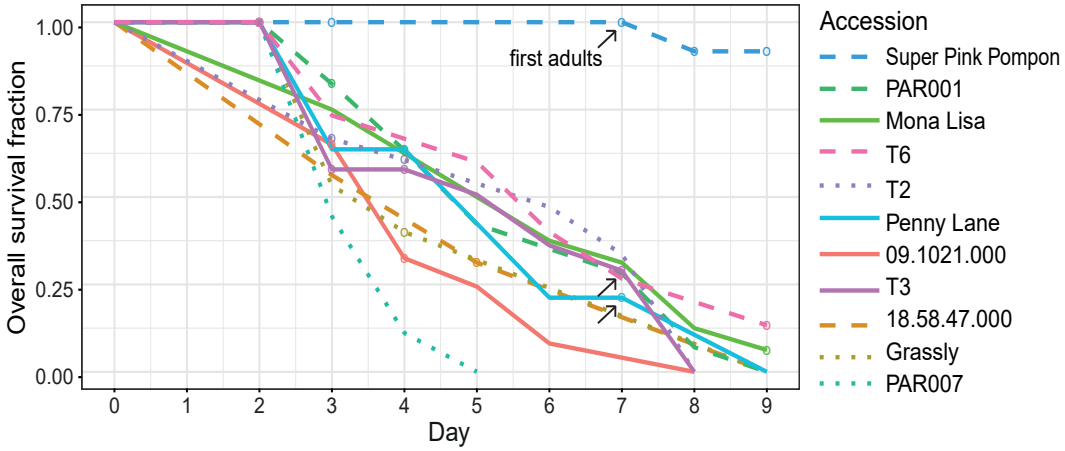

Figure 4. Kaplan-Meier survival curves of *Frankliniella occidentalis* on 11 *Chrysanthemum* accessions. The fraction of thrips surviving is plotted over time. The average number of replicates per accession was 17. Censored events, i.e. lost individuals, are indicated by "o". The survival curves differed significantly (Chi square= 58.2, 10 d.f., $p < 0.001$). The time on which the first adults emerged on Super Pink Pompon, T6, and Mona Lisa are indicated with arrows. On other accessions, no adults emerged.

Discussion

Variation in resistance to thrips *F. occidentalis* among accessions of *C. x morifolium*

Our study focused on evaluating thrips population build-up and life table variables that contribute to it, i.e. larval development and survival, reproduction rate of female thrips. We screened 70 accessions of chrysanthemum for resistance against *F. occidentalis* and found variation for both population build-up and larval development. In our screening, cultivars Penny Lane and Super Pink Pompon were included as respectively the resistant and susceptible reference cultivars. Super Pink Pompon consistently supported high population build-up, larval development and survival (Table S1, Table 2), confirming previous found results by Leiss et al. (2009). Penny Lane had a consistently low population development, and supported no survival of larvae past the first L1 stage. This cultivar was already considered resistant to thrips and the leafminer *Liriomyza trifolii* (De Jong and van de Vrie, 1987; Leiss et al., 2009b; Van Dijken et al., 1994). In this study, we also identified new sources of resistance against thrips: from the first screenings, we selected two susceptible accessions and 11 putatively resistant ones, whose thrips-resistance

levels were further investigated in subsequent experiments. In a whole plant assay, we confirmed the reduced population build-up of 9 accessions, identifying eight accessions as novel sources of resistance to thrips.

The variation in population build-up among accessions that we recorded in the whole plant assays did not correlate with the reproduction rate we assessed in the leaf disc assay, neither for eggs nor for larvae. Moreover, we did not find a correlation between reproduction rates based on number of eggs and number of larvae per female. Since we did not find lower number of larvae than eggs, a possible egg-mortality effect cannot be deduced from this experiment. Instead, the absence of correlation between the two could indicate that the number of larvae is not a good indicator for oviposition in chrysanthemum, whereas it is in cucumber (De Kogel et al., 1997b). However, we cannot draw a strong conclusion from our reproduction experiment. In fact, the discrepancy between the whole plant assay and the reproduction assay may be due to the different conditions that affected the thrips in the two assays. The reproduction rate was assessed on detached leaves, but the females used in this assay were reared under optimal conditions on flowering chrysanthemum plants, and their reproduction in the next few days may have been influenced more by this rearing than by the plants on which they oviposited (Murai and Loomans, 2001; Trichilo and Leigh, 1988). On the other hand, in the whole plant assay the females were feeding on non-flowering plants. Therefore, the accession and generally the feed quality might have a stronger effect in the whole plant assay than in the detached leaf assay. Nevertheless, previous studies showed that thrips reproduction was affected on resistant chrysanthemum accessions, also in detached leaf assays performed with females reared on the same host (De Kogel et al., 1998; Ohta, 2002). In any case, it seems likely that the thrips population build-up may be more influenced by factors impacting other stages of the life cycle than reproduction rate of the adults, such as survival and development of the larvae.

Thrips development from L1 to L2 stage is affected by *C. x morifolium* host plant resistance

A significant finding of this study was the impact of chrysanthemum host plant resistance on the development of thrips from the first to the second larval stage (L1 to L2), and on their survival. On susceptible accessions, the majority of the larvae developed into L2 during the first two days after hatching. On resistant accessions thrips exhibited delayed transition from L1 to L2, alongside reduced larval survival: a significant proportion of larvae died during the L1 stage on all resistant accessions (Figure 3, 4). Daily observations indicated that assessing larval development after four days is optimal, as during this period larvae either develop into L2 or die, with

clear differences between accessions. Our observations on larval development and survival are in line with previous research on resistant *Capsicum* spp. and other resistant chrysanthemum accessions, on which the larval development was indeed hampered (De Jager et al., 1995b; Maharijaya et al., 2012; Ohta, 2002; Van Haperen et al., 2019). In this study, a weak but significant positive correlation was found between population build-up and larval development. In a thrips resistance study in pepper, higher positive correlation was found between resistance level, evaluated as plant damage, in whole plant assays, detached leaf assays, and leaf disc assays (Maharijaya et al., 2011). The weakness of the correlation we found suggests that arresting of the larval development is indeed one of the factors affecting the population build-up on whole plants, but not the only one.

In this experiment, we observed high larval mortality, and this could lead to an overestimation of the resistance of chrysanthemum plants. We observed larvae developing into pupal stages and adults emerging only on two accessions, Super Pink Pompon and Grassly. This is in contrast with our expectation of an overall higher adult emergence rate in the susceptible accessions, especially on other accessions such as T3 that showed a similar population build-up in the validation whole plant assay. The phenotyping method used could have affected thrips development. In fact, leaf discs may be susceptible to deterioration and desiccation, thus for long experiments like this study it was necessary to transfer the larvae onto fresh leaf discs. However, leaf discs still have a different physiology than whole plants, and insects respond differently to conditions in leaf discs, whole leaves and whole plants (Risch, 1985). A high larval mortality was also observed in previous studies in which thrips larval development and survival were assessed using either detached leaf assays or clip-cages on intact plants (De Jager et al., 1995b; Ohta, 2002). However, leaf disc assays allow for a reduction in the number of factors affecting the outcome of an experiment and a close observation of individual thrips. In contrast, whole plant assays, which mimic actual chrysanthemum growing conditions, often require further investigations to pinpoint which life stage of thrips is affected. Although larval mortality was high also on the susceptible accessions, the difference between the resistant and the susceptible accession was evident. Importantly, larval mortality on the susceptible plants was consistently lower than on the resistant ones.

Penny Lane and Super Pink Pompon as reference cultivars and the effect of season

As previously discussed, in our assays Penny Lane and Super Pink Pompon consistently showed thrips resistance and susceptibility respectively. Despite this, we noticed that the thrips population build-up varied per batch. In fact, the average number of thrips per plant in batch 4 and 5 of the initial screening (summer 2020) was at

least double the number of thrips found in batch 1 (winter 2019) (Table S1). We hypothesize that this is due to seasonal effects. Over the course of our experiments, plants of both resistant and susceptible accessions allowed a higher population development in summer than in winter. However, this contradicts previous findings that found higher thrips resistance in summer (i.e. lower number of larvae, lower survival of adults) attributed to increased light intensity (De Kogel et al., 1997a). It is difficult to determine why in winter the plants allowed a lower population development, because of the many factors that influence both the experiments and the cuttings' production stage such as lower outside temperature, effect of the lighting environment and photoperiod (Moosavi-Nezhad et al., 2022). Moreover, also the *F. occidentalis* population was affected by the season. In fact, we observed a reduction in thrips number in the mass-rearing in winter despite the presence of abundant flowers on the host plants and stable climate conditions. Further research is needed to determine the cause of this seasonal variation. Altogether, this may also lead to the conclusion that when screening in winter, thrips resistance may appear stronger, but is in fact overestimated. Therefore, in this study we performed a validation whole plant assay in October 2020, to compare accessions evaluated in different batches, filter out the seasonal effect, and select resistant accessions.

In conclusion, we developed phenotyping methods for thrips resistance in chrysanthemum, focusing on population build-up and life table variables. Using these methods, we found variation in thrips resistance among *C. x morifolium* accessions, confirmed the resistance level of cultivar Penny Lane and identified novel resistance sources. Moreover, we show the importance of performing multiple and different type of assays across different seasons when screening for thrips resistance to distinguish between several experimental factors as well as resistance factors possibly involved. The identified resistant accessions may be used to study the genetics of thrips resistance in cultivated hexaploid chrysanthemum and to introgress the trait in breeding programs.

Acknowledgements

We thank Sean Geurts and Jorik Smits for taking care of the cuttings in the greenhouse. We thank the involved breeding companies for providing the plant material for the experiments. This research was financially supported by a grant (TU-18043) from the Ministry of Economic Affairs of the Netherlands and the companies Dekker Breeding, Deliflor Chrysanten, Floritec Breeding, Gediflora, Inochio Holdings, Syngenta Seeds and Royal van Zanten.

Supplementary materials

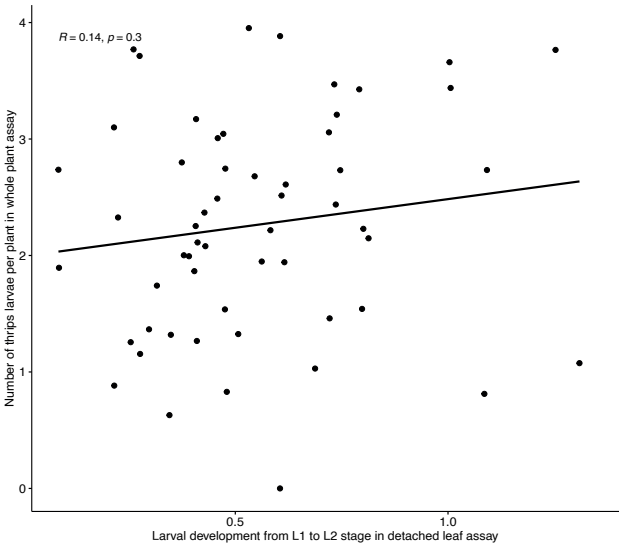

Figure S1. Scatter plot of number of larvae per plant and larval development (L1 to L2). Individual data points for number of thrips per plant and larval development per leaf disc were respectively transformed as y=sqrt(x) and y=arcsin(sqrt(x)), Pearson correlation test; R = 0.14, p > 0.05.

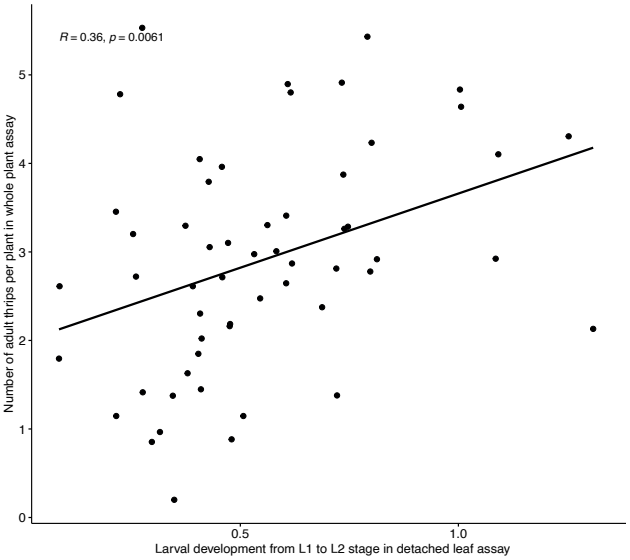

Figure S2. Scatter plot of number of adult thrips per plant and larval development from L1 to L2. Individual data points for number of thrips per plant and larval development per leaf disc were respectively transformed as y=sqrt(x) and y=arcsin(sqrt(x)), Pearson's correlation test; R = 0.36, p < 0.01.

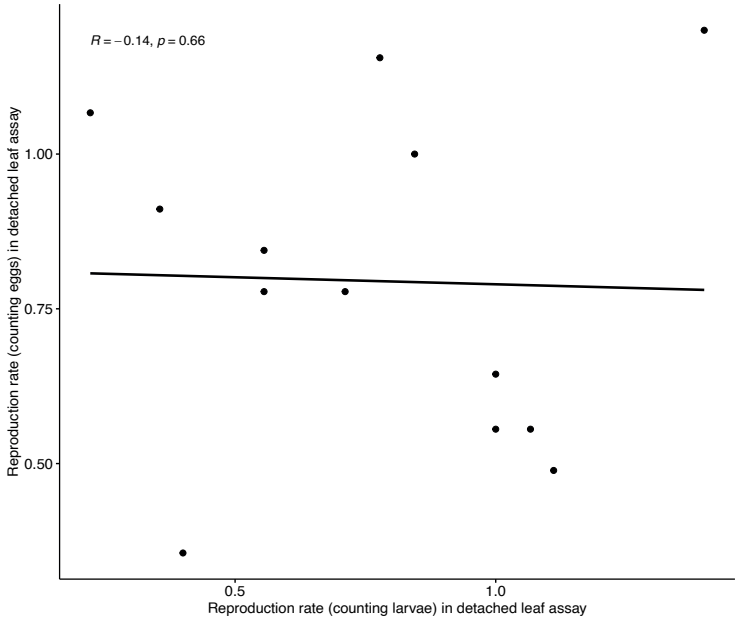

Figure S3. Scatter plot of reproduction rate based on number of eggs and number of larvae (Spearman's correlation test; rho =-0.14, p > 0.05).

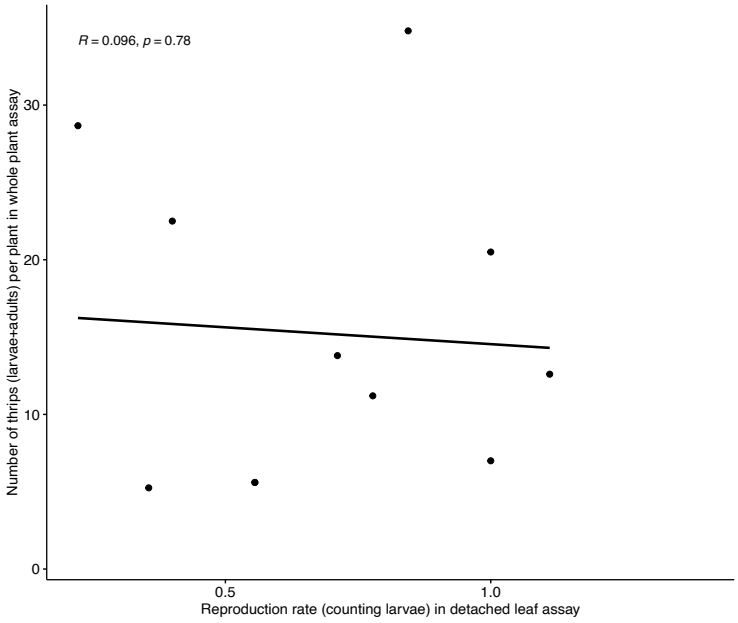

Figure S4. Scatter plot of total number of thrips per plant and reproduction rate based on number of hatched larvae (Spearman's correlation test; rho = 0.096, p > 0.05).

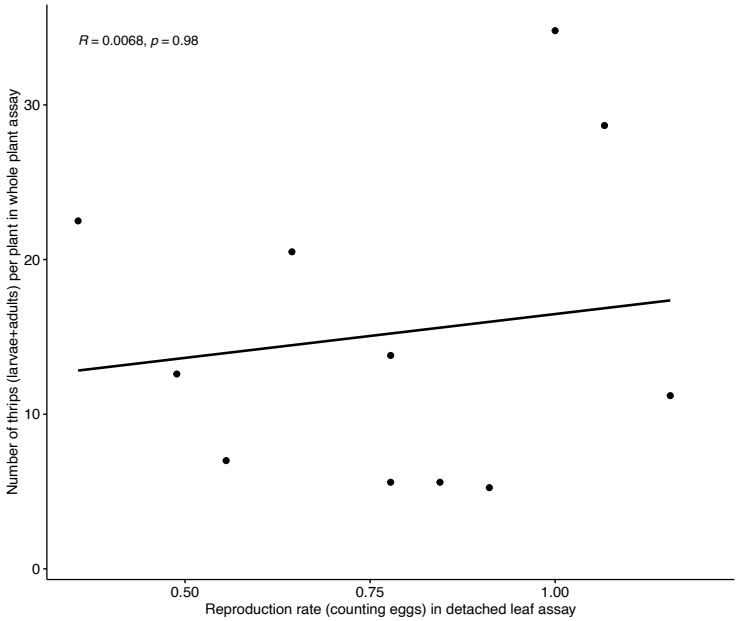

Figure S5. Scatter plot of total number of thrips per plant and reproduction rate based on number of eggs (R = 0.0068, p > 0.05).

Table S1. Results for 70 accessions tested in the initial screening experiments.

				Initial screening, whole plant assays				Initial screening, detached leaf assays			
Batch (*)	Accession name	Company (**)	Type (***)	Number of thrips larvae	Number of adult thrips	Total number of thrips ± SE	N (Plants)	Fraction of L2 - young leaves (4dpi) ± SE	N (L2 - young leaves)	Fraction of L2 - old leaves (4dpi) ± SE	N (L2 - old leaves)
1	DB 60408	A	a			20.0 ± 1.5	4	0.75 ± 0.12	5	0.83 ± 0.13	5
1	Cologne	A	a			17.0 ± 4.3	4	0.64 ± 0.17	6	0.78 ± 0.13	6
1	Baltica	A	a			15.8 ± 3.8	4	0.77 ± 0.11	6	0.96 ± 0.04	6
1	Penny Lane	A	a			9.0 ± 1.6	4	0.63 ± 0.13	5	0.88 ± 0.09	6
1	Super Pink Pompon	A	a			26.0 ± 6.7	4	0.84 ± 0.07	6	0.93 ± 0.07	6
1	Vibe	A	a			25.0 ± 3.6	4	0.78 ± 0.11	6	0.96 ± 0.04	5
1	Radost	A	a			22.5 ± 3.8	4	0.88 ± 0.06	6	0.53 ± 0.10	6
1	DB 60580	A	a			9.8 ± 1.0	4	0.71 ± 0.07	6	0.65 ± 0.13	6
1	DB 60330	A	a			10.3 ± 1.0	4	0.50 ± 0.12	6	0.66 ± 0.16	6

Table S1. continued.

Batch (*)	Accession name	Company (**)	Type (***)	Initial screening, whole plant assays				Initial screening, detached leaf assays			
				Number of thrips larvae	Number of adult thrips	Total number of thrips ± SE	N (Plants)	Fraction of L2 - young leaves (4dpi) ± SE	N (L2 - young leaves)	Fraction of L2 - old leaves (4dpi) ± SE	N (L2 - old leaves)
1	DB 24715	A	a			20.8 ± 1.7	4	0.79 ± 0.14	4	0.52 ± 0.16	5
2	S5	B	a	28.6	17.2	45.8 ± 1.8	5				
2	T5	B	a	2.8	8.2	11.0 ± 0.7	5				
2	T8	B	a	6.8	25.0	31.8 ± 1.9	5	0.30 ± 0.10	5	0.43 ± 0.13	4
2	T1	B	a	6.4	14.8	21.2 ± 1.6	5	0.18 ± 0.05	5	0.26 ± 0.12	4
2	S1	B	a	13.0	25.6	38.6 ± 2.8	5	0.45 ± 0.12	4		
2	S4	B	c	2.4	7.8	10.2 ± 0.6	5	0.51 ± 0.07	5		
2	T3	B	c	3.5	7.3	8.6 ± 3.1	5				
2	T4	B	c	1.4	6.0	7.4 ± 1.0	5	0.30 ± 0.14	5	0.56 ± 0.15	5
2	T6	B	b	4.4	6.6	11.0 ± 1.2	5	0.00 ± 0.00			
2	T2	B	b	3.2	5.4	8.6 ± 2.2	5	0.25 ± 0.12	5	0.33 ± 0.16	4
2	Dark Pink Pompon	C	a	8.6	21.0	29.6 ± 1.9	5				
2	Euro Speedy	C	a	6.6	18.0	24.6 ± 4.2	5	0.24 ± 0.12	5	0.24 ± 0.12	5
2	Euro Sunny	C	a	4.8	23.8	28.6 ± 1.6	5	0.32 ± 0.06	5	0.36 ± 0.07	5
2	Grassly	C	a	4.8	11.6	16.4 ± 1.9	5	0.20 ± 0.08	5	0.47 ± 0.18	5
2	Mona Lisa	C	a	7.4	17.0	24.4 ± 3.2	5				
2	Mona Lisa Sunny	C	a	6.4	10.4	16.8 ± 1.3	5	0.05 ± 0.05	4	0.45 ± 0.16	5
2	Morreno Pink	C	a	14.0	32.0	46.0 ± 2.4	5	0.16 ± 0.07	5	0.12 ± 0.07	5
2	Penny Lane	C	a	2.2	10.8	13.0 ± 1.6	5	0.13 ± 0.08	5	0.13 ± 0.08	4
2	Tiger	C	a	5.8	23.4	29.2 ± 1.8	5	0.00 ± 0.00	4	0.33 ± 0.33	3
2	Vesuvio Green	C	a	5.6	18.4	24.0 ± 1.4	5	0.58 ± 0.08	2	0.47 ± 0.12	3
2	Penny Lane	A	a	3.0	6.2	9.2 ± 1.6	5	0.39 ± 0.14	5	0.56 ± 0.10	5
2	Super Pink Pompon	A	a	5.8	31.2	37.0 ± 1.3	5	0.80 ± 0.20	2	0.90 ± 0.10	4
3	070-2186	D	a	10.5	11.8	22.3 ± 3.7	4	0.11 ± 0.11	6	0.77 ± 0.11	6

Table S1. continued.

Batch (*)	Accession name	Company (**)	Type (***)	Initial screening, whole plant assays				Initial screening, detached leaf assays			
				Number of thrips larvae	Number of adult thrips	Total number of thrips ± SE	N (Plants)	Fraction of L2 - young leaves (4dpi) ± SE	N (L2 - young leaves)	Fraction of L2 - old leaves (4dpi) ± SE	N (L2 - old leaves)
3	071-5013	D	a	9.4	5.0	14.4 ± 4.9	5	0.10 ± 0.06	6	0.48 ± 0.10	6
3	090-6038	D	a	12.8	9.6	22.4 ± 6.8	5	0.17 ± 0.12	6	0.79 ± 0.08	6
3	110-6064	D	a	5.6	7.0	12.6 ± 3.5	5	0.18 ± 0.06	6	0.23 ± 0.10	6
3	120-0480	D	a	3.6	3.6	7.2 ± 1.2	5	0.39 ± 0.09	6	0.07 ± 0.07	6
3	120-6054	D	a	8.6	4.4	13.0 ± 3.8	5	0.03 ± 0.03	6	0.12 ± 0.08	6
3	120-5062	D	a	8.8	7.4	16.2 ± 6.2	5	0.00 ± 0.00	6	0.68 ± 0.10	5
3	130-5122	D	a	16.6	6.2	22.8 ± 11.0	5	0.22 ± 0.12	6	0.24 ± 0.12	6
3	130-5524	D	a	4.6	7.0	11.6 ± 2.2	5	0.00 ± 0.00	6	0.07 ± 0.07	6
3	140-5020	D	a	8.0	9.8	17.8 ± 4.9	5	0.21 ± 0.07	6	0.37 ± 0.14	6
3	11- Valentino	E	a	9.8	13.6	23.4 ± 6.5	5	0.00 ± 0.00	6	0.47 ± 0.19	6
3	12- Mahon	E	a	22.2	9.0	31.2 ± 13.6	5	0.32 ± 0.17	6	0.40 ± 0.11	6
3	13-	E	a	12.8	13.0	25.8 ± 7.7	5	0.34 ± 0.19	5	0.15 ± 0.10	6
3	14-	E	a	28.2	8.0	36.2 ± 23.9	5	0.00 ± 0.00	6	0.37 ± 0.14	6
3	15-	E	a	10.6	10.0	20.6 ± 3.9	5	0.12 ± 0.08	6	0.36 ± 0.12	6
3	16- Promiss Apple	E	a	11.2	8.0	19.2 ± 7.6	5	0.11 ± 0.07	6	0.47 ± 0.14	6
3	17-	E	a	4.6	7.2	11.8 ± 3.8	5	0.28 ± 0.16	6	0.36 ± 0.14	6
3	18 – Amethyst 09.1021.000	E	a	20.6	13.4	34.0 ± 11.0	5	0.35 ± 0.09	6	0.44 ± 0.11	5
3	19- Hardwell	E	a	7.4	10.8	18.2 ± 7.4	5	0.50 ± 0.22	4	0.41 ± 0.10	6
3	20-	E	a	3.6	3.4	7.0 ± 3.1	5	0.33 ± 0.21	5	0.61 ± 0.16	6
3	Penny Lane	A	a	11.4	6.0	17.4 ± 4.9	5	0.69 ± 0.13	6	0.77 ± 0.08	5
3	Super Pink Pompon	A	a	10.4	3.2	13.6 ± 5.5	5	0.73 ± 0.16	4	0.95 ± 0.05	4
4	PAR001	F	b	1.2	1.0	2.2 ± 0.4	5	0.26 ± 0.06	6	0.29 ± 0.11	6
4	PAR002	F	b	4.4	2.8	7.2 ± 0.3	5	0.30 ± 0.10	6	0.09 ± 0.06	5
4	PAR003	F	b	1.0	1.8	2.8 ± 0.2	5	0.22 ± 0.10	6	0.00 ± 0.00	4

Table S1. continued.

Batch (*)	Accession name	Company (**)	Type (***)	Initial screening, whole plant assays				Initial screening, detached leaf assays			
				Number of thrips larvae	Number of adult thrips	Total number of thrips ± SE	N (Plants)	Fraction of L2 - young leaves (4dpi) ± SE	N (L2 - young leaves)	Fraction of L2 - old leaves (4dpi) ± SE	N (L2 - old leaves)
4	PAR004	F	b	4.6	1.2	5.8 ± 0.4	5	0.19 ± 0.11	6	0.17 ± 0.07	6
4	PAR005	F	b	1.0	2.0	3.0 ± 0.2	5	0.22 ± 0.11	6	0.17 ± 0.07	5
4	PAR006	F	b	2.5	1.0	3.5 ± 0.5	4	0.12 ± 0.07	5	0.20 ± 0.09	5
4	PAR007	F	b	2.4	0.2	2.6 ± 0.4	5	0.14 ± 0.05	6	0.25 ± 0.16	4
4	PAR008	F	b	3.2	2.2	5.4 ± 0.6	5	0.40 ± 0.23	4	0.32 ± 0.18	4
4	PAR009	F	b	2.0	2.0	4.0 ± 0.3	3	0.18 ± 0.11	5	0.10 ± 0.10	2
4	PAR010	F	b	2.3	3.0	5.3 ± 0.7	4	0.19 ± 0.12	4	0.28 ± 0.11	6
4	Penny Lane	A	a	1.8	5.4	7.2 ± 0.2	5	0.20 ± 0.08	6	0.09 ± 0.06	6
4	Super Pink Pompon	A	a	26.0	26.0	52.0 ± 0.9	5	0.55 ± 0.13	4	0.77 ± 0.08	5
5	MP023	G	c	8.0	17.4	25.4 ± 3.1	5	0.73 ± 0.16	6	0.69 ± 0.12	6
5	MP581	G	c	12.6	29.8	42.4 ± 3.0	5	0.56 ± 0.17	4	0.56 ± 0.19	5
5	MG216	G	b	0.0	7.0	7.0 ±-	1	0.42 ± 0.20	5	0.52 ± 0.18	5
5	MG156	G	b	8.0	12.2	20.2 ± 4.1	5	0.85 ± 0.06	5	0.31 ± 0.18	5
5	MG035	G	b	1.8	8.8	10.6 ± 1.4	5	0.80 ± 0.05	5	0.78 ± 0.12	6
5	MP091	G	c	14.8	22.8	37.6 ± 5.4	5	0.83 ± 0.08	6	0.56 ± 0.14	5
5	MG163	G	b	2.2	6.2	8.4 ± 2.5	5	1.00 ± 0.00	6	0.67 ± 0.14	6
5	MP019	G	c	13.8	25.8	39.6 ± 8.0	5	0.80 ± 0.20	4	0.91 ± 0.06	5
5	MG368	G	b	7.0	8.3	15.3 ± 1.3	3	0.47 ± 0.18	6	0.50 ± 0.18	4
5	MP471	G	c	8.8	15.4	24.2 ± 2.4	5	0.22 ± 0.10	5	0.92 ± 0.05	5
5	Penny Lane	A	a	8.8	22.6	31.4 ± 5.7	5				
5	Super Pink Pompon	A	a	37.0	32.3	69.3 ± 5.2	3				

* Experiment, Time of experiments, ANOVA whole plant assay, ANOVA detached leaf assay

Batch 1; Dec 2019; F = 4.1605, num df = 9, denom df = 30, p-value < 0.01; F = 1.6259, num df = 9, denom df = 103, p-value > 0.05

Batch 2; Jun 2020; F = 8.4281, num df = 21, denom df = 88, p-value < 0.0001; F = 4.122, num df = 15.000, denom df = 38.575, p-value < 0.001

Table S1. continued.

Batch 3; Jul 2020; F = 1.0795, num df = 21, denom df = 87, p-value > 0.05; F = 6.0262, num df = 21.000, denom df = 68.797, p-value < 0.0001

Batch 4; Aug 2020; F = 3.7429, num df = 11, denom df = 16.411, p-value < 0.01; F = 3.0637, num df = 11, denom df = 110, p-value < 0.01

Batch 5; Jul 2020; F = 5.3628, num df= 11, denom df = 40, p-value < 0.0001; F = 1.9634, num df = 9, denom df = 108, p-value > 0.05

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** Companies

'A' Deliflor Chrysanten B.V., Korte Kruisweg 163, 2676BS, Maasdijk, NL

'B' Royal van Zanten, Lavendelweg 15, 1435EW, Rijsenhout, NL

'C' Dekker Chrysanten B.V., Julianaweg 6-A, 1711 RP, Hensbroek, NL

'D' Inochio Holdings Inc., 95 Kitashinkiri, Mukokusama-cho, Toyohashi-city, Aichi, JP

'E' Floritec, Van Hemessenkade 7, 2481BG, Woubrugge, NL

'F' Gediflora, Schierveldestraat 14, 8840 Oostnieuwkerke, BE

'G' Syngenta, Cornelis Kuinweg 28A, 1619PE, Andijk, NL

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*** Chrysanthemum type

'a' cut chrysanthemum

'b' garden mum

'c' pot chrysanthemum

Table S2. Duration of L1 stage per accession of larvae that developed into L2 stage in larval survival and development experiment. Number of replicates (count), mean duration of L1 stage in days (mean) and its standard error (SE) per accession.

Accession	count	mean	SE
09.1021.000	20	1.42	0.115
18.5847.000	16	3	0.224
Grassly	18	1.25	0.107
Mona Lisa	16	1.55	0.172
PAR001	18	1.64	0.198
PAR007	11	1.33	0.156
Penny Lane	13	1.67	0.196
Super Pink Pompon	21	1.29	0.14
T2	18	1.33	0.154
T3	20	1.85	0.272
T6	17	1.31	0.117

Chapter 3

Wild relatives of *Chrysanthemum* suppress population build-up of thrips *Frankliniella occidentalis*

Marcella Bovio, Ruilin Huang, Miriam Strijker, Roeland E. Voorrips,
Joop J. A. van Loon, Ben Vosman, Lotte Caarls

Abstract

Chrysanthemum is one of the most important ornamental crops and its cultivation is affected by thrips, of which in Europe *Frankliniella occidentalis* is the most important. Although genetic variation for thrips resistance in *Chrysanthemum* cultivars has been observed, no thrips resistance has been successfully and extensively introduced in breeding programs. Crop wild relatives are often considered as sources of resistance traits, but no information is yet available on the thrips-resistance level of wild species in the *Chrysanthemum* genus or related *Artemisia* species. Therefore, we screened forty-seven accessions from different *Chrysanthemum* species and related genera in whole plant assays for thrips population build-up and in leaf disc assays for larval development, larval survival, and reproduction. We identified wild *Chrysanthemum* and *Artemisia* accessions that reduce thrips population build-up as well as larval survival and development. On five highly resistant accessions the development larvae was suppressed, effectively interrupting the life cycle of thrips. Comparing 4-week-old to 8-week-old plants, we observed that the resistance increased with plant age. Adult reproduction on leaves did not differ between accessions, but the reproduction on flowers was accession dependent. The accessions that showed the highest leaf-based resistance did not show flower-based resistance. The resistance might depend on specialized metabolites. The identified resistant and susceptible wild relatives of *Chrysanthemum* may be used in further studies to elucidate the mechanism and the genetics of thrips resistance.

Introduction

Plants constantly face pathogen and insect attacks without the possibility of walking away from the threat. To counteract these attacks and avoid being damaged, plants evolved complex and diverse defence mechanisms (Fürstenberg-Hägg et al., 2013; War et al., 2012). However, during domestication many defence traits have not been selected for and are not present in modern crop varieties (Chen et al., 2015; Whitehead et al., 2017). Fortunately, many of these defence traits can still be found in the crop wild relatives, that can now act as sources of resistance (Dempewolf et al., 2017; Warschefsky et al., 2014; Zhang et al., 2017). These resistances against insects previously found in wild relatives of crops can affect one or more stages of the pest life cycle. For instance, in wild *Solanum* species, quantitative trait loci for both reduced adult survival and reduced oviposition of whiteflies have been identified (Firdaus et al., 2013; Maliepaard et al., 1995; Momotaz et al., 2010).

Chrysanthemum plants are susceptible to various pest species, i.e. two-spotted spider mites, aphids, and thrips. Thrips are one of the most destructive pests in chrysanthemum cultivation, in particular the species *Frankliniella occidentalis*, or western flower thrips (WFT). They cause economic losses by damaging the crop, not only directly through feeding, causing silver damage and malformations, but also indirectly as virus vectors (He et al., 2020; Rotenberg et al., 2015). Differences in resistance levels among chrysanthemum cultivars have been reported (Chen et al., 2020b; De Jager et al., 1995a; De Jager et al., 1993; De Kogel et al., 1998; Ohta, 2002), and have also been described in this thesis (Chapter 2), where we identified cultivars with reduced population build-up and reduced larval development. However, thrips resistance in cultivated chrysanthemum is so far an accidental occurrence and insufficient to prevent economic damage. As chrysanthemums are grown for ornamental purposes, tolerance for visual damage is very low. In addition, the presence of insects on plant material in international trade is prohibited.

Novel resistance to thrips may be found in the wild relatives of the cultivated chrysanthemum. However, no information is available on the thrips-resistance level of wild species in the *Chrysanthemum* genus or related *Artemisia* species that have been found and collected in China, Japan and Korea, the areas of origin of these species (Zeven and Zhukovskiĭ, 1975). Mechanisms of resistance vary depending on both the plant and the pest. Plants have evolved direct defences, which affect the pest by interfering with life history traits, and indirect defences, which attract the natural enemies of the pest. Direct defences can be chemical, i.e. specialized compounds that are toxic or repellent for the pest, or physical barriers, such as waxes and trichomes (Fürstenberg-Hägg et al., 2013). An example of chemical direct

defence are glycoalkaloids with tetrose side chains that are found in wild relatives of potato and are toxic to the Colorado potato beetle (Tai et al., 2014; Wolters et al., 2023). Other examples of identified pest resistance depend on trichomes, such as resistance to aphids in *Solanum* (Cho et al., 2017), and resistance to whitefly, aphids, thrips, and two-spotted spider mites in tomato, which has been associated mainly with glandular trichomes type IV density and acyl sugar production (Alba et al., 2009; Firdaus et al., 2013; McDaniel et al., 2016; Vosman et al., 2019). The role of trichomes in thrips resistance in cultivated chrysanthemum has been investigated (Chen et al., 2020b), but it has not been studied in *Chrysanthemum* wild relatives.

Resistance may be introgressed into cultivated chrysanthemum through intergeneric and interspecific hybridization, followed by backcrossing (Su et al., 2019). Hybridization of cultivated chrysanthemum (*Chrysanthemum x morifolium*) with *Ajania*, *Artemisia*, and other *Chrysanthemum* spp. has already been successful (Deng et al., 2010, 2011; Cheng et al., 2011). Moreover, the distant relative of chrysanthemum, *Artemisia vulgaris* '*Variegata*' is highly resistant to the aphid *Macrosiphoniella sanbourni* and its intergeneric hybrid with cultivated chrysanthemum showed enhanced resistance to aphids (Deng et al., 2010).

Despite these successful hybridization experiments and the fact that wild relatives were shown to be interesting sources of resistance traits, not much research has been done on the use of wild relatives for insect resistance breeding, especially not for thrips resistance. Here, we report the screening of wild accessions of *Chrysanthemum* relatives for thrips resistance in their non-flowering stage. We investigated which thrips life-cycle parameters are affected by the resistance and the possible role of trichomes. Then, we investigated the effect of plant age on the resistance and whether flowers of accessions that had showed resistance in leaves of non-flowering plants, differed in resistance level. The outcomes of this study provide further insights into the potential of *Chrysanthemum* wild relatives as a new source of thrips resistance that may be exploited in breeding programmes of cultivated chrysanthemums.

Materials and Methods

Plant material

Forty-seven accessions of *Chrysanthemum* spp. and related genera (Table 1) were obtained from Thompson and Morgan (United Kingdom), the Genetic Resources Center National Agriculture and Food Research Organization (NARO), Tsukuba, the National BioResource Project (NBRP) (Japan Agency for Medical Research

and Development), Higashi-Hiroshima City, and Hiroshima University, Hiroshima, Japan. Mother plants were maintained and cuttings were produced at several locations in the Netherlands by the breeding companies involved in the project. During maintaining of mother plants insecticides were routinely applied. For experiments, the accessions were vegetatively propagated without the application of insecticides: the growing tips were cut and rooted twice over a six week period at the breeding company to reduce the possible after-effects of insecticides, after which they were shipped to the test location in Wageningen, the Netherlands. The first round of evaluations was carried out between October 2020 and February 2021. After this first round, 26 accessions were selected for validation experiments, carried out in October 2021. To reduce possible after-effects of location-specific non-genetic factors, the cuttings for the validation experiment were pinched again after transportation, rooted for two weeks, and then transplanted in pots. All test plants were grown in 11 cm pots at 25/21°C, 16:8 h light:dark period, 70% RH in a glasshouse at Wageningen University and Research Centre, Wageningen, the Netherlands, without the application of insecticides.

Insect population

The population of *F. occidentalis* was acquired from Greenhouse Horticulture of Wageningen University, Bleiswijk. It was maintained in a mass rearing for several years on commercial yellow-flowering pot chrysanthemums, *C. x morifolium* at ~25 °C, 16:8 h light:dark period with LED lighting. Twice a week, two new plants with open flowers were added and the two oldest were removed from the rearing, maintaining the number of plants at about 16 plants. Female adult thrips were collected directly from the mass rearing with an aspirator. Synchronized thrips larvae (L1) were obtained by letting females oviposit for 24 h on snack cucumbers (*Cucumis sativus*). After removal of the females, the snack cucumbers were placed on paper in glass jars and incubated for four days in a climate cabinet (25°C, 16-8 h day-night and 70% RH) after which L1 larvae hatched and were handled with a fine brush (Mollema et al., 1993).

Screening methods

The 47 accessions were evaluated in four batches in an initial screening consisting of a whole plant and a leaf disc assay (Table 2). Based on the results from the thrips population development assays described in Chapter 2, two cultivars were included as references: Penny Lane as moderately resistant and Super Pink Pompon as susceptible. Based on the initial screening we selected four of the most susceptible and 22 putatively resistant accessions, which were compared in a validation experiment that consisted of one whole plant assay for population build-up and three leaf disc assays for larval survival and development.

Table 1. Plant species, code, ploidy and supplier of selected accessions. HU: Hiroshima University, Hiroshima, Japan. NARO: Genetic Resources Center, National Agriculture and Food Research Organization, Tsukuba. NBRP: National BioResource Project (Japan Agency for Medical Research and Development), Higashi-Hiroshima City. Ploidy indicates the ploidy level of the accession, or if that is not known of the species.

Species	Accession code	Ploidy	Selected for validation experiments	Supplier
Artemisia feddei	PB-MB101	2x	*	HU
Artemisia keiskeana	PB-MB102	2x	*	HU
Chrysanthemum arcticum	PB-MB103	2x		NARO
Chrysanthemum boreale	PB-MB104	2x		Deliflor
Chrysanthemum carinatum	PB-MB105	na		Thompson & Morgan
Chrysanthemum crassum	PB-MB106	10x	*	NARO
Chrysanthemum crassum	PB-MB107	10x		HU
Chrysanthemum indicum	PB-MB108	4x, 6x	*	NARO
Chrysanthemum indicum	PB-MB109	6x	*	NBRP
Chrysanthemum indicum	PB-MB110	4x		NBRP
Chrysanthemum indicum	PB-MB111	6x	*	NBRP
Chrysanthemum indicum	PB-MB112	6x		NBRP
Chrysanthemum indicum	PB-MB113	4x	*	NBRP
Chrysanthemum japonense	PB-MB114	6x	*	NARO
Chrysanthemum japonense	PB-MB115	6x	*	NARO
Chrysanthemum japonense	PB-MB116	6x		NARO
Chrysanthemum japonense	PB-MB117	6x		HU
Chrysanthemum lavendulifolium	PB-MB118	2x		Deliflor
Chrysanthemum makinoi	PB-MB119	2x	*	NARO
Chrysanthemum makinoi	PB-MB120	2x		HU
Chrysanthemum nankingense	PB-MB121	2x		Deliflor
Chrysanthemum nipponicum	PB-MB122	2x		NARO
Chrysanthemum nipponicum	PB-MB123	2x	*	NARO
Chrysanthemum ornatum	PB-MB124	8x		HU
Chrysanthemum ornatum	PB-MB125	8x		HU
Chrysanthemum ornatum	PB-MB126	8x		HU
Chrysanthemum pacificum	PB-MB127	10x	*	NARO

Table 1. continued.

Species	Accession code	Ploidy	Selected for validation experiments	Supplier
Chrysanthemum pacificum	PB-MB128	8x	*	NARO
Chrysanthemum pacificum	PB-MB129	10x		HU
Chrysanthemum segetum	PB-MB130	na		Syngenta
Chrysanthemum segetum	PB-MB131	na		Syngenta
Chrysanthemum seticuspe	PB-MB132	2x	*	HU
Chrysanthemum seticuspe	PB-MB133	2x	*	HU
Chrysanthemum seticuspe	PB-MB134	2x	*	HU
Chrysanthemum seticuspe	PB-MB135	2x		HU
Chrysanthemum seticuspe	PB-MB136	2x		HU
Chrysanthemum seticuspe	PB-MB137	2x	*	HU
Chrysanthemum shiwogiku	PB-MB138	8x	*	HU
Chrysanthemum vestitum	PB-MB139	6x		Deliflor
Chrysanthemum vulgare	PB-MB140	na	*	NARO
Chrysanthemum yezoense	PB-MB141	10x		HU
Chrysanthemum yosinaganthum	PB-MB-142	4x		HU
Chrysanthemum yosinaganthum	PB-MB-143	4x	*	HU
Chrysanthemum zawadskii	PB-MB-144	4x, 6x, 8x	*	NARO
Chrysanthemum x morifolium	Penny Lane	6x	*	Deliflor
Chrysanthemum x morifolium	Super Pink Pompon	6x		Deliflor
Tanacetum cinerariifolium	PB-MB145	2x	*	HU

Table 2. Overview of experiments, screening methods, measured variables and time of experiments.

Experiment	Screening method	Measured variables	Time of experiment
Initial screening	Whole plant assay	Population build-up	Oct 2020 – Feb 2021
Initial screening	Leaf disc assay	Larval survival, larval development	Oct 2020 – Feb 2021
Validation experiment	Whole plant assay	Population build-up	Oct 2021
Validation experiment	Leaf disc assay	Larval survival, larval development	Nov 2021 – Dec 2021
Plant age experiment	Leaf disc assay	Reproduction as number of larvae (L1)	November 2021
Plant age experiment	Leaf disc assay	Larval survival, larval development	January 2022
Reproduction in flowers	Detached flower assay	Reproduction as number of larvae (L1, L2)	June 2022
Trichome quantification		Trichome density	September 2021

Whole plant assay for population development

In the whole plant assays, we estimated the thrips population build-up on non-flowering plants over three weeks, as described in Chapter 2. Five three-week-old plants per accession were assayed in a completely randomized design in a greenhouse compartment. Each plant was enclosed in a thrips-proof sleeve and a water seal was used to assure that no thrips escaped or entered, i.e. the sleeve's edges were always immersed in water which acted as barrier. Thrips were collected from the mass rearing and sedated with CO^2. Fifteen female thrips were placed in a glass vial and then transferred onto each plant. The climate conditions were set at 25/21 °C, 16:8 h light:dark period, 70% RH. Three weeks after infestation, sleeves were removed and each plant was shaken out so that thrips fell onto a white paper sheet, and the number of thrips larvae and adults were counted. The pupal stages and their numbers were not recorded because pupation occurs in the soil.

Leaf disc assay for larval survival and development

For the larval survival and development validation experiments, we collected the 1st and 2nd youngest fully expanded leaves from three five-week-old cuttings and punched 1.5 cm diameter leaf discs. Each leaf disc was placed with the abaxial side upward on 1.5% agar before it solidified in a 50*9 mm Falcon Tight-Fit Lid Petri dish, whose lid had a 1 cm diameter hole covered with 70-micron fine mesh to allow ventilation but prevent thrips escape. We placed one newly hatched L1 larva per leaf disc and observed its survival and development to a later stage daily during 14 days. The Petri dishes were arranged in a completely randomized design and

incubated in a climate cabinet at 25 °C, 16:8 h light:dark period, and 70% RH. The observations were stopped when the thrips was not found for two consecutive days, was found dead, or reached the adult stage.

Plant age experiment

Two groups of cuttings were produced with a four week interval. When the first group was 8 weeks old and the second was 4 weeks old, the 1st and 2nd youngest fully expanded leaves of three plants were collected and leaf discs of 1.5 cm diameter were punched and placed abaxial side upward in Petri dishes on 1.5% water agar.

To assess the effect of plant age on larval survival and development, newly hatched L1 larvae were individually placed on leaf discs of 4- and 8-week-old plants and their development was observed daily for 14 days. Each accession and plant age combination was replicated 36 times.

To evaluate the effect of plant age on thrips reproduction, two female adult *F. occidentalis* were placed on a single leaf disc for 48 h. There were six replicate discs for each accession and plant age. Petri dishes were arranged in a completely randomized design, and incubated in a climate cabinet at 25 °C, 16:8 h light:dark period for the duration of the whole experiment. Reproduction was assessed by counting the hatched larvae under a stereo-microscope at seven days post-infestation (dpi) (McDonald et al., 1998).

Reproduction in flowers

After four weeks in long-day conditions, three cuttings of six accessions were transferred to short-day conditions (8:16 light:dark) to induce flowering. When the plants were 12 weeks old, the first flowers started to open. Two weeks later, two to six flowers per accession were collected and individually placed with their petiole in water agar in Petri dishes. Each detached open flower was infested with five female *F. occidentalis* and incubated at 25 °C, 16:8 h light:dark period, and 70% RH. The Petri dishes were arranged following a completely randomized design. The females were left on the flowers until the end of the experiment. At day 7 dpi the number of offspring per flower was counted by dissecting the flower.

Trichome density

Trichomes were counted on young and old leaves of four-week-old plants of 24 *Chrysanthemum* and related genera accessions. The 1st and 2nd, and the 7th and 8th fully expanded leaves counting from the top of the plant were collected and considered respectively as young leaves and old leaves. Five leaves were collected per leaf age per accession; six areas of 0.78 mm² were marked for counting with the

tip of a glass Pasteur pipette in the region near the midvein on the adaxial and abaxial side for each collected leaf. T-shaped and glandular trichomes were quantified with a Zeiss Stemi SV8 stereomicroscope magnification X8-X64 (Zeiss, Germany), or the VHX-7000 digital microscope equipped with lens Z20:X100 (Keyence, Japan) for accessions that had a high trichome density.

Statistical analysis

Thrips population build-up, whole plant assays

To estimate thrips population build-up, the total number of thrips per plant was calculated by adding the number of larvae to the number of adults. The total number was transformed to y=sqrt(x). Accession means were analysed with ANOVA, Dunnett's post-hoc test with cv. Penny Lane as reference cultivar in the first, second and fourth whole plant assay, and Tukey's HSD in the third whole plant assay because cv. Penny Lane was not available in that assay. In the validation whole plant assay, mean numbers of thrips at 21 dpi were analysed with ANOVA and Tukey's HSD post-hoc procedure.

Larval survival and development, leaf disc assay

Daily observations of thrips survival on leaf discs were analysed with Kaplan-Meier survival analysis. Death of the monitored individual was recorded as event (1) and lost larva were recorded as censored events (0). Emerged adults were considered alive individuals. The time from the start of the experiment (infestation, day 0) to the event or the censored event was calculated in days.

The data were analysed using the "survival" package in R, specifically the functions "survfit()" and "survdiff()" to test for differences in survival over time per accession (Chi-square test) (R Core Team, 2022; Therneau, 2022). Additionally, we used the "survminer" package and its functions "ggsurvplot()" and "pairwise_survdiff()" to plot the survival curves and perform pairwise comparisons between accessions using the Log-Rank test with Benjamini and Hochberg (BH) corrections for multiple testing (Kassambara et al., 2021).

Reproduction, plant age experiment and flowers

The number of offspring was transformed to y=sqrt(x). In the plant age experiment, the effects of accession and plant age on the number of hatched larvae were analysed with a two-way ANOVA. In the experiment quantifying reproduction on flowers, the effect of accession on the number of offspring on flowers was analysed with an ANOVA and Dunnett's post-hoc test with cv. Penny Lane as reference.

Trichome density and correlation between trichome density and resistance levels

The effect of accession, leaf side, and leaf age as well as their interactions on the density of glandular and T-shaped trichomes were analysed with a three-way ANOVA. Spearman's correlation analysis was performed between trichome densities and thrips variables (population build-up, reproduction and larval survival).

Results

Population development on wild accessions of *Chrysanthemum*

Forty-seven accessions of *Chrysanthemum* and close relatives were screened for thrips population development in four whole plant assays. The total number of thrips per plant was assessed three weeks after infestation. In the initial screening the mean number of thrips per plant differed significantly among accessions in all four assays (ANOVA; $p < 0.001$ in all four assays). Overall, the number of thrips found per plant ranged between 0 and 100 (Figure 1).

We compared the wild accessions with the resistant reference cv. Penny Lane and identified the accessions that were more resistant as the ones with a significantly lower population development than this reference. In the first experiment, the most resistant accessions were PB-MB109, PB-MB133, PB-MB134, PB-MB111, and PB-MB119. In the second and third experiment, the most resistant accessions were PB-MB141, PB-MB138, PB-MB102, PB-MB143, and PB-MB101. In the fourth experiment, the most resistant accessions were PB-MB128, PB-MB116, and PB-MB123. Highly susceptible accessions, i.e. allowing a high population development, were also identified as well, including PB-MB132, PB-MB137, PB-MB113, and PB-MB129.

Validation of thrips population build-up on 23 selected *Chrysanthemum* accessions

In the validation whole plant assay, we re-evaluated the population build-up not only of the most resistant accessions of the first screening, but also of the accessions with a resistance level comparable to Penny Lane, and of a few susceptible accessions (Table 1). The accessions differed significantly for the mean number of thrips per plant (ANOVA (unequal variance); $p < 0.001$) (Figure 2). Some of the accessions previously selected as resistant appeared to be more susceptible when re-evaluated (i.e. PB-MB115, PB-MB140, PB-MB134, PB-MB127, PB-MB111), but most of the accessions showed a thrips population build-up comparable to the first screenings. Considering the mean population build-up, PB-MB113 and PB-MB132 were confirmed as highly susceptible, and PB-MB102, PB-MB143, and PB-MB101 were confirmed as highly resistant.

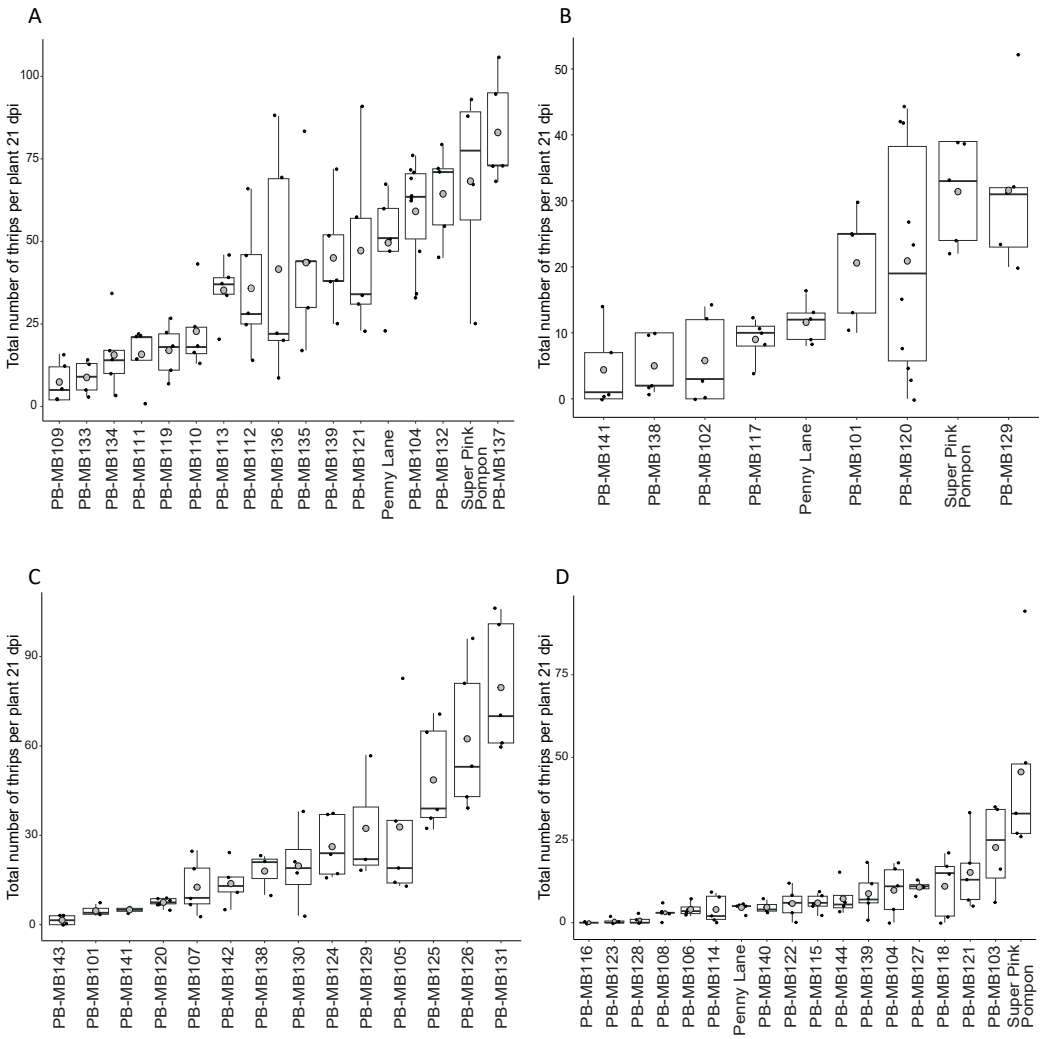


Figure 1. Screening of accessions of *Chrysanthemum* and related genera for thrips population development. Accessions were evaluated in four whole plant assays (A, B, C, D). Five plants per accession were infested with 15 female thrips and the number of thrips (adults + larvae) per plant was counted after 21 days. Super Pink Pompon and Penny Lane were included as susceptible and resistant reference cultivars, respectively, in the assays A, B, and D. Black dots indicate the number of thrips found per individual plant. Boxplots show the distribution of the observations (number of thrips per plant per accession): the box represents the interquartile range, the horizontal line inside the box represents the median, and the whiskers indicate the range of data. Grey dots indicate the mean numbers of thrips per plant per accession. Differences in population development between accessions were significant in all four assays (ANOVA; $p < 0.001$).

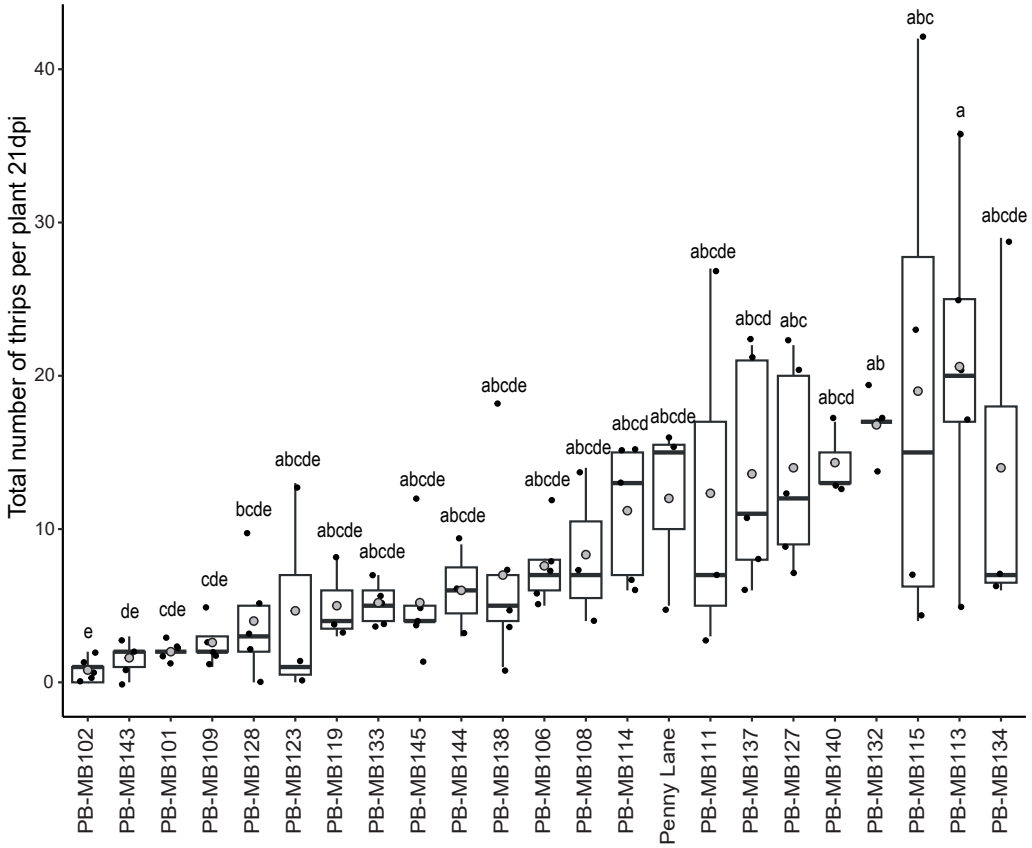


Figure 2. Validation of thrips population build-up on the 23 *Chrysanthemum* and related genera accessions selected after the first screening. Five plants per accessions were infested with 15 female thrips and the total number of thrips (adults + larvae) per plant was counted after 21 days. Penny Lane was included as reference cultivar. Black dots indicate the total number of thrips found on individual plants. Boxplots show the distribution of the observations (number of thrips per plant for each accession): the box represents the interquartile range, the horizonal line inside the box represents the median, and the whiskers indicate the range of data. Grey dots indicate the mean number of thrips per plant. Different letters indicate accessions that significantly differ according to Tukey's HSD post-hoc test ($p < 0.05$).

Larval survival and development on 26 *Chrysanthemum* wild relatives

The survival and development of immature stages of *F. occidentalis* on 26 *Chrysanthemum* and related genera accessions were evaluated in three leaf disc assays until adult emergence or death of all individuals. The thrips survival curves differed significantly between accessions in all three experiments (Kaplan-Meier tests: $p < 0.001$ for all three assays). From day 2 or 3, higher larval mortality was observed on the most resistant accessions than on susceptible accessions. Most of the accessions considered resistant based on population build-up also showed a low larval survival (Spearman's rho (number of thrips per plant- larval mortality day 2 and day 3) = -0.24) (Table S1). This was particularly evident in accessions PB-MB-123 (Figure 3a), PB-MB102, PB-MB109, PB-MB133 (Figure 3b), and PB-MB145 (Figure 3c).

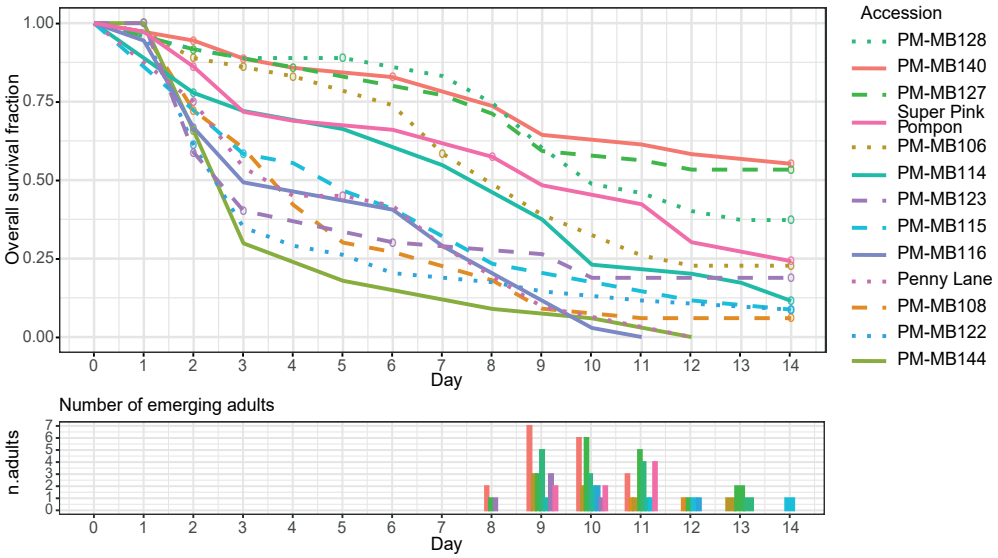


Figure 3a. In the top graph, Kaplan-Meier survival curves of *Frankliniella occidentalis* on 13 *Chrysanthemum* accessions. The fraction of 36 thrips surviving is plotted over time. Censored events, i.e. lost individuals, are indicated by "o". The survival curves differed (Chi square = 133, 12 d.f., $p < 0.001$). In the bottom graph 'Number of emerging adults', the number of adults that emerged per day is plotted.

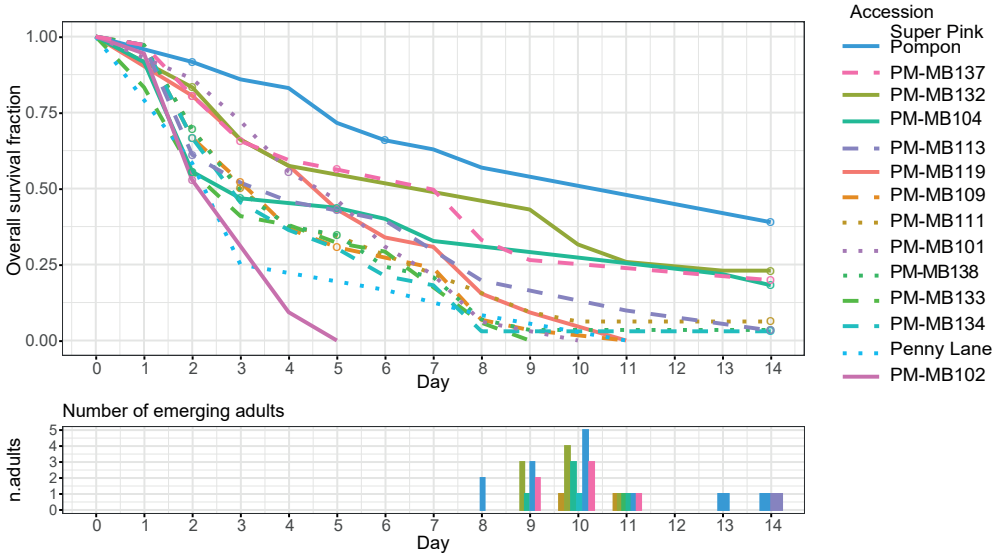


Figure 3b. In the top graph, Kaplan-Meier survival curves of *Frankliniella occidentalis* on 14 *Chrysanthemum* and *Artemisia* accessions. The fraction of 36 thrips surviving is plotted over time. Censored events, i.e. lost individuals, are indicated by "o". The survival curves differed (Chi square = 98.8, 13 d.f., $p < 0.001$). In the bottom graph 'Number of emerging adults', the number of adults that emerged per day is plotted.

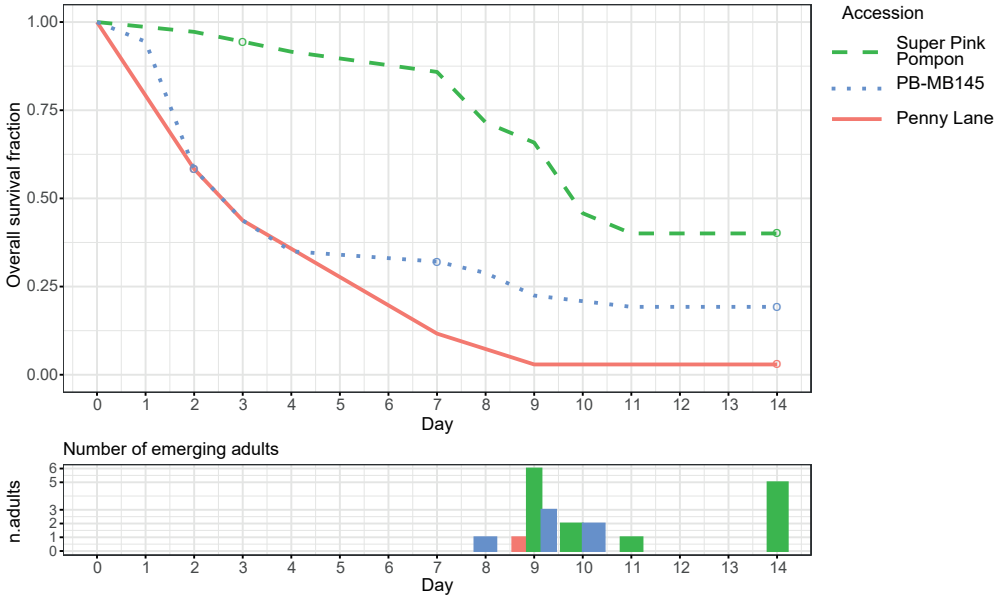


Figure 3c. In the top graph, Kaplan-Meier survival curves of *Frankliniella occidentalis* on two *Chrysanthemum* accessions and *Tanacetum cinerariifolium*. The fraction of 36 thrips surviving is plotted over time. Censored events, i.e. lost individuals, are indicated by "o". The survival curves differed (Chi square = 30.3, 2 d.f., $p < 0.001$). In the bottom graph 'Number of emerging adults', the number of adults that emerged per day is plotted.

During this experiment we also recorded the developmental stages of the thrips and their survival. The survival from L1 to L2 stage ranged from 14% (on PB-MB102) to 92% (on Super Pink Pompon) (Table 3). The survival from L2 to the prepupal stage was 0% on PB-MB116, PB-MB102, PB-MB109, PB-MB133 and PB-MB101, and on the accessions that allowed some development, it varied from 4% (on PB-MB119) to 69% (on PB-MB127). The development from prepupa onwards could only be studied on more susceptible genotypes. On these, the survival from prepupal to pupal stage was high (67% to 100%). Similarly, the fraction of pupae from which adults emerged was also high (50% to 100%). Adult emergence was recorded starting from day 8 (Figure 3a, 3b, 3c, bottom graphs).

Effect of plant age on larval survival

We investigated the effect of plant age on the survival and development of immature stages of *F. occidentalis* on 11 *Chrysanthemum* and *Artemisia* accessions. Four-week-old plants and 8-week-old plants were compared in a leaf disc assay using the 1st and 2nd youngest fully expanded leaves. Both the effect of plant age and accession on thrips survival were significant (Chi-square test; p=<0.0001). Older plants seemed to be more resistant than younger plants of all accessions. On accessions Penny Lane, PB-MB101 and PB-MB102 (Figure 4a), and PB-MB119, PB-MB109, and PB-MB132 (Figure 4b), significantly higher thrips survival was observed on 4-week-old plants than on 8-week-old plants (Log-Rank test for pairwise comparison with BH correction; $p < 0.05$). On 4-week-old plants also a higher number of emerging adults was found than on 8-week-old plants (Figure 4a, 4b). Only on accessions PB-MB113 and PB-MB144, the survival was higher on 8-week-old than on 4-week-old plants, but the difference was not significant (Log-Rank test for pairwise comparison with BH correction; $p > 0.05$).

Plant age effect on thrips reproduction

We investigated the effect of plant age on thrips reproduction by counting the number of hatched larvae on leaf discs. Leaf discs of 4-week-old plants and 8-week-old plants of 11 *Chrysanthemum* and related genera accessions were compared (Figure S1). We did not find significant differences in number of hatched larvae among accessions but 4-week-old plants had significantly more larvae than 8-week-old plants (Two-way ANOVA; accession $p > 0.05$, plant age $p < 0.0001$).

Table 3. Survival (%) of immature stages of *Frankliniella occidentalis* on leaf discs of 26 *Chrysanthemum* and related genera accessions. Survival (%) and the number of individuals between brackets are presented for each developmental stage.

Validation exp	Accession	Survival rate (%)			
		L1 to L2	L2 to prepupa	prepupa to pupa	pupa to adult
1	PB-MB144	19 (7)[1]	14 (1)	100 (1)	0 (0)
	PB-MB122	31 (11)	45 (5)	80 (4)	75 (3)
	Penny Lane	33 (12)	0 (0)	-[2]	-
	PB-MB123	33 (12)	58 (7)	86 (6)	83 (5)
	PB-MB108	39 (14)	14 (2)	100 (2)	100 (2)
	PB-MB116	44 (16)	0 (0)	-	-
	PB-MB104	53 (19)	32 (6)	100 (6)	83 (5)
	PB-MB115	58 (21)	14 (3)	100 (3)	100 (3)
	PB-MB114	67 (24)	29 (7)	86 (6)	67 (4)
	Super Pink Pompon	67 (24)	38 (9)	89 (8)	100 (8)
	PB-MB106	78 (28)	25 (7)	100 (7)	100 (7)
	PB-MB127	81 (29)	69 (20)	95 (19)	95 (18)
	PB-MB140	83 (30)	67 (20)	95 (19)	95 (18)
	PB-MB128	89 (32)	50 (16)	88 (14)	93 (13)
2	PB-MB102	14 (5)	0 (0)	-	-
	Penny Lane	28 (10)	0 (0)	-	-
	PB-MB109	39 (14)	0 (0)	-	-
	PB-MB133	39 (14)	0 (0)	-	-
	PB-MB111	44 (16)	19 (3)	67 (2)	100 (2)
	PB-MB113	44 (16)	19 (3)	67 (2)	50 (1)
	PB-MB138	44 (16)	6 (1)	100 (1)	100 (1)
	PB-MB134	47 (17)	6 (1)	100 (1)	100 (1)
	PB-MB137	64 (23)	30 (7)	86 (6)	100 (6)
	PB-MB119	67 (24)	4 (1)	100 (1)	-
	PB-MB132	67 (24)	42 (10)	90 (9)	89 (8)
	PB-MB101	69 (25)	0 (0)	-	-
	Super Pink Pompon	86 (31)	52 (16)	81 (13)	100 (13)
3	Penny Lane	28 (10)	10 (1)	100 (1)	100 (1)
	PB-MB145	33 (12)	50 (6)	100 (6)	100 (6)
	Super Pink Pompon	92 (33)	45 (15)	100 (15)	87 (13)

[1] Number in parentheses are live individuals at each developmental stage. The starting number was 36 first instar (L1) individuals.

[2] '-' indicates that no individuals reached the previous stage, no data available.

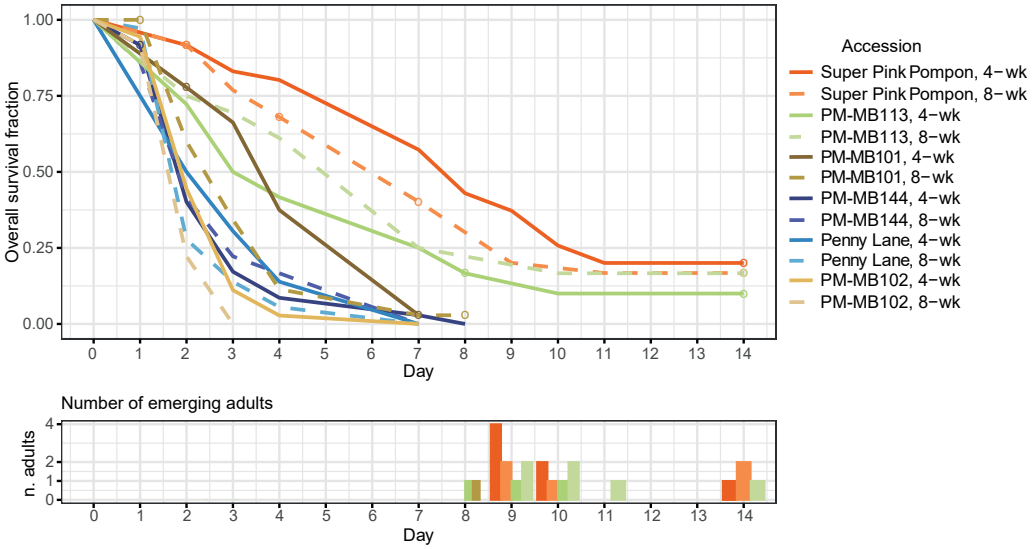


Figure 4a. In the top graph, Kaplan-Meier survival curves of *Frankliniella occidentalis* on six *Chrysanthemum* and *Artemisia* accessions and two plant ages. The fraction of 36 thrips surviving is plotted over time. Solid lines represent 4-week-old plants and dashed lines represent 8-week-old plants. Censored events, i.e. lost individuals, are indicated by "o". The survival curves differed per accession and plant age (Chi square = 396, 21 d.f., p = < 0.0001). In the bottom graph 'Number of emerging adults', the number of adults that emerged per day is plotted.

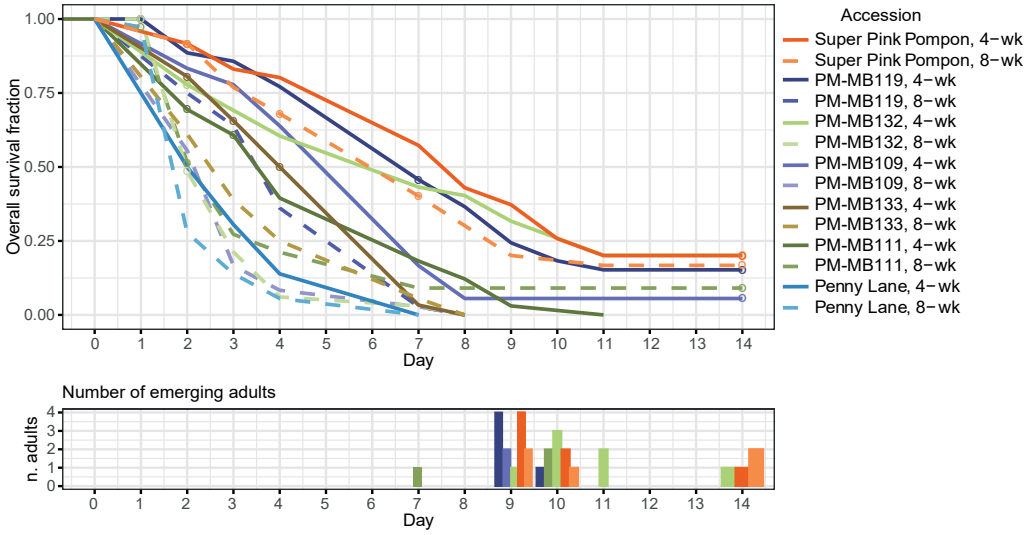


Figure 4b. In the top graph, Kaplan-Meier survival curves of *Frankliniella occidentalis* on seven *Chrysanthemum* and two plant ages. The fraction of 36 thrips surviving is plotted over time. Solid lines represent 4-week-old plants and dashed lines represent 8-week-old plants. Censored events, i.e. lost individuals, are indicated by "o". The survival curves differed per accession and plant age (Chi square =396, 21 d.f., p = < 0.0001). In the bottom graph 'Number of emerging adults', the number of adults that emerged per day is plotted.

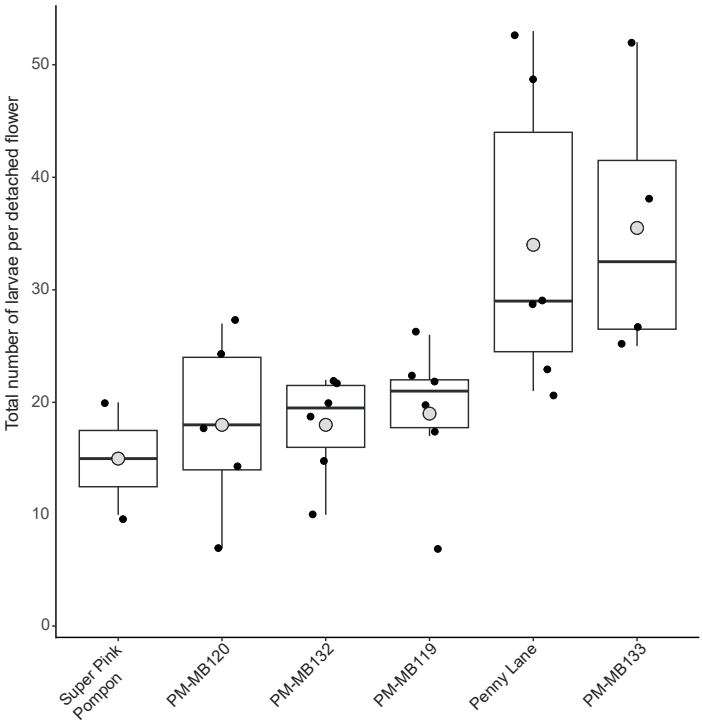


Figure 5. Number of hatched *Frankliniella occidentalis* larvae on detached flowers of six *Chrysanthemum* accessions. Each detached open flower was infested with five females of *F. occidentalis* and the number of larvae was counted after 7 days. The mean numbers of larvae per flower are plotted as grey dots, the individual observations are plotted as black dots. Boxplots show the distribution of the observations: the box represents the interquartile range, the horizonal line inside the box represents the median, and the whiskers indicate the range of data. The number of hatched larvae differed between accessions (ANOVA; $p < 0.01$).

Reproduction on flowers

Thrips reproduction was assessed on detached flowers of six *Chrysanthemum* accessions. The number of larvae counted per flower differed between accessions (ANOVA; $p < 0.01$). Flowers of Penny Lane and PB-MB133 allowed higher reproduction than Super Pink Pompon, ABP23, PB-MB119, and PB-MB132. On some of the flowers of Penny Lane and PB-MB133 more than 50 larvae were counted (Figure 5).

Trichome density and thrips resistance

T-shaped and glandular trichomes were quantified on the abaxial and adaxial side of young and old leaves of 24 *Chrysanthemum* and related genera (Figure S2, S3). For T-shaped trichome density the interactions between accession and leaf side and between accession and leaf age were significant, as well as the main effects of accession, leaf side and leaf age (Three-way ANOVA; Accession $p < 0.0001$;

Leafside p <0.0001; Leafage p < 0.0001; Accession:Leafside p < 0.0001; Accession:Leafage p < 0.0001; Leafage:Leafside p > 0.05). For glandular trichome density the main effects of leaf side and leaf age were not significant; only the accession effect was significant (Three-way ANOVA; Accession p < 0.0001; Leafside p > 0.05; Leafage p > 0.05).

No significant correlations were found between T-shaped trichome density on accessions and thrips resistance traits. Weak significantly positive correlations were found between the duration of the L1 stage and four glandular trichomes density parameters (glandular trichome density, Spearman's rho = 0.559, p < 0.01; density on old leaves, Spearman's rho = 0.533, p < 0.01; density on adaxial side of old leaves, Spearman's rho = 0.585, p < 0.01; and density on adaxial side of young leaves, Spearman's rho = 0.524, p < 0.01). Some thrips resistant accessions had low glandular and T-shaped trichome density while others had trichome densities higher than some susceptible accessions. No or rare glandular trichomes on the adaxial side of leaves were observed in four accessions (PB-MB119, PB-MB111, PB-MB132, and PB-MB102).

Discussion

Thrips-resistant wild relatives of *Chrysanthemum* show a reduced thrips population build-up, larval survival and development

Variation for thrips resistance in cultivated chrysanthemum was previously found by us (Chapter 2) and was also reported in other studies (Chen et al., 2020b; De Jager et al., 1995a; De Jager et al., 1993; De Kogel et al., 1998; Ohta, 2002). Given the variation already present in cultivated chrysanthemum, large variation was expected also among the wild relatives of *Chrysanthemum*. In the present study, we screened wild relatives of *Chrysanthemum* in whole plant assays and showed that a large variation in population build-up of WFT is indeed present among these wild relatives. We validated the reduced population build-up of thrips on a subset of resistant wild accessions. Further, using leaf disc assays assessing survival of each thrips stage we found that there was a correlation between population build-up and larval survival. In fact, we found a decreased larval survival on the resistant wild accessions. Significant differences in survival probability (Figure 3a, 3b, 3c) between resistant and susceptible reference cultivars were found, as well as between susceptible and resistant wild accessions, which resulted in high and low population build-up, respectively. The most resistant wild accessions belong to different species, namely *Chrysanthemum seticuspe* (PB-MB133), *Chrysanthemum*

indicum (PB-MB109), *Artemisia feddei* (PB-MB101), *Artemisia keiskeana* (PB-MB102), and *Chrysanthemum makinoi* (PB-MB119). Within some of these species we also identified susceptible accessions, i.e. in *C. seticuspe* (PB-MB132) and *C. indicum* (PB-MB113). On most of the resistant wild *Chrysanthemum* accessions the larval transition from stage L1 to L2 was significantly inhibited, and no development into pupal and adult stages was observed, thereby preventing the continuation of the life cycle. Similarly, in previously identified resistant *Chrysanthemum* cultivars and also in thrips-resistant *Capsicum* accessions the development from L1 to L2 is completely inhibited (Maharijaya et al., 2012; Ohta, 2002). Clearly, a high larval mortality negatively affects the population build-up on whole plants. Therefore, the selection and use of these resistant accessions in breeding programs could inhibit build-up of thrips populations that may be kept below economic damage threshold through biocontrol agents.

The role of trichomes in thrips resistance in *Chrysanthemum*

This study demonstrated that there is variation for trichome density, of both T-shaped and glandular type, among wild relatives of *Chrysanthemum*. The variation is especially large for the T-shaped trichomes, for which significant differences were found among accessions, leaf-side and leaf-age. Yet, we did not find a correlation between T-shaped trichome density and thrips resistance level. On the other hand, we found a weak correlation between the density of glandular trichomes and the duration of the first thrips larval stage. Trichomes, together with leaf surface wax, thorns and cell wall thickness form the first physical barriers that affect insect infestation of plants, and the effect of both T-shaped and glandular trichomes on insect pests has been investigated (Chen et al., 2020b; Cheng et al., 2011; Stavrinides and Skirvin, 2003). A dense layer of trichomes usually negatively affects landing, feeding, oviposition and movement of insects (Cho et al., 2017; Stavrinides and Skirvin, 2003; Tian et al., 2012). Moreover, glandular trichomes may produce and/or accumulate defensive metabolites that repel or trap arthropods and/or are toxic to them. In other plant species it has been shown that trichomes are a key factor for resistance against insects. Glandular trichomes, and their exudates, confer resistance to aphids, whiteflies and two-spotted spider mites in *Solanum* spp. (Alba et al., 2009; Cho et al., 2017; Firdaus et al., 2013; McDaniel et al., 2016; Vosman et al., 2019). In *Solanum lycopersicum*, type VI glandular trichome density was increased after thrips infestation, suggesting a defensive role (Escobar-Bravo et al., 2017). In *Chrysanthemum*, non-glandular trichome density seems to play a role in aphid resistance (Cheng et al., 2011). Chen et al. (2020) showed a large variation in both glandular and non-glandular trichomes among chrysanthemum accessions but did not find a correlation with thrips resistance. Similarly, in *Capsicum*, trichome

density and thrips resistance were not linked (Maharijaya et al., 2015). Although trichomes play a role in some plant-pest relations we found that thrips resistance in non-flowering chrysanthemum is independent of T-shaped trichomes. However, the role of glandular trichomes in conferring resistance to thrips deserves to be studied further.

If not based on trichome density, the defence mechanisms against thrips in wild relatives of *Chrysanthemum* may be based on specialized metabolites. Several metabolites have been suggested to be involved in insect resistance in wild relatives of *Chrysanthemum*. Monoterpenes have been discovered in and isolated from *A. keiskeana* (Kwak et al., 2001, 1997). Interestingly, essential oils of *A. keiskeana* show acute lethal toxicity on larvae of *Artemia salina*, an aquatic crustacean (Suleimen et al., 2019). Terpenes represent 90% of essential oils of *Artemisia vulgaris*, and it has been suggested that they are involved in resistance against the aphid *Macrosiphoniella sanbourni* (Deng et al., 2010). However, abundance of terpenes is not in all cases correlated with toxicity towards arthropods. In fact, essential oils of *A. feddei* have been found to be rich in oxygenated monoterpenes and sesquiterpenes, but did not show larvicidal activity on *Aedes aegypti* (Kim, 1997; Özek et al., 2014). Because of their abundance and their known biological activity, it is possible that terpenes are also involved in the WFT resistance in the two *Artemisia* spp. tested. Also, numerous sesquiterpenoids and monoterpenoids have been identified in various *C. indicum* and *C. makinoi* accessions (Jiang et al., 2021).

Even when considering specialized metabolites as a potential cause of thrips resistance, glandular trichomes should still be considered in metabolite studies. This is because several compounds are produced and accumulated in glandular trichomes. For instance, insecticidal pyrethrins, which are terpenoid esters, are produced and accumulated in the glandular trichomes of disc ray florets of *Tanacetum cinerariifolium* flowers (Zito et al., 1983). In fact, natural pyrethrin insecticides are traditionally applied in the form of powder of dried flowers of Dalmatian pyrethrum (*T. cinerariifolium*), and they are effective against a broad spectrum of insect species, including thrips (Lybrand et al., 2020; T. Yang et al., 2012). Moreover, the production of monoterpenoids, sesquiterpenoids and volatile terpenoids seem to correlate with the number of glandular trichomes on leaves of *C. morifolium* and *C. indicum*, and the emission of volatile terpenoids correlates with the abundance of glandular trichomes on flowers (Guan et al., 2022). Thus, further research is needed to elucidate the thrips-resistance mechanism in *Chrysanthemum* relatives, and the role of leaf terpenoids and other compounds in thrips-resistance, and to establish if a higher glandular trichome density leads to higher resistance levels.

Plant age affects thrips resistance: young plants are more susceptible than older plants

We showed that the resistance level of most of the *Chrysanthemum* accessions increased with plant age in two leaf disc assays. In 9 of the 11 tested accessions, both thrips-susceptible as well as thrips-resistant, thrips larval survival was lower on 8-week-old plants than on 4-week-old plants, as was adult emergence. Similarly, we observed lower thrips reproduction on older plants. Only on two susceptible accessions higher survival probability was observed as well as higher adult emergence on 8-week-old plants. Young plants are in general more susceptible than older plants, and the accession effect on thrips survival is already clear for 4-week-old plants. Although only detached young leaves were tested, these results suggest that thrips resistance in young leaves generally increases with plant age. The effect of plant age on thrips resistance has been studied in pepper and shown to depend on the resistance level of the accession. Four-week-old thrips-resistant *Capsicum* plants were less resistant than 8-week-old plants, but thrips-susceptible *Capsicum* accessions did not show an increase in thrips resistance over time (Van Haperen et al., 2019), in contrast to our observations in *Chrysanthemum*. Also, resistance to the whitefly *Trialeurodes vaporariorum* in *Solanum habrochaites*, to diamondback moth *Plutella xylostella* in *Brassica oleracea*, and to *Bemisia tabaci* in *Solanum pennellii* increases with plant age (Bas et al., 1992; Campos et al., 2003; Van den Oever-van den Elsen et al., 2016). Young plants could be more susceptible to insect pests because their tissues are more nutritious and less fibrous, facilitating intake and digestion, or they might not yet have built up the necessary levels of defence metabolites. Given this plant-age effect, it is important to validate that young whole plants are also more susceptible to thrips, in which case extra protection might be needed to avoid feeding damage on developing organs. At the same time, even if young plants are more susceptible than older plants, thrips-resistant accessions showed almost no development into L2 stage already at 4-weeks-old. Thus, these identified resistance sources could be exploited to prevent severe damage on the growing crop.

Flowers of resistant accessions allow high population development

Flowers are the final product of chrysanthemum production and they are particularly affected by thrips damage, thus thrips resistance in flowers would also be relevant for chrysanthemum cultivation. We assessed the reproduction of WFT on detached flowers of four wild *Chrysanthemum* relatives and two cultivated chrysanthemums. Thrips reproduction on flowers did not reflect the resistance in the vegetative parts of the plants: for instance, the flowers of the susceptible reference cultivar Super Pink Pompon showed the lowest reproduction, while those of the resistant cultivar

Penny Lane and the resistant wild *C. seticuspe* PB-MB133 showed the highest. This difference in thrips resistance between the leaves and the flowers could be caused by the presence of pollen in the latter. In fact, pollen is known to be a good food source for thrips: it positively affects reproduction and longevity, and it shortens the developmental time from egg to adult (Murai and Loomans, 2001; Trichilo and Leigh, 1988). The susceptible cultivar supported relatively lower thrips reproduction on flowers, likely because it has a pompon type flower that produces less pollen. Nevertheless, the number of hatched larvae counted on flowers was at least double the number generally counted on leaf discs, indicating that no flower resistance is present among the small set of wild relatives of *Chrysanthemum* accessions we tested. This is in accordance with other studies showing that flowering chrysanthemum plants are more suitable hosts than non-flowering plants (Katayama, 1997; Van Dijken, 1992). On the other hand, the flowers of the wild relative *T. cinerariifolium* are reported to be resistant to thrips because of the accumulated pyrethrins, and other *Chrysanthemum* relatives could harbour a similar mechanism (T. Yang et al., 2012). We mainly focused on non-flowering chrysanthemum to tackle early thrips infestations, which affect the worldwide distribution of chrysanthemum cuttings, both because of the damage and because of the strict regulations against thrips presence on transported plants. In conclusion, we identified thrips-resistant wild relatives of *Chrysanthemum* on which thrips larval development was completely suppressed. Further research is needed to characterize the resistance mechanism and its genetic background.

Acknowledgements

We are grateful to the National Agriculture and Food Research Organization (NARO), the National BioResource Project (NBRP) and Hiroshima University, Hiroshima, Japan for providing plant material. We thank Martijn Verweij for taking care of the cuttings in the greenhouse. This research was financially supported by a grant (TU-18043) from the Ministry of Economic Affairs of the Netherlands and the companies Dekker Breeding, Deliflor Chrysanten, Floritec Breeding, Gediflora, Inochio Holdings, Syngenta Seeds and Royal van Zanten.

Supplementary materials

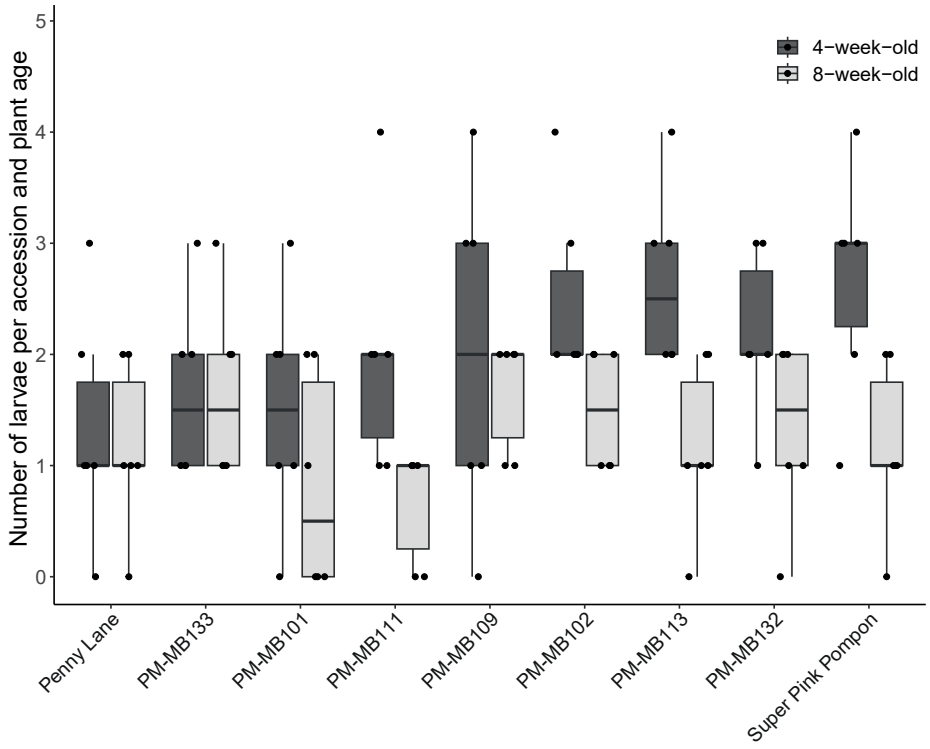


Figure S1. Number of hatched *Frankliniella occidentalis* larvae per plant age and *Chrysanthemum* accession on leaf discs. Two female thrips were allowed to oviposit on 4-week-old or 8-week-old plant leaf discs for 48 hours. There were six replicate discs for each plant age and accession. The hatched larvae were counted at day 7 dpi. Black points reflect individual observations. Boxplots show the distribution of the observations: the box represents the interquartile range, the horizonal line inside the box represents the median, and the whiskers indicate the range of data. Differences between accessions were not significant ($p > 0.05$), but 4-week-old plants showed a significantly higher number of hatched larvae than 8-week-old plants ($p < 0.0001$).

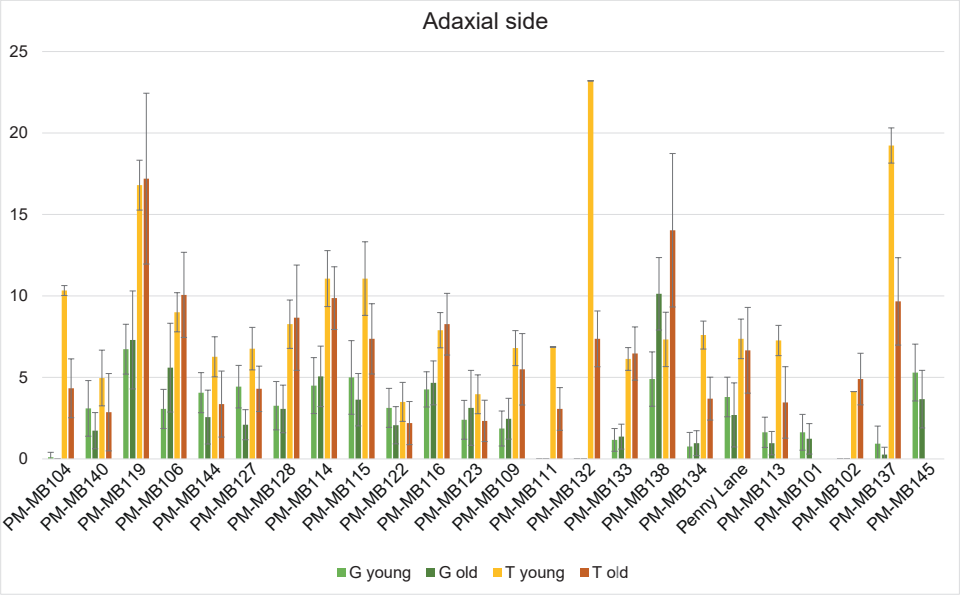


Figure S2. Number of Glandular (G) and T-shaped (T) trichomes on the adaxial side of young and old leaves of 24 *Chrysanthemum* and related genera accessions. The average number of trichomes counted in 6 fields of 0.78 mm² per leaf is plotted per accession. Light green bars represent glandular trichomes on young leaves, dark green bars represent glandular trichomes on old leaves. Yellow bars represent T-shaped trichomes on young leaves and brown bars represent T-shaped trichomes on old leaves. Error bars are standard deviation.

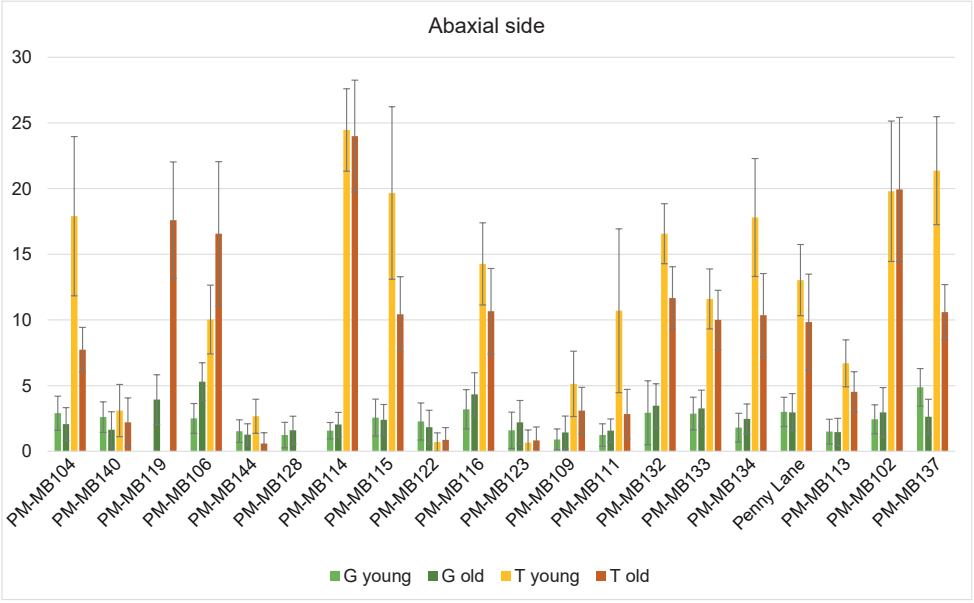
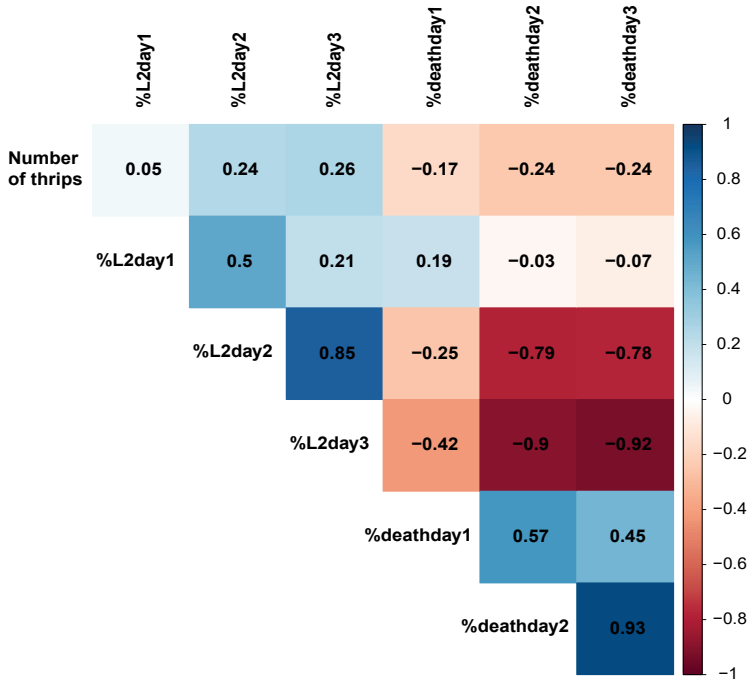


Figure S3. Number of Glandular (G) and T-shaped (T) trichomes on the abaxial side of young and old leaves of 20 *Chrysanthemum* accessions. The average number of trichomes counted in 6 fields of 0.78 mm² per leaf is plotted per accession. Light green bars represent glandular trichomes on young leaves, dark green bars represent glandular trichomes on old leaves. Yellow bars represent T-shaped trichomes on young leaves and brown bars represent T-shaped trichomes on old leaves. Error bars are standard deviation.

Table S1. Spearman correlation matrix for validation experiment. 'Number of thrips': mean number of thrips per plant per accession. '%L2day1', '%L2day2', '%L2day3': proportion of larvae that developed into L2 stage over the total number of larvae on day 1, 2, 3, respectively. '%deathday1', '%deathday2', '%deathday3': proportion of larvae that died over the total number of larvae on day 1, 2, 3, respectively.



'Number of thrips': mean number of thrips per plant per accession.

'%L2day1', '%L2day2', '%L2day3': proportion of L2 larvae over the total number of larvae on day 1, 2, 3, respectively.

'%deathday1', '%deathday2', '%deathday3': proportion of larvae that died over the total number of larvae on day 1, 2, 3, respectively.

Chapter 4

Variation in virulence of thrips species and populations on wild *Chrysanthemum*

Marcella Bovio, Roeland E. Voorrips, Ben Vosman,
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Abstract

Populations of pest insects can differ in their responses to resistant plants, which can significantly impact the durability of plant resistance. Differential fitness of biotypes or populations of thrips *Frankliniella occidentalis* has been reported previously and, furthermore, new invasive thrips species, such as *Thrips parvispinus*, may not be affected by an identified resistance. In this study, we assessed the virulence of *F. occidentalis* populations collected in the Netherlands on various *Chrysanthemum* accessions and characterized the genetic diversity of these populations. We also examined the resistance of the *Chrysanthemum* accessions against *Thrips tabaci* and *T. parvispinus*. Significant differences in the development of thrips larvae (from the L1 to L2 stage) across five *F. occidentalis* populations on the five evaluated *Chrysanthemum* accessions were found. Two accessions, *Chrysanthemum seticuspe* PB-MB133 and *Chrysanthemum x morifolium* Penny Lane, were consistently resistant, exhibiting low larval development for all *F. occidentalis* populations. Mitochondrial CO1 gene analysis revealed five distinct haplotypes among *F. occidentalis* individuals from different populations, belonging to both the glasshouse and lupin strains. Furthermore, when comparing thrips larval performance on various *Chrysanthemum* accessions for the three thrips species, *F. occidentalis*, *T. tabaci*, and *T. parvispinus*, we found significant effects of plant accession, thrips species, and their interactions on larval development. Penny Lane exhibited suppression of larval development for only *F. occidentalis*, whereas *C. seticuspe* PB-MB133 suppressed larval development for all three thrips species tested. Interestingly, *C. seticuspe* PB-MB132, previously identified as susceptible to *F. occidentalis*, suppressed *T. parvispinus* development, indicating that in *C. seticuspe* multiple mechanisms of resistance might be present. In conclusion, our findings demonstrate that thrips populations infesting *Chrysanthemum* differ in virulence, and they highlight the importance of screening with multiple populations. Moreover, our study identified *Chrysanthemum* accessions exhibiting resistance against multiple thrips species.

Introduction

Populations of pest insects can differ in their responses to resistant plants and these differences have consequences for the durability of resistance (De Kogel et al., 1998, 1997; Taggar and Arora, 2017). Insect populations or biotypes that have overcome the resistance are referred to as virulent, while those whose survival or reproduction is affected are defined as avirulent (Smith, 2005). Examples of differential survival or fitness of biotypes on resistant plants have been reported for aphids and thrips (De Kogel et al., 1998, 1997; Kim et al., 2008; Smith and Chuang, 2014). Virulence can rapidly evolve like in the case of aphid *Aphis glycines* on soybean, which was reported four years after the identification of the resistance source, and before this resistance was widely distributed (Hill et al., 2004; Kim et al., 2008). Furthermore, new invasive insect species and biotypes that turn out to be virulent on resistant plant genotypes can disperse.

Frankliniella occidentalis and *Thrips tabaci* (Thysanoptera: Thripidae) are two thrips pest species widely distributed. In Europe, chrysanthemum cultivation is heavily affected by *F. occidentalis*, but other species are also present in greenhouses. *Frankliniella occidentalis*, also known as Western Flower Thrips (WFT), is considered a species complex of two strains, the lupin strain and the glasshouse strain (Nielsen et al., 2010; Rugman-Jones et al., 2010). They both originate from Western North America, but only the more aggressive glasshouse strain spread all over Europe starting from the Netherlands in 1983. It is now the main thrips species in many greenhouse crops (Kirk and Terry, 2003). Its rapid spread is favored by its short life cycle, high reproduction rate, and wide host range. *Thrips tabaci*, also known as onion thrips, is a cryptic species complex consisting of three species or lineages, one tobacco lineage and two leek lineages, that differ in several aspects, such as reproductive mode, geographical distribution and host plants (Brunner et al., 2004). In Europe, all three lineages are widely distributed. The leek lineage 1 and the tobacco lineage are both reported on a restricted number of hosts, *Allium* spp. and tobacco, respectively. Both lineages are associated with mainly arrhenotokous reproduction, in which females develop from fertilized eggs, and males develop from unfertilized eggs and are haploid. The leek lineage 2 is the most common with hundreds of host species reported, including *Chrysanthemum* (Loredo Varela and Fail, 2022). It is characterized by thelytokous reproduction, in which females develop from unfertilized eggs, and the males are absent or present in small numbers.

New (invasive) species such as *Thrips parvispinus* (Thysanoptera: Thripidae) may also pose a threat to European horticulture in the coming years (Thorat et al., 2022). *Thrips parvispinus*, or pepper thrips, originates from tropical Asia. It affects many

crops, both vegetables like pepper and ornamentals like rose and chrysanthemum (The Netherlands Plant Protection Organization, 2019). *Thrips parvispinus* showed an important extension of its geographical distribution in the past 20 years (Thorat et al., 2022). In India, it has displaced *Scirtothrips dorsalis* on chilli cultivation; in Indonesia, it has displaced *Thrips palmi* (Sridhar et al., 2021). Recently, it has been reported also in Spain and the Netherlands (Lacasa et al., 2019; The Netherlands Plant Protection Organization, 2019), and climate change may increase the potential impact of this tropical pest in Europe.

Climate warming creates suitable environmental conditions for the migration of pests from tropical to temperate regions expanding their distribution range and, thus, increasing the chances of establishment of these invasive species (Skendžić et al., 2021). Moreover, an increase in mean temperature favors higher developmental rates, shorter generation time, increased population size, and higher outbreak frequencies (Schneider et al., 2022). To obtain durable and broad-spectrum host plant resistance, it is important to study different pest species and populations when screening for suitable donor material to be used in breeding. The aim of this paper is to evaluate differences in virulence of *F. occidentalis* populations collected across the Netherlands on *Chrysanthemum* accessions, as well as to characterize the genetic diversity and structure of the F. occidentalis populations used for screening. Moreover, we assessed the resistance of *Chrysanthemum* accessions to *T. tabaci* and *T. parvispinus*, intending to identify an accession with broad-spectrum thrips resistance.

Materials and Methods

Thrips populations collection and rearing

One population of thrips *F. occidentalis* (OCC1) was started from females collected randomly from the standing *F. occidentalis* rearing on chrysanthemum plants kept at Plant Breeding, Wageningen University & Research, Wageningen. Three populations (OCC2, OCC3, OCC5) of *F. occidentalis* were collected from three locations in the Netherlands representing areas where chrysanthemums are grown (Table 1). The thrips were collected from *Chrysanthemum* flowers and were confirmed via microscopic observation to be *F. occidentalis*. A fifth *F. occidentalis* population (OCC4) was obtained from a rearing maintained on green beans (Wageningen, NL). Further, females were collected randomly from a *T. tabaci* rearing on leek at Wageningen University & Research, Wageningen (Porta et al., 2023). Finally, a population of *T. parvispinus* was obtained from the Greenhouse Horticulture & Flower Bulbs group,

Wageningen University and Research, Bleiswijk. All populations were maintained on snack cucumbers, each in a separate climate cabinet, at 25°C, 70% RH and 16-8 h light-dark for at least six weeks prior to the experiments.

Synchronized thrips larvae (L1) were obtained by letting females oviposit for 24 h on fresh snack cucumbers. After removal of the females, the snack cucumbers were placed on paper in glass jars and incubated for four days in a climate cabinet after which L1 larvae were transferred with a fine brush onto leaf material (25°C, 16-8 h light-dark and 70% RH) (Mollema et al., 1993).

Table 1. Thrips species and populations (with code) used in assays. Location of collection, host, collection date, and number of individuals transferred to cucumbers are presented for each population. NL: The Netherlands

Species	Location of collection	Collected on	Collection date of original population	Number of individuals placed on cucumbers	Code
Frankliniella occidentalis [1,2]	Wageningen, NL	Chrysanthemum (rearing)	~8 years ago	> 100	OCC1
F. occidentalis [1]	Honselersdijk, NL	Chrysanthemum	June 2022	> 100	OCC2
F. occidentalis [1]	Bleiswijk, NL	Chrysanthemum	June 2022	> 100	OCC3
F. occidentalis [1]	Wageningen, NL	Green beans (rearing)	2020	~30	OCC4
F. occidentalis [1]	Andijk, NL	Chrysanthemum, gerbera	July 2022	~5	OCC5
Thrips tabaci [1,2]	Groningen, NL	Onion, leek (rearing)	August 2021	~30	TAB1
Thrips parvispinus [2]	Bleiswijk, NL	Collected on anthurium, green beans (rearing)	2020	~30	PAR1

[1] populations used in leaf disc assays in 2022

[2] populations used in leaf disc assays in 2023

Plant material and thrips assays

Thrips performance was assessed on six chrysanthemum accessions: *Chrysanthemum x morifolium* cultivars Penny Lane and Super Pink Pompon, *Chrysanthemum seticuspe* accessions PB-MB132 and PB-MB133, and *Chrysanthemum makinoi* accessions PB-MB119 and PB-MB120. Penny Lane and Super Pink Pompon were obtained from Dekker Chrysanten BV, the Netherlands. Accession PB-MB119 was obtained from the National Agriculture and Food Research Organization (NARO),

Tsukuba, and PB-MB120, PB-MB132, and PB-MB133 were obtained from University of Hiroshima, Hiroshima, Japan. The accessions were selected based on their thrips-resistance level assessed with the *F. occidentalis* OCC1 population maintained on chrysanthemum at WUR Plant Breeding (Chapter 3). Cuttings of the accessions were grown without pesticides for at least 6 weeks prior to the experiments. The thrips assays were conducted on 6-week-old plants. The plants were grown at 25-21 °C, 16-8 h light-dark conditions from August to October 2022, and at 23-21 °C, 16-8 h light-dark conditions from May to June 2023, in the greenhouse facilities of Unifarm, Wageningen.

Larval development and survival

We used leaf disc assays to determine larval survival and development. Leaf discs of 1.5 cm diameter were punched from the 1st and 2nd fully expanded leaves counting from the top of the three 6-week-old plants. The leaf discs were placed with the abaxial side upward on a drop of 1.5% water agar just before it solidified. The leaf discs were placed in 50*9 mm Falcon Tight-Fit Lid Petri dishes, with 1 cm diameter holes in the lids covered with 70-micron fine mesh to allow ventilation but prevent thrips escape. Five newly hatched L1 larvae (less than 2 h old) were placed on each leaf disc and the Petri dishes were incubated in a climate cabinet at 25 °C, 16:8 h light:dark period, and 70% RH (Maharijaya et al., 2012; Van Haperen et al., 2019). After four days, the larvae were observed with a stereomicroscope and the number of L1 larvae, the number of L2 larvae, and their survival were determined. Two experiments were performed, one in September 2022 with five replicates of each combination of plant accession and thrips population, and one in 2023 with six replicates of each combination of plant accession and thrips species.

Thrips genotyping

After we obtained the larvae for the thrips assays in 2022, we randomly collected 30 female thrips per population from the six rearings we established on cucumber and stored them in 96% ethanol. Each individual was crushed with a plastic grinder in a single 1.5 ml Eppendorf tube. We extracted mitochondrial DNA from individual thrips with the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the suppliers' recommendations. DNA was stored at −20 °C prior to PCR amplification. The populations were analysed for variation in a 433 bp DNA segment from the 5' end of the mitochondrial Cytochrome Oxidase subunit 1 (*CO1*) gene sequenced by the universal *CO1* primer pairs mtD7.2F (5' ATTAGGAGCHCCHGAYATAGCATT 3') and mtD9.2R (5' CAGGCAAGATTAAAATATAAACTTCTG 3') (Brunner et al., 2002). Polymerase chain reaction (PCR) was conducted using the QIAGEN Multiplex PCR Kit (Qiagen, Germany) in 12 µl reaction volume composed of 6 µl Multiplex PCR

kit solutions, 0.25 µl 10 nM mtD7.2F primer, 0.25 µl 10 nM mtD9.2R primer, 3.5 µl thrips DNA, 2 µl ddH2O. The thermocycling profile was 15 min at 95 °C; 35 cycles of 1 min at 95 °C, 1 min at 50 °C, and 1 min at 72 °C; and a final extension of 10 min at 72 °C. Amplification products were checked on agarose gel, and 4 µl of the reaction product was used for sequencing by Macrogen Europe B.V. No purification step was performed because only one band was visible on the agarose gel.

Thrips genetic diversity analysis and phylogenetic analysis

Nucleotide sequences from both strands of the 433 bp *CO1* gene fragment were assembled with highest sensitivity settings, primer sequences were trimmed and aligned with ClustalW in Geneious R10.0.9 (Kearse et al., 2012; Thompson et al., 1994). The *CO1* haplotypes, their number, their frequency in each population (f), number of polymorphic sites (S), fraction of nucleotide differences (k), haplotype diversity (Hd), and nucleotide diversity (Pi) were analysed in MEGA7 with standard settings (Kumar et al., 2016). Nucleotide differences (k) is the average number of pairwise nucleotide differences within a population. Haplotype diversity (Hd) was calculated as Hd = 1 - Σ(f_i)2, where f_i is the frequency of the i-th haplotype. Hd is a measure of genetic diversity that takes into account both the number of haplotypes and their frequencies in a population. Nucleotide diversity (Pi), or Nei's genetic diversity, represents the degree of genetic variation within a population at the nucleotide level. Pi is the average number of nucleotide differences per site between two sequences in all possible combinations in the population.

A Neighbor-joining tree of consensus *CO1* fragment sequences was constructed to scale, with the number of branches at bootstrap values > 50% obtained with Kimura's two-parameter (K2P) distance model and 1000 bootstraps. The percentage of replicate trees in which the associated haplotypes clustered together is presented next to the branch in MEGA7 (Kumar et al., 2016; Saitou and Nei, 1987). Species confirmation was done through BLASTn analysis with the sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/blast/). Five *CO1* sequences of *F. occidentalis* (accessions JN790696.1-JN790700.1) from X. M. Yang et al. (2012) were retrieved from the NCBI database, aligned with the haplotypes found in our study, and trimmed to the same length. Based on the analysis of 10 sequences of *F. occidentalis*, a Neighbor-joining tree of consensus *CO1* fragment sequences was constructed to scale in MEGA7 with the number of branches at bootstrap values >50% obtained with Kimura's two-parameter (K2P) distance model and 1000 bootstraps. The percentage of replicate trees in which the associated haplotypes clustered together is shown next to the branch.

Statistical analysis

Larval development was expressed as (number of L2)/(number of L1+L2) for the five thrips in each Petri dish and transformed to y=arcsin(sqrt(x)). The effects on larval development of thrips population or species, chrysanthemum accession, and their interactions were analyzed with two-way ANOVAs, and means were compared with Tukey multiple comparisons with adjusted p-value ($\alpha = 0.05$). Pearson's correlation analysis was performed between *F. occidentalis* haplotype frequencies and thrips resistance expressed as larval development on five *Chrysanthemum* accessions.

Results

Development of thrips larvae differs among plant genotypes and *F. occidentalis* populations

Development of thrips larvae from the first (L1) to second (L2) stage from five *F. occidentalis* populations was evaluated on five *Chrysanthemum* accessions (Figure 1). The effects of accession and population on larval development were both significant, but their interaction was not (ANOVA; accession: $p < 0.0001$; population: $p < 0.0001$; accession:population: $p > 0.05$; Table S1). For all *F. occidentalis* populations, larval development was consistently low on two accessions, *C. seticuspe* PB-MB133 and Penny Lane, while it was high on cultivar Super Pink Pompon, which was included as susceptible control. On *C. makinoi* accessions PB-MB119 and PB-MB120, larval development varied depending on the thrips population tested: more larvae from the OCC3, and OCC5 populations developed into L2 stage than from the OCC1 populations. In addition, the performance of *T. tabaci* larvae was evaluated on the five *Chrysanthemum* accessions. Super Pink Pompon allowed high larval development of *T. tabaci*, similar to that of *F. occidentalis* (Figure 2). On *C. makinoi* PB-MB119 and PB-MB120, more larvae of *T. tabaci* than of *F. occidentalis* OCC1 developed into the L2 stage. On Penny Lane development of *F. occidentalis* OCC1 larvae was suppressed but not that of *T. tabaci*. On *C. seticuspe* PB-MB133 larval development of both *F. occidentalis* OCC1 and *T. tabaci* were suppressed.

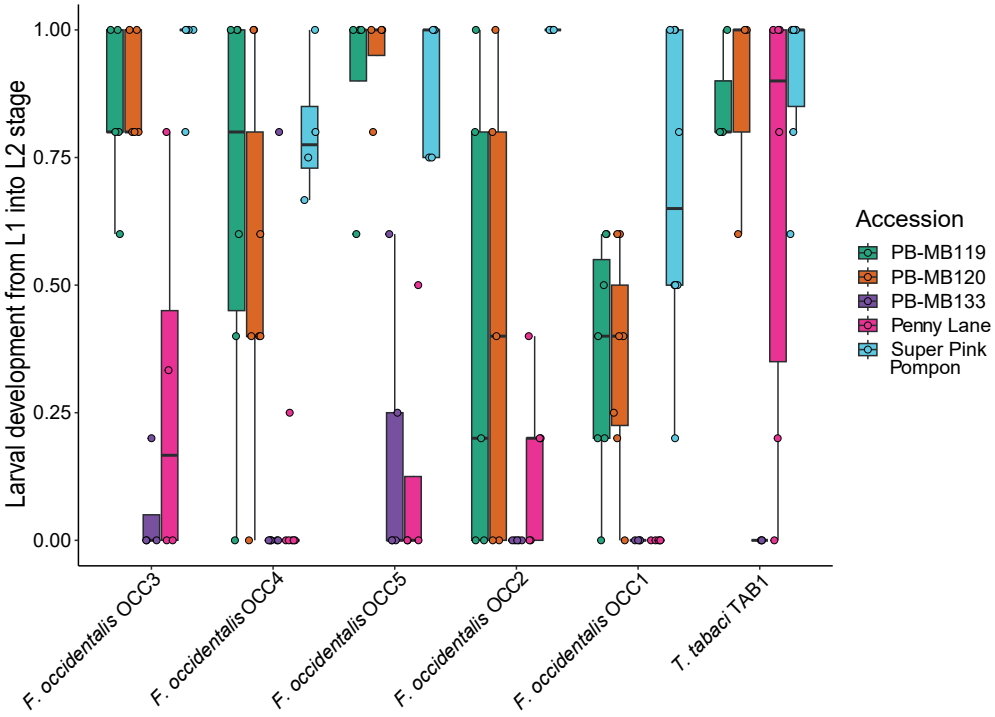


Figure 1. Larval development of thrips from five *F. occidentalis* populations and one *T. tabaci* population on five *Chrysanthemum* accessions. Larval development is expressed as the fraction of larvae that developed from L1 to L2 stage in 5 days. Dots indicate single observations of larval development per plant accession (N=5). Boxplots show the quartiles, min and max) of the observations of larval development per plant accession. Super Pink Pompon (in light blue) was included as susceptible reference.

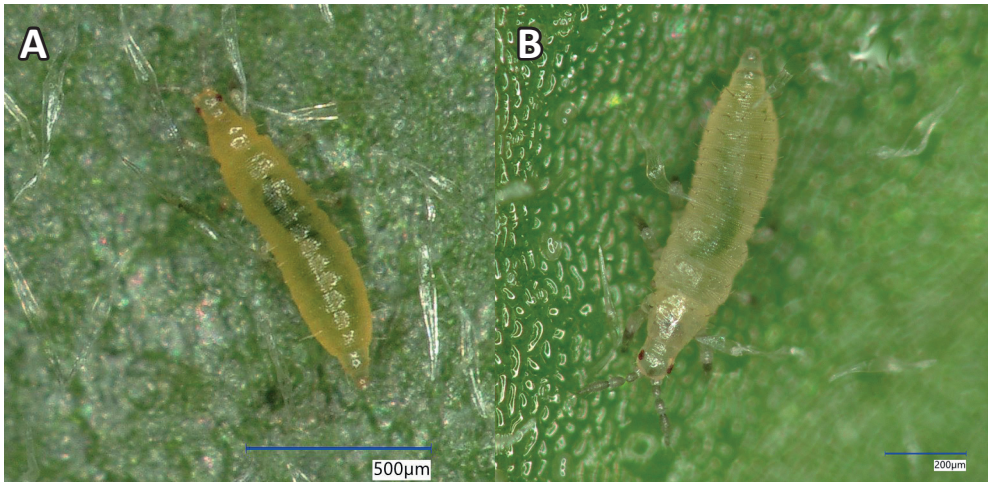


Figure 2. L2 larvae of A) *F. occidentalis* and B) *T. tabaci* feeding on Super Pink Pompon (abaxial side of leaf disc). T-shaped trichomes are also visible.

Frankliniella occidentalis populations differ in *CO1* haplotype composition

The alignment of the mitochondrial *CO1* gene fragment showed five haplotypes among the 150 individuals of *F. occidentalis* from five populations (Hap_1, 2, 3, 4, 9, Table S3), and was based on 20 polymorphic sites (S). The number of haplotypes per population (h) varied from 3 to 5 (Table 2, Figure 3). The fraction of nucleotide differences (k) varied depending on the populations compared and ranged between 0.00 to 0.01 (Table 3), whereas the maximum nucleotide difference for 433 base pairs would be 0.046. Nucleotide diversity, or Nei's genetic diversity, within population averaged at Pi = 0.005, while overall Pi was 0.006. Distinct patterns of haplotype diversity (Hd) among the five populations of *F. occidentalis* were found, Hd values varied between 0.1310 and 0.7057 (Table 2). Haplotype 1 was the most frequent haplotype across all populations, except in the OCC4 population (Figure 3). Haplotype 3 was the second most common, and it was found mainly in OCC4, OCC3, and OCC1 populations. Haplotype 4 was co-dominant in the OCC4 population, in a minor frequency present in the OCC3 population, but absent in the other populations. Haplotype 2 was present in a low frequency in all populations. Haplotype 9 was found in only two populations (OCC1 and OCC3), with a total of 8 individuals out of 150.

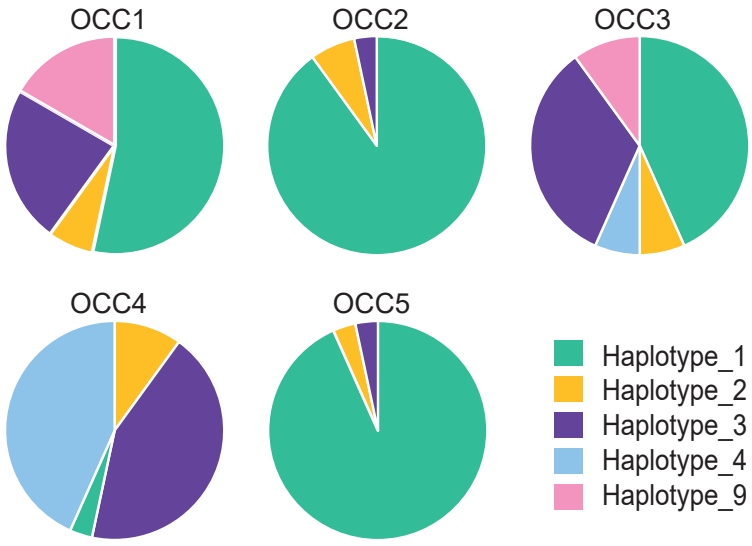


Figure 3. *CO1* mitochondrial haplotype frequencies per *Frankliniella occidentalis* population. Thirty thrips were individually genotyped per population.

Table 2. Haplotype diversity (Hd) and number of haplotypes (h) per *Frankliniella occidentalis* population.

Population	Hd	h
OCC1	0.6506	4
OCC2	0.1908	3
OCC3	0.7057	5
OCC4	0.6345	4
OCC5	0.1310	3

Table 3. Estimates of evolutionary divergence over sequence pairs between populations. The fraction of nucleotide differences (k), the average number of pairwise nucleotide differences between populations are shown. The analysis involved 150 sequences of 433 nucleotides. Analyses were conducted in MEGA7 with standard settings (Kumar et al., 2016).

Nucleotide differences (k)	OCC1	OCC2	OCC3	OCC4
OCC2	0.007			
OCC3	0.010	0.005		
OCC4	0.010	0.004	0.008	
OCC5	0.007	0.000	0.005	0.004

Thrips tabaci population genetic diversity

Four *CO1* mitochondrial haplotypes were found in the genotyped *T. tabaci* population, presented as haplotype 5, 6, 7 and 8. The *T. tabaci* population consisted mainly of haplotype 5 (27 out of 30 individuals), the other haplotypes were present with one individual each (Table S4). The haplotype diversity (Hd) in this population was 0.1931.

Phylogeny of *F. occidentalis* and *T. tabaci* haplotypes

Based on the homology between the sequences and the data reported in the NCBI database, Haplotype 1 to 4, and 9 were confirmed to be *F. occidentalis*, whereas haplotype 5 to 8 were confirmed to be *T. tabaci*. In the unrooted NJ tree constructed using the *CO1* mitochondrial haplotypes from our study (Figure 4), the four *T. tabaci* haplotypes separate from the five *F. occidentalis* haplotypes. Moreover, the *T. tabaci* haplotypes form two lineages. Among the *F. occidentalis* haplotypes, haplotype 9 forms a different lineage. This haplotype_9 was 100% identical (BLASTn) to JN790699.1 from X. M. Yang et al. (2012) (Figure 5).

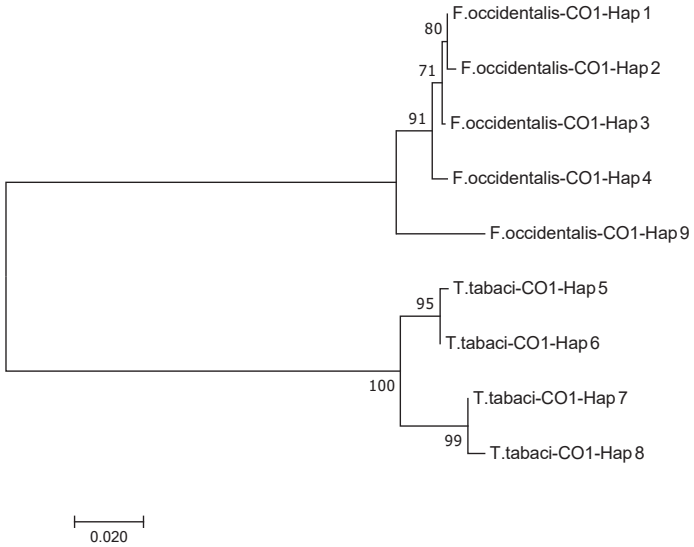


Figure 4. Relationship among *CO1* haplotypes of *Frankliniella occidentalis* and *Thrips tabaci* individuals collected in the Netherlands. Neighbor-joining tree showing genetic relationship based on *CO1* sequences among 9 identified haplotypes. The tree is drawn to scale; the number of branches determined by bootstrap values > 50% obtained with K2P model distance (1000 bootstrap replicates). The percentage of replicate trees in which the associated taxa clustered together are shown next to the branches.

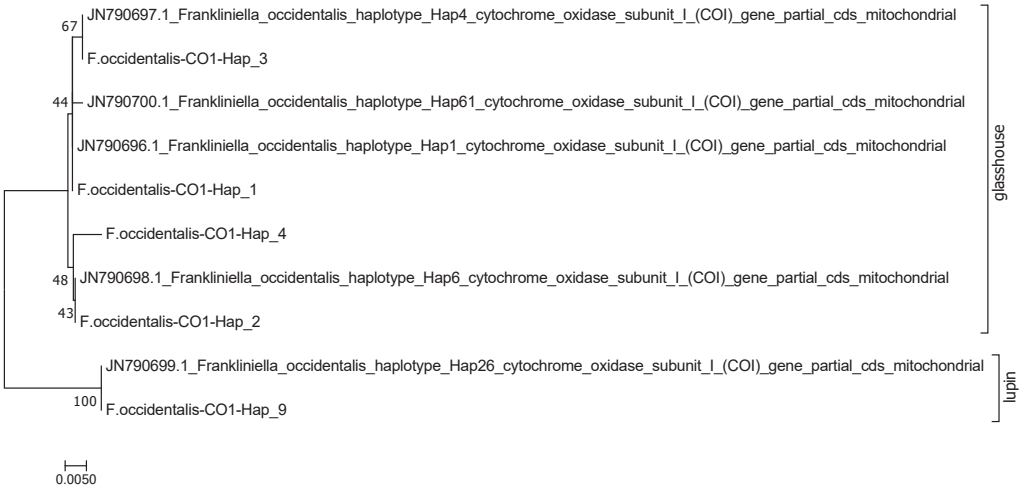
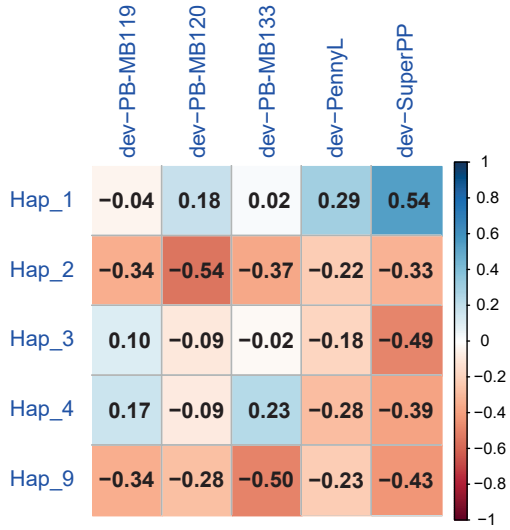


Figure 5. Relationship among *CO1* haplotypes of *Frankliniella occidentalis* collected in the Netherlands and those previously reported in literature (X. M. Yang et al., 2012). Neighbor-joining tree showing genetic relationship based on *CO1* sequences between the 5 haplotypes identified in this study and the 5 haplotypes identified in China. The tree is drawn to scale; the number of branches are bootstrap values > 50% obtained with K2P model distance (1000 bootstrap replicates). The percentage of replicate trees in which the associated taxa clustered together are shown next to the branches. Indicated on the right is the *F. occidentalis* strain, glasshouse or lupin, to which the haplotypes are associated.

No correlation between haplotype composition and larval development

There was no significant correlation between haplotype frequency and resistance measured as larval development (Table 4, S5).

Table 4. Correlation matrix between *Frankliniella occidentalis CO1* haplotype frequencies and thrips resistance measured as larval development on five chrysanthemum accessions. Pearson's correlation coefficients are presented, none of the correlations was significant ($p > 0.05$).



Larval development of *F. occidentalis*, *T. tabaci*, and *T. parvispinus* on *Chrysanthemum* accessions

The larval performance of three species, *F. occidentalis* OCC1, *T. tabaci* and *T. parvispinus*, was evaluated on five *Chrysanthemum* accessions, three accessions that were previously identified as thrips resistant (Chapter 3, Figure 1) and two, including PB-MB132, previously identified as thrips-susceptible (Chapter 3) (Figure 6, S1). The accession, thrips species and their interaction all had a significant effect on larval development (Figure 6, Table S2, ANOVA; accession: $p < 0.0001$; species: $p < 0.001$; accession:species: $p < 0.0001$). On both *C. makinoi* PB-MB119 and Super Pink Pompon accessions, the larvae of *T. parvispinus*, similarly to the ones of *T. tabaci* successfully developed into the L2 stage. Penny Lane suppressed the larval development of *F. occidentalis* OCC1, but not that of *T. tabaci* and *T. parvispinus*. In contrast, *C. seticuspe* PB-MB133 suppressed larval development of *T. parvispinus*, *T. tabaci*, as well as *F. occidentalis* OCC1. *Chrysanthemum seticuspe* PB-MB132 allowed development of both *F. occidentalis* and *T. tabaci*, but it suppressed that of *T. parvispinus*.

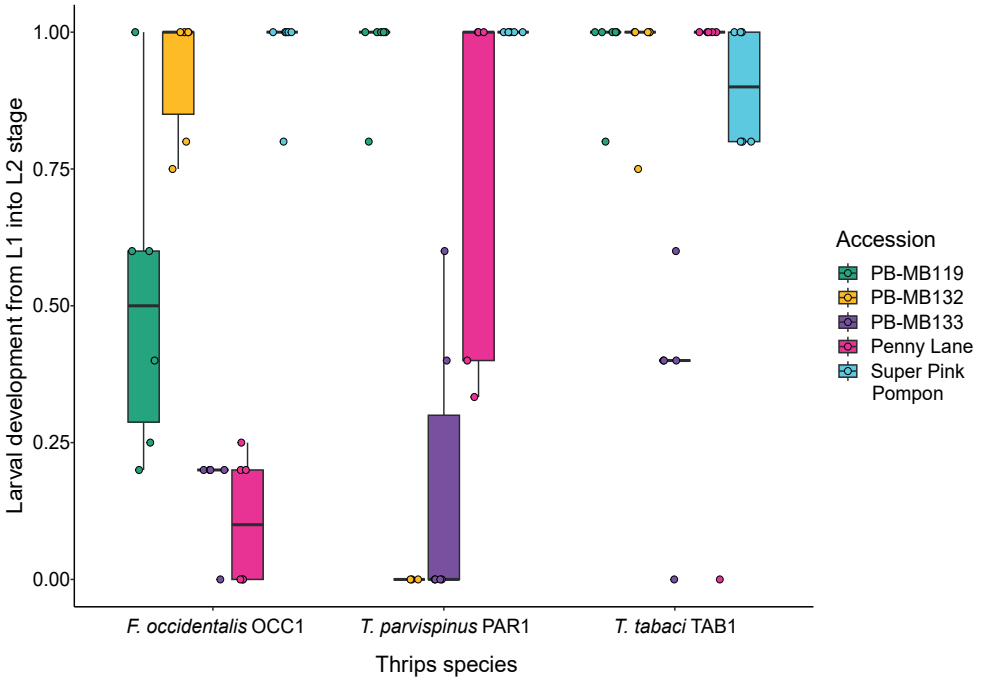


Figure 6. Larval development of thrips from *Frankliniella occidentalis* (OCC1), *Thrips parvispinus* and *Thrips tabaci* on five *Chrysanthemum* accessions. Larval development was expressed as the fraction of larvae that developed from L1 to L2 stage in 5 days. Dots indicate single observations of larval development per plant accession. Six replicates per thrips species and plant accession combination were included. Boxplots show the distribution (median, 25 and 75% quantiles, min and max) of the observations of larval development per plant accession. Super Pink Pompon (in light blue) was included as susceptible reference.

Discussion

Wild *Chrysanthemum* accessions as sources of broad-spectrum resistance

Larval development of five *F. occidentalis* populations was evaluated on five *Chrysanthemum* accessions. *Chrysanthemum seticuspe* PB-MB133 and Penny Lane, both previously identified as resistant (Chapter 2, 3) consistently suppressed *F. occidentalis* larval development of all populations, whereas Super Pink Pompon was consistently susceptible to all populations. Interestingly, the resistance level of *C. makinoi* seemed dependent on the thrips population tested, indicating that the resistance initially identified with population OCC1 (Chapter 3) has been overcome by populations OCC3, OCC4, and OCC5. Furthermore, the contrasting performance

of the three thrips species, *F. occidentalis*, *T. tabaci*, and *T. parvispinus*, on different *Chrysanthemum* accessions highlights the complexity of thrips resistance (Figure 6, S1). While cultivar Penny Lane suppressed *F. occidentalis* development, no effect was found on larval development of *T. tabaci* and *T. parvispinus*. In contrast, the low larval development of all three thrips species on *C. seticuspe* PB-MB133 suggests the presence of broad-spectrum resistance, so far unique among the tested accessions. Interestingly, also *C. seticuspe* PB-MB132 showed resistance against *T. parvispinus*, despite being highly susceptible to both *F. occidentalis* and *T. tabaci* (Figure 6). Thus, *C. seticuspe* might be a source of multiple resistance mechanisms that could be studied further to understand thrips resistance in *Chrysanthemum*. While in this study the focus was on larval development from L1 to L2 stage, in future studies it is important to consider other life-cycle variables as well. This broader approach may provide a more comprehensive understanding of thrips resistance in *Chrysanthemum* and its mechanism(s).

Genetic diversity of *F. occidentalis* populations and their virulence on wild *Chrysanthemum*

We investigated the divergence and genetic distance of five *F. occidentalis* populations from the Netherlands using the mitochondrial Cytochrome Oxidase subunit I (*CO1*) gene. Haplotype 1 was the most frequent haplotype in our study and predominant in all the populations reared or collected on *Chrysanthemum* (Table 1, Figure 2). This was not unexpected as haplotype 1 seems the most common, in fact, it was also one of the most frequent haplotypes reported in *F. occidentalis* populations across China (GenBank accession JN790696.1) (X. M. Yang et al., 2012). We further observed variation in haplotype diversity across the five populations. The *F. occidentalis* OCC1 population is one of the populations with the highest diversity, despite being laboratory-reared on chrysanthemum plants for several years. This could be due to the involuntary introduction of new thrips in this rearing via flowering plant material, which is particularly difficult to check for infestation by eggs. Among the populations collected in the Netherlands in 2022 on flowers, some populations exhibited high genetic diversity, while others show a lower diversity, specifically OCC2 and OCC5 (Table 2). A low population diversity can have several causes; it may indicate possible historical bottlenecks, genetic isolation or founder effects. The latter may have been the case for population OCC5, which derived from a small number of collected individuals. However, it may also depend on the collection method used. In fact, in most cases a limited number of thrips was collected (about 30) on a single location, probably resulting in the collection of closely related individuals. Pest populations from recently invaded regions are expected to have lower genetic diversity than populations from regions closer to the

centre of origin, due to founder effects. Range of haplotype diversity values similar to ours have been reported by comparable studies on *F. occidentalis* populations in non-native areas (Cao et al., 2017; X. M. Yang et al., 2012), whereas the analysis done on thrips collected in Western North America, the centre of origin for thrips *F. occidentalis*, resulted in higher haplotype diversity (Hd = 0.897) (Brunner and Frey, 2010).

We found no correlation between *CO1* haplotype and thrips larval performance on different *Chrysanthemum* accessions. Although no direct effect of the mitochondrial gene *CO1* was expected on the performance of thrips larvae, the correlation analysis was performed to investigate whether a certain *CO1* haplotype could explain the varying levels of virulence towards *Chrysanthemum*. The rationale behind this analysis was to investigate whether the evolutionary history of thrips, which could have affected the frequency of *CO1* haplotypes, might have played a role in potentially breaking down the resistance of *Chrysanthemum* to thrips. The absence of a correlation between *CO1* haplotypes and virulence could indicate that gene flow or migration among different thrips populations led to mixing of *CO1* haplotypes.

Evidence of the presence of two strains of *F. occidentalis* in the Netherlands

Frankliniella occidentalis is currently considered a cryptic species consisting of two distinct and reproductively isolated but morphologically indistinguishable species, with different host-plant preference and pest-status. The glasshouse strain, also known as the hot/dry ecotype, is considered the most invasive and aggressive of the two. On the other hand, the lupin strain, also known as the cool/moist ecotype, is generally considered less widespread and causing less damage (Brunner and Frey, 2010; Nielsen et al., 2010; Rugman-Jones et al., 2010). A previous study determined 20 individuals collected from four populations in the Netherlands to consist of only the glasshouse strain (Mirnezhad et al., 2012). In contrast, in our study we detected haplotypes of both the glasshouse, haplotype 1, 2, 3, and 4, and lupin strains, haplotype 9. The glasshouse strain was predominant, similarly to the *F. occidentalis* populations screened in China (X. M. Yang et al., 2012). In the NJ tree, Haplotype 9 is distinct from the other four haplotypes we detected (Figure 4). Haplotype 9 was detected in 8 individuals in two distinct *F. occidentalis* populations, OCC1 and OCC3, and was 100% identical to the haplotype JN790699.1 which was previously identified as belonging to the lupin strain (X. M. Yang et al., 2012) (Figure 5). Before this study and the ones on the recent introduction of *F. occidentalis* in China (Cao et al., 2017; X. M. Yang et al., 2012), it was thought that the lupin strain was restricted to its native distribution range and New Zealand, and therefore, the glasshouse strain was the only one relevant for pest studies in

the rest of the world. It is possible that previous studies like the one by Mirnezhad et al., (2012) did not detect the lupin strain because of the smaller sample size, or because it was more recently introduced. No clear indications of virulence on *Chrysanthemum* accessions of the lupin strain become evident from our study. In fact, no correlation between *CO1* haplotype and larval development was found. This could be because only a small number of thrips belonged to the lupin strain, and the two populations in which we found these individuals, OCC1 and OCC3, did not show the same virulence on different *Chrysanthemum* accessions. Therefore it is important to investigate how virulent the lupin strain is on plants cultivated in Europe, especially chrysanthemums, and what its current distribution and host range is.

In conclusion, our study sheds light on the genetic diversity of *F. occidentalis* populations in the Netherlands, based on the *CO1* locus. *Chrysanthemum seticuspe* PB-MB133 and Penny Lane were consistently resistant to all *F. occidentalis* populations studied but only *C. seticuspe* PB-MB133 was shown to also suppress the larval development of *T. tabaci* and *T. parvispinus*. These results highlight the importance of studying multiple thrips populations and species when assessing resistance, especially considering current climate change and the possibility of invasive species spreading. Further research is needed to elucidate the genetic basis and mechanisms behind the identified resistance(s) and to explore the potential application of such resistance traits in thrips management strategies for *Chrysanthemum* cultivation.

Acknowledgements

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Supplementary materials

Table S1. Tukey multiple comparisons of means (95% family-wise confidence level) of larval development of five *F. occidentalis* populations on different accessions. Comparisons and relative adjusted p-values are presented, for both accession and thrips population. aov(formula = dev ~ acc * pop, data = R_6thrips).

Accession	p adj
PB-MB120-PB-MB119	> 0.05
PB-MB133-PB-MB119	< 0.0001
Penny Lane-PB-MB119	< 0.0001
Super Pink Pompon-PB-MB119	< 0.01
PB-MB133-PB-MB120	< 0.0001
Penny Lane-PB-MB120	< 0.0001
Super Pink Pompon-PB-MB120	< 0.01
Penny Lane-PB-MB133	> 0.05
Super Pink Pompon-PB-MB133	< 0.0001
Super Pink Pompon-Penny Lane	< 0.0001

Population	p adj
OCC4-OCC3	> 0.05
OCC5-OCC3	> 0.05
OCC2-OCC3	> 0.05
OCC1-OCC3	< 0.01
TAB1-OCC3	> 0.05
OCC5-OCC4	> 0.05
OCC2-OCC4	> 0.05
TAB1-OCC4	> 0.05
TAB1-OCC4	< 0.05
OCC2-OCC5	> 0.05
OCC1-OCC5	< 0.001
TAB1-OCC5	> 0.05
OCC1-OCC2	> 0.05
TAB1-OCC2	< 0.05
TAB1- OCC1	< 0.0001

Table S2. Tukey multiple comparisons of means (95% family-wise confidence level) of larval development of *F. occidentalis*, *T. tabaci* and *T. parvispinus* on different accessions. Only significant comparisons (and relative adjusted p-values) are presented. aov(formula = dev ~ acc * spp, data = R_species)

Tukey multiple comparisons of means	
$Accession:Species	**p adj**
Super Pink Pompon:*T. parvispinus*-Penny Lane:*F. occidentalis*	< 0.0001
Super Pink Pompon:*T. parvispinus*-PB-MB133:*T. parvispinus*	< 0.0001
Super Pink Pompon:*T. parvispinus*-PB-MB132:*T. parvispinus*	< 0.0001
Super Pink Pompon:*F. occidentalis*-Penny Lane:*F. occidentalis*	< 0.0001
PB-MB119:*T. parvispinus*-Penny Lane:*F. occidentalis*	< 0.0001
PB-MB119:*T. tabaci*-Penny Lane:*F. occidentalis*	< 0.0001
PB-MB132:*T. tabaci*-Penny Lane:*F. occidentalis*	< 0.0001
PB-MB133:*T. parvispinus*-Super Pink Pompon:*F. occidentalis*	< 0.0001
PB-MB133:*T. parvispinus*-PB-MB119:*T. parvispinus*	< 0.0001
PB-MB119:*T. tabaci*-PB-MB133:*T. parvispinus*	< 0.0001
PB-MB132:*T. parvispinus*-Super Pink Pompon:*F. occidentalis*	< 0.0001
PB-MB132:*T. parvispinus*-PB-MB119:*T. parvispinus*	< 0.0001
PB-MB119:*T. tabaci*-PB-MB132:*T. parvispinus*	< 0.0001
PB-MB132:*T. tabaci*-PB-MB133:*T. parvispinus*	< 0.0001
PB-MB132:*T. tabaci*-PB-MB132:*T. parvispinus*	< 0.0001
Penny Lane:*F. occidentalis*-PB-MB132:*F. occidentalis*	< 0.0001
PB-MB132:*T. parvispinus*-PB-MB132:*F. occidentalis*	< 0.0001
PB-MB133:*T. parvispinus*-PB-MB132:*F. occidentalis*	< 0.0001
Super Pink Pompon:*T. parvispinus*-PB-MB133:*F. occidentalis*	< 0.0001
Super Pink Pompon:*T. tabaci*-Penny Lane:*F. occidentalis*	< 0.0001
Super Pink Pompon:*T. tabaci*-PB-MB132:*T. parvispinus*	< 0.0001
Super Pink Pompon:*T. tabaci*-PB-MB133:*T. parvispinus*	< 0.0001
Super Pink Pompon:*F. occidentalis*-PB-MB133:*F. occidentalis*	< 0.0001
PB-MB119:*T. parvispinus*-PB-MB133:*F. occidentalis*	< 0.0001
PB-MB119:*T. tabaci*-PB-MB133:*F. occidentalis*	< 0.0001
Penny Lane:*T. tabaci*-PB-MB132:*T. parvispinus*	< 0.0001
Penny Lane:*T. tabaci*-Penny Lane:*F. occidentalis*	< 0.0001
PB-MB132:*T. tabaci*-PB-MB133:*F. occidentalis*	< 0.0001
Penny Lane:*T. tabaci*-PB-MB133:*T. parvispinus*	< 0.0001
PB-MB133:*F. occidentalis*-PB-MB132:*F. occidentalis*	< 0.001
Penny Lane:*T. parvispinus*-PB-MB132:*T. parvispinus*	< 0.001

Table S2. continued.

Tukey multiple comparisons of means	
$Accession:Species	**p adj**
PB-MB133:*T. tabaci*-Super Pink Pompon:*T. parvispinus*	< 0.001
Super Pink Pompon:*T. tabaci*-PB-MB133:*F. occidentalis*	< 0.001
Penny Lane:*T. parvispinus*-Penny Lane:*F. occidentalis*	< 0.001
Penny Lane:*T. parvispinus*-PB-MB133:*T. parvispinus*	< 0.001
Penny Lane:*T. tabaci*-PB-MB133:*F. occidentalis*	< 0.001
PB-MB133:*T. tabaci*-Super Pink Pompon:*F. occidentalis*	< 0.01
PB-MB133:*T. tabaci*-PB-MB119:*T. parvispinus*	< 0.01
PB-MB133:*T. tabaci*-PB-MB119:*T. tabaci*	< 0.01
PB-MB133:*T. tabaci*-PB-MB132:*T. tabaci*	< 0.01
PB-MB133:*T. tabaci*-PB-MB132:*F. occidentalis*	< 0.01
Penny Lane:*T. parvispinus*-PB-MB133:*F. occidentalis*	< 0.01
Super Pink Pompon:*T. parvispinus*-PB-MB119:*F. occidentalis*	< 0.05
Super Pink Pompon:*T. tabaci*-PB-MB133:*T. tabaci*	< 0.05
Penny Lane:*T. tabaci*-PB-MB133:*T. tabaci*	< 0.05
PB-MB132:*T. parvispinus*-PB-MB119:*F. occidentalis*	< 0.05
Super Pink Pompon:*F. occidentalis*-PB-MB119:*F. occidentalis*	< 0.05
PB-MB119:*T. parvispinus*-PB-MB119:*F. occidentalis*	< 0.05
PB-MB119:*T. tabaci*-PB-MB119:*F. occidentalis*	< 0.05

Table S3. Nucleotide sequences of *CO1* haplotypes found in 5 *F. occidentalis* and one *T. tabaci* populations. The *CO1* sequences had 20 polymorphic sites, at positions 16, 58, 75, 99, 138, 162, 171, 183, 190, 204, 213, 216, 307, 312, 339, 351, 366, 396, 406, and 414.

Hap_1 – *Frankliniella occidentalis*

TCCTCGACTTAATAACATAAGATTTTGACTTCTTCCACCCTCTTTAACATTGTTAATTATAGGTTTATCAAAA-
GATGGTGCAGGAACAGGATGAACAGTTTACCCACCTTTGTCAACTTTTTATCACTCTGGACCATCAG-
TAGATTTAACTATTTTTTCCCTTCATTTAGCAGGTATTTCTTCAATTCTAGGAGCTTTAAATTTTATTA-
CAACGATTTTAAATTTAAAGATCAAAAAATTAACAACGGAAAAGATAACTTTATTTGTTTGATCAGTTAT-
TTTAACAGCTATTTTATTATTATTGTCGTTACCAGTTTTAGCAGGAGCTATTACAATATTATTAACAGAC-
CGAAACTTAAATACATCCTTCTTTGATCCGAGAGGAGGTGGGGACCCAGTTTTATACCAGCACTTGTTTT-
GATTTTTTGGGCACC

Hap_2 – *F. occidentalis*

TCCTCGACTTAATAACATAAGATTTTGACTTCTTCCACCCTCTTTAACATTGTTAATTATAGGTTTATCAAAA-
GATGGTGCAGGAACAGGATGAACAGTTTACCCACCTTTGTCAACTTTTTATCACTCTGGACCATCAG-
TAGATTTAACTATTTTTTCCCTTCATTTAGCAGGTATTTCTTCAATTCTAGGAGCTTTAAATTTTATTA-
CAACGATTTTAAATTTAAAGATCAAAAAATTAACAACGGAAAAGATAACTTTATTTGTTTGATCAGTTAT-
TTTAACAGCTATTTTATTATTATTGTCGTTACCAGTTTTAGCAGGAGCTATTACGATATTATTAACAGAC-
CGAAACTTAAATACATCCTTCTTTGATCCGAGAGGAGGTGGGGACCCAGTTTTATACCAGCACTTGTTTT-
GATTTTTTGGGCACC

Table S3. continued.

Hap_3 – *F. occidentalis*
TCCTCGACTTAATAACATAAGATTTTGACTTCTTCCACCCTCTTTAACATTGTTAATTATAGGTTTATCAAAA-GATGGTGCAGGAACAGGATGAACAGTTTACCCACCTTTGTCAACTTTTTATCACTCTGGACCATCAG-TAGATTTAACTATTTTTTCCCTTCATTTAGCAGGTATTTCTTCAATTCTAGGAGCTTTAAATTTTATTA-CAACGATTTTAAATTTAAAGATCAAAAAATTAACAACGGAAAAGATAACTTTATTTGTTTGATCAGTTAT-TTTAACAGCTATTTTATTATTATTGTCGTTACCAGTTTTAGCAGGAGCTATTACAATATTATTAACAGAC-CGAAACTTAAATACATCCTTCTTTGATCCGAGAGGAGGTGGAGACCCAGTTTTATACCAGCACTTGTTTT-GATTTTTTGGGCACC
Hap_4 – *F. occidentalis*
TCCTCGACTTAATAACATAAGATTTTGACTTCTTCCACCCTCTTTAACATTGTTAATTATAGGTTTATCAAAA-GATGGTGCAGGAACAGGATGAACAGTTTACCCACCTTTGTCAACTTTTTATCACTCTGGACCCTCAG-TAGATTTAACTATTTTTTCCCTTCATTTAGCAGGTATTTCTTCAATTCTAGGAGCTTTAAATTTTATTACGA-CAATTTTAAATTTAAAGATCAAAAAATTAACAACGGAAAAGATAACTTTATTTGTTTGATCAGTTATTTTAA-CAGCTATTTTATTATTATTGTCGTTACCAGTTTTAGCAGGAGCTATTACAATATTATTAACAGACCGAAACT-TAAATACATCCTTCTTTGATCCGAGAGGAGGTGGAGACCCAGTTTTATACCAGCACTTGTTTTGATTTTTTG-GGCACC
Hap_9 – *F. occidentalis*
TCCTCGACTTAATAATATAAGATTTTGACTTCTTCCACCCTCTTTAACATTGTTAATCATAGGTTTATCAAA-GGATGGTGCAGGAACAGGATGAACGGTTTACCCACCTTTGTCAACTTTTTATCACTCTGGACCATCAG-TAGATTTAACTATTTTTTCTCTTCATTTGGCAGGTATTTCCTCAATTTTAGGAGCTTTAAACTTTATTACAA-CAATTTTAAATTTAAAGATCAAAAAATTAACAACGGAAAAGATAACTTTATTTGTTTGATCAGTTATTTTAA-CAGCTATTTTATTATTACTGTCATTACCAGTTTTAGCAGGAGCTATTACAATATTATTAACGGACCGAAACT-TAAACACATCCTTCTTTGATCCGAGAGGAGGTGGGGACCCAGTTCTATACCAACACTTGTTTTGATTTTTTG-GGCACC
Hap_5 – *Thrips tabaci*
CCCTCGATTAAATAATATAAGATTCTGACTTTTACCCCCTTCTCTGGGATTATTAATTATAGGACTTTATAAA-GAAGGAGCGGGAACGGGATGAACAGTATATCCACCTTTATCAACGTTTTATCATTCAGGACCTTCAGTA-GACTTAACAATTTTTTCTTTACACCTTGCAGGGATTTCTTCAATTTTAGGTGCCTTAAATTTTATTACTACAAT-TATTAATCTTAAAGCAAAAAACCTTTCAGCAGAAAAAATTAGACTATTTGTCTGATCAGTTATTTTAACAGC-CATTCTTCTTCTTTTATCTTTGCCAGTGTTAGCGGGAGCTATCACAATACTTTTAACTGACCGAAACTTAAAT-ACCTCTTTTTTTGACCCTAGAGGGGGAGGGGACCCTGTTTTATATCAACACCTTTTTTGATTTTTTGGGT-CACC
Hap_6 – *T. tabaci*
CCCTCGATTTAATAATATAAGATTCTGACTTTTACCCCCTTCTCTGGGATTATTAATTATAGGACTTTATAAA-GAAGGAGCGGGAACGGGATGAACAGTATATCCACCTTTATCAACGTTTTATCATTCAGGACCTTCAGTA-GACTTAACAATTTTTTCTTTACACCTTGCAGGGATTTCTTCAATTTTAGGTGCCTTAAATTTTATTACTACAAT-TATTAATCTTAAAGCAAAAAACCTTTCAGCAGAAAAAATTAGACTATTTGTCTGATCAGTTATTTTAACAGC-CATTCTTCTTCTTTTATCTTTGCCAGTGTTAGCGGGAGCTATCACAATACTTTTAACTGACCGAAACTTAAAT-ACCTCTTTTTTTGACCCTAGAGGGGGAGGGGACCCTGTTTTATATCAACACCTTTTTTGATTTTTTGGGT-CACC

Table S3. continued.

Hap_7 – *T. tabaci*
CCCTCGATTAAATAATATAAGATTCTGACTTTTACCCCCTTCTCTAGGATTATTAATTATAGGACTTTATAAA- GAAGGAGCGGGAACAGGATGAACAGTGTATCCACCTTTATCAACATTTTATCATTCAGGACCTTCAGTA- GACTTAACAATTTTTTCTTTACACCTTGCAGGGATTTCTTCAATTTTGGGTGCCTTAAATTTTATTACTACAAT- TATTAATCTTAAAGCAAAAAACCTTTCAGCAGAAAAAATTAGACTATTTGTCTGATCAGTTATTTTAACAGC- TATTCTCCTTCTTTTATCTCTGCCAGTGTTAGCAGGAGCTATCACAATACTTTTAACTGACCGAAATTTAAAT- ACTTCTTTTTTTGACCCTAGGGGGGGAGGAGACCCTGTCTTATATCAACACCTTTTTTGATTTTTTGGTCACC
Hap_8 – *T. tabaci*
CCCTCGATTAAATAATATAAGATTCTGACTTTTACCCCCTTCTCTGGGATTATTAATTATAGGACTTTATAAA- GAAGGAGCGGGAACAGGATGAACAGTGTATCCACCTTTATCGACATTTTATCATTCAGGACCTTCAGTA- GACTTAACAATTTTTTCTTTACACCTTGCAGGGATTTCTTCAATTTTGGGTGCCTTAAATTTTATTACTACAAT- TATTAATCTTAAAGCAAAAAACCTTTCAGCAGAAAAAATTAGACTATTTGTCTGATCAGTTATTTTAACAGC- TATTCTCCTTCTTTTATCTCTGCCAGTGTTAGCAGGAGCTATCACAATACTTTTAACTGACCGAAATTTAAAT- ACTTCTTTTTTTGACCCTAGGGGGGGAGGAGACCCTGTCTTATATCAACACCTTTTTTGATTTTTTGGTCACC

Table S4. *CO1* haplotypes found in 5 *F. occidentalis* and one *T. tabaci* populations. Per haplotype, number of individuals per species (N) and frequency are presented.

Haplotype	N	Frequency
Hap_1 – *Frankliniella occidentalis*	85	0.472222
Hap_2 **–** *F. occidentalis*	10	0.055556
Hap_3 **–** *F. occidentalis*	32	0.177778
Hap_4 **–** *F. occidentalis*	15	0.083333
Hap_9 **–** *F. occidentalis*	8	0.044444
Hap_5 – *Thrips tabaci*	27	0.15
Hap_6 – *T. tabaci*	1	0.005556
Hap_7 – *T. tabaci*	1	0.005556
Hap_8 – *T. tabaci*	1	0.005556

Table S5. P-values of Pearson's correlation coefficients from Table 4.

p-values	dev-PB-MB119	dev-PB-MB120	dev-PB-MB133	dev-Penny Lane	dev- Super Pink Pompon
Hap_1	0.947	0.771	0.974	0.632	0.343
Hap_2	0.570	0.344	0.541	0.720	0.589
Hap_3	0.867	0.884	0.969	0.771	0.407
Hap_4	0.791	0.889	0.708	0.644	0.516
Hap_9	0.571	0.650	0.394	0.711	0.465

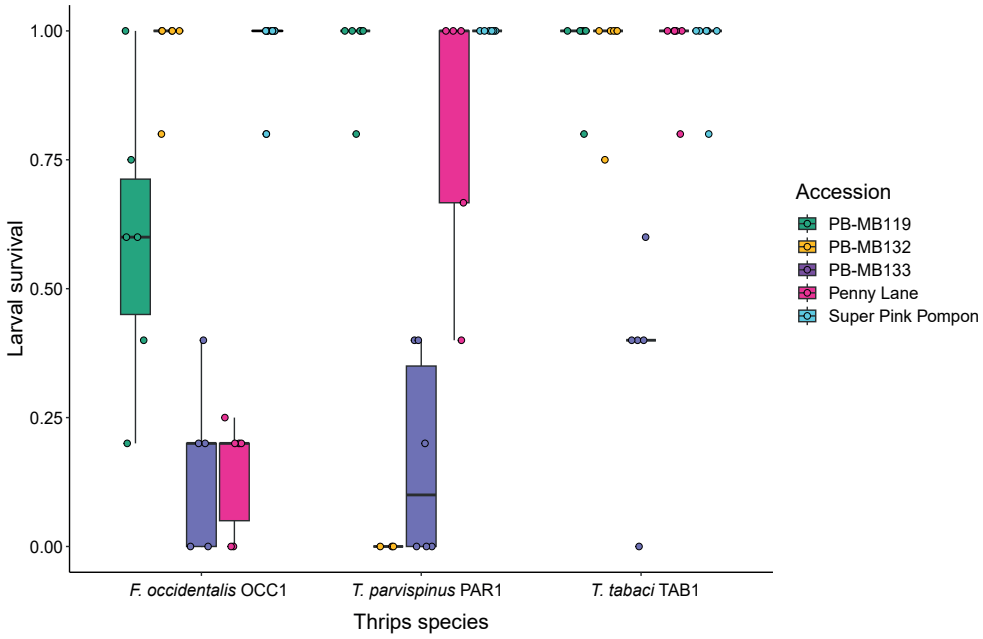

Figure S1. Larval survival of *F. occidentalis* (OCC1), *T. parvispinus* and *T. tabaci* on five *Chrysanthemum* accessions. The thrips assay was carried out in June 2023. Larval survival was expressed as the fraction of larvae that survived at 5 dpi. Dots indicate single observations of larval survival per plant accession. Six replicates per thrips species and plant accession combination were included. Boxplots show the distribution (median, 25 and 75% quantiles, min and max) of the observations of larval survival per plant accession. Super Pink Pompon (in light blue) was included as susceptible reference.

Chapter 5

Exploring plant metabolites associated with thrips resistance in *Chrysanthemum x morifolium* cultivars

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Abstract

Chrysanthemum cultivation is hampered by thrips, notably *Frankliniella occidentalis*, causing visible damage and virus transmission. However, thrips control is challenging due to their fast reproduction, short life cycle, and insecticide resistance. Therefore, we aimed to unravel mechanisms of thrips resistance found in a *Chrysanthemum x morifolium* cultivar, focusing on secondary plant metabolites. Our goal was to identify metabolites in *Chrysanthemum* leaves associated with both constitutive and induced thrips resistance, expressed as reduced thrips larval development. Therefore, we determined plant resistance levels of a thrips-resistant (Penny Lane) and a thrips-susceptible (Super Pink Pompon) cultivar and quantified their leaf metabolite profiles by an untargeted LC-MS-based metabolomics approach, both prior to and after thrips infestation. Thrips larval development significantly differed between Penny Lane and Super Pink Pompon, while a previous thrips infestation had no effect on thrips resistance level in both cultivars. Ten metabolites were significantly induced by thrips infestation, two in Super Pink Pompon and eight in Penny Lane. We subsequently examined an F1 population of 48 genotypes from a cross between Super Pink Pompon and Penny Lane for their thrips resistance levels and selected 18 contrasting genotypes for determining differences in leaf metabolite profiles. In this F1 population, we identified 27 metabolites significantly correlating with resistance and 11 with susceptibility. The majority of these 38 metabolites were putatively annotated as phenolic compounds, terpenoids, and flavonoids. Next to chlorogenic acid and luteolin, known from other studies to be associated with thrips resistance, we identified a series of other metabolites also to be associated with resistance against thrips other insects, suggesting a role in thrips resistance in *Chrysanthemum*. However, validation of the role of these metabolites in causing resistance to thrips is necessary.

Introduction

Chrysanthemum cultivation is heavily affected by the thrips species *Frankliniella occidentalis*. Thrips are tiny, 1-2 mm long, insects that easily get transported by wind gusts from host to host (Mound, 2005). They feed through piercing-sucking mouthparts (Hunter and Ullman, 1992), with which they empty the leaf mesophyll cells from their content (Kindt et al., 2003). This results in not only visual damage, usually described as silver damage and growth damage, but also in virus transmission (Riley et al., 2011; Rotenberg et al., 2015). Therefore, thrips control is extremely important. However, due to their high reproduction rate, short life cycle, cryptic behaviour, and the development of resistance to insecticides, thrips control is challenging.

In response to this challenge, considerable efforts have been directed towards identifying thrips-resistant genetic sources and unravelling the mechanism(s) of host plant resistance to thrips in various species. Such host plant resistance can manifest itself through an array of physical or biochemical plant characteristics and may be either constitutive or induced by thrips feeding and ovipositing (War et al., 2012). Many metabolites that are constitutively produced by plants have been associated with thrips resistance. For example, acyl sugars have been linked to thrips resistance in tomato (Mirnezhad et al., 2010; Vosman et al., 2018), whereas pyrrolizidine alkaloids, specifically jacobine and jaconine, along with the flavonoid kaempferol glucoside were found in resistant varieties of *Senecio* (Leiss et al., 2009a). Moreover, thrips-resistant carrot cultivars were found to contain elevated levels of the flavonoid luteolin, the phenylpropanoid sinapic acid, and the amino acid ß-alanine (Leiss et al., 2013). A flavonoid and several capsianosides (diterpene glycosides) were associated with thrips resistance in pepper (Macel et al., 2019; Maharijaya et al., 2019).

Next to constitutive defence metabolites, plants can also upregulate the production of specific metabolites upon thrips-infestation. Thrips feeding has been shown to activate the biosynthesis of jasmonic acid (JA) and the expression level of genes in the JA-pathway in *Arabidopsis thaliana* (Abe et al., 2008). Moreover, application of JA to *A. thaliana* and *Brassica rapa* plants resulted in higher thrips resistance, characterized by reduced oviposition rates and lower population build-up (Abe et al., 2009). In tomato, eggplant, and pepper plants resistance against thrips was induced and associated with biosynthesis of phenolic compounds (Leiss et al., 2009b; Liu et al., 2022; Maharijaya et al., 2012; Papadaki et al., 2008; War et al., 2012). In tomato plants, thrips infestation also increased glandular trichome densities on the adaxial

surfaces of young leaves and increased terpene production in these trichomes (Escobar-Bravo et al., 2017). Lastly, in leaves of susceptible pepper, thrips infestation induced the production of alkanes and fatty acids (Maharijaya et al., 2012).

Sources of thrips resistance have been identified within the *Chrysanthemum* genus (Leiss et al., 2009b; Ohta, 2002; Chapter 2; Chapter 3). In *Chrysanthemum*, constitutive thrips resistance does not correlate with trichome density (Chapter 3; Chen et al., 2020b), but JA-treatment can enhance thrips resistance, measured as reduced silver damage, and increase in the density of T-shaped trichomes (Chen et al., 2020a). Thrips resistance in *Chrysanthemum* is therefore more likely dependent on the presence or induction of specific metabolites. Research into the mechanism of resistance has identified several compounds, i.e. unsaturated isobutylamide, which was suggested to repel thrips (Tsao et al., 2005), and chlorogenic acids (caffeoyl quinic acid and feruloyl quinic acid), which were found at higher levels in a set of resistant cultivars (Leiss et al., 2009b). However, metabolites conferring resistance may vary depending on the source of host plant resistance examined and resistance may depend on combinations of metabolites.

A promising approach to identify metabolites contributing to resistance is performing large-scale metabolite analysis on a population that segregates for thrips resistance. The presence of varying compound combinations within such populations due to genetic segregation offers a unique opportunity to discern the mixture of metabolites necessary for effective resistance. In this study we investigated the effect of previous thrips infestation on the resistance level of a thrips-resistant and a thrips-susceptible cultivar of *Chrysanthemum x morifolium*. We characterized the metabolite profiles of leaves and resistance level of both thrips-infested and mock-treated plants of the thrips-resistant Penny Lane and the thrips-susceptible Super Pink Pompon (Chapter 2; Leiss et al., 2009b). Moreover, we characterized an F1 population derived from a cross between Super Pink Pompon x Penny Lane for variation in thrips resistance levels and leaf metabolites by an untargeted comprehensive metabolomics approach (LC-MS). Through this combined approach, we aimed to identify metabolites potentially reducing thrips larval development in *C. x morifolium* leaves.

Materials and Methods

Thrips rearing and synchronization

A population of *F. occidentalis* was maintained on flowering pot chrysanthemums at Wageningen University and Research, Wageningen, the Netherlands. Synchronized first instar larvae (L1) were obtained by allowing female adults to oviposit on snack cucumbers in glass jars. The cucumbers were incubated at 25 °C in a climate cabinet. The females were brushed off after 24 h and the synchronized L1 larvae emerged after four days.

Larval development assay (Leaf disc assay)

Leaf discs of 1.5 cm diameter were punched from *C. x morifolium* leaves. Individual leaf discs were placed on 1 ml of 1.5% agar in Falcon Tight-Fit Lid Petri dishes with a ventilation hole in the lid. Each leaf disc was infested with five L1 larvae. The Petri dishes were randomized and placed at 25 °C in a climate cabinet. After four days, the number of larvae that developed from L1 into L2 stage was determined. Larval development was determined as the fraction of larvae developing into the L2 stage, and calculated as (number of L2)/(number of L1 + L2). This fraction was transformed as y = arcsin(sqrt(x)) and analysed with a one-way-ANOVA and Tukey's HSD post-hoc procedure.

Larval development on thrips-infested and mock-treated leaves

Six cuttings of the thrips-resistant Penny Lane and the thrips-susceptible Super Pink Pompon were tested in August 2022 to study the effect of a pre-infestation with adult thrips on the thrips resistance level and metabolite induction. Cuttings were grown at 25-23 °C, with a photoperiod of 16:8 h day:night, and 70% RH. No insecticides were applied.

The cuttings of Penny Lane and Super Pink Pompon were randomized and enclosed in thrips-proof sleeves, after which three cuttings per cultivar were infested with 20 adult female thrips for 48 h and three cuttings per cultivar were kept clean (hereafter referred to as "thrips-infested" and "mock-treated"). The youngest fully expanded leaves were then collected and used in the thrips larval development assay. Two leaf discs were punched from each leaf and infested with five L1 larvae, resulting into six replicates per combination of cultivar and treatment. The second youngest fully expanded leaves of each plant were individually frozen in liquid nitrogen and stored in -80 °C until further analysis.

Larval development on the Super Pink Pompon x Penny Lane F1 population

The thrips-susceptible cultivar Super Pink Pompon and the resistant cultivar Penny Lane were crossed to obtain an F1 population. Seeds were sown and mother plants of 48 individuals and the parents were grown and maintained. Three cuttings per genotype, including the parents, were randomized in a greenhouse and tested for thrips larval development in June 2022. Cuttings were grown at 25-23 °C, with a photoperiod of 16:8 h day:night, and 70% RH. No insecticides were applied.

The level of thrips resistance of the 48 individuals of the F1 population and the parents was determined in a leaf disc assay. One leaf disc (1.5 cm diameter) per plant of three plants per genotype was obtained from the youngest fully expanded leaf. Then, the leaf discs were infested with five L1 larvae.

Validation of larval development on 18 selected F1 individuals

Next, nine resistant and nine susceptible F1 genotypes were selected for validation of thrips resistance level and metabolite profiling. In September 2022, three cuttings per selected F1 genotype and both parents were randomized in a greenhouse. First, for LC-MS profiling, the second youngest fully expanded leaf of each plant was collected, and individually frozen in liquid nitrogen and stored at -80 °C until further analysis. Three days after, the resistance level of 18 selected F1 genotypes and the parents was validated. Two leaf discs were punched from the youngest fully expanded leaf per plant, resulting in 6 leaf discs per genotype which were then infested with thrips larvae. Cuttings were grown at 25-23 °C, with a photoperiod of 16:8 h day:night, and 70% RH. No insecticides were applied.

Metabolite extraction and profiling using LC-MS

The thrips pre-infested and mock-treated leaves of Penny Lane and Super Pink Pompon were ground into fine powder in liquid nitrogen and 300 mg fresh weight of powder was used per sample in the LC-MS analysis. Three quality control samples (QC) consisting of a mix of powders from all samples were included to determine analytical robustness.

The leaves collected from the 18 selected F1 genotypes and parents were ground into fine powder in liquid nitrogen and 100 mg fresh weight powder per sample was used to extract metabolites. Five technical quality control samples (QC) of mixed powder from all samples were included in the analysis.

Metabolites were extracted by adding 300 µl 98.7% MeOH with 0.13% formic acid (FA), followed by 15 min sonication and centrifugation. The resulting clear supernatant was transferred into an HPLC vial with a 180 µl glass insert. In the

induction experiment, LC-MS of leaf extracts was performed on a LC-PDA-Orbitrap FTMS (Van Treuren et al., 2018), while for the subsequent analysis of the F1 plants we used a LC-PDA-Q Exactive Orbitrap FTMS (Van Haperen et al., 2021). In both series 5 µl of extracts was injected, and a Luna C18 reversed phase 2.1 x 150 mm column (Phenomenex) at 40 °C with a gradient of 5-75% water-acetonitrile (both acidified with 0.1% FA) in 45 min were used for chromatographic separation of the extracted leaf metabolites. Negative electrospray ionization was used to detect the eluting compounds in the mass range of m/z 90-1350 at a mass resolution of 60,000 FWHM.

LC-MS raw data files were processed in an untargeted manner. MetAlign software was used for baseline correction to pick mass peaks (Lommen, 2009) and MSClust software was used to group mass peaks that likely belong to the same metabolite, including isotopes, adducts, and fragments, based on their corresponding retention times and relative abundance pattern across all samples (ClusterIDs) (Tikunov et al., 2012). Hereafter, these mass peak clusters (ion source mass spectra) will be referred to as putative "metabolites", in which each metabolite consists of at least 2 mass features and its relative abundance value is calculated as the sum of intensities of all mass features with a cluster membership of at least 0.90.

Metabolite data statistical analysis

Non-detects in the metabolite intensity data (i.e. an intensity value less than 1000 ion counts, which is the threshold set in Metalign for peak picking) were assigned a random value between 250 and 750. The metabolite intensity data were subsequently log10-transformed. Student's t-tests and Pearson's correlation coefficients and p-values, with false discovery rate correction (FDR) with $\alpha = 0.10$ (Benjamini and Hochberg) were calculated in Excel. Pearson's correlation coefficients were calculated between metabolite intensity values and thrips larval development.

Principal component analyses (PCA) were carried out using SIMCA version 17.0.2 (Umetrics, Umea, Sweden) with Pareto scaling.

Selected metabolites were putatively annotated manually based on the accurate mass of the presumed [M-H]- ion as well as in-source fragments, if present, within the clustered masses and considering a max mass deviation of 5 ppm. Molecular ion masses were matched with open metabolite libraries including the HMDB (https://hmdb.ca/spectra/ms/search) and KNAPSACK (http://www.knapsackfamily.com/knapsack_core/top.php) databases, as well as in-house databases from previous LC-MS analyses on Asteraceae plants.

Results

Larval development on thrips-infested and mock-treated leaves

Thrips larval development significantly differed between the two cultivars tested, Penny Lane and Super Pink Pompon, but larval development did not differ between thrips-infested and mock-treated *C. x morifolium* plants (ANOVA; genotype $p < 0.001$, infestation treatment $p > 0.05$) (Figure 1). On the thrips-resistant Penny Lane a low fraction of larvae developed from L1 to L2. The opposite was true for thrips-susceptible Super Pink Pompon plants.

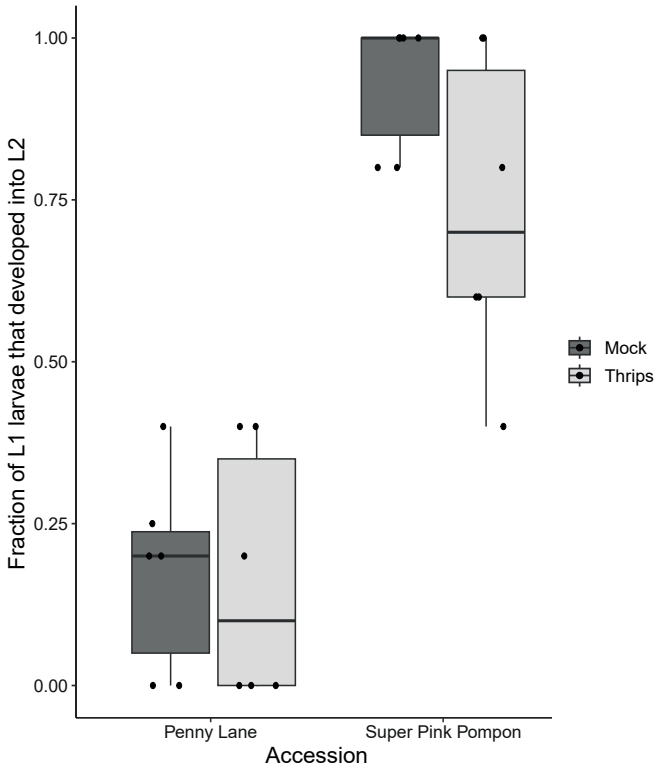

Figure 1. Larval development on thrips-induced and mock-treated leaf material of thrips-resistant Penny Lane and thrips-susceptible Super Pink Pompon. Six replicates per treatment and cultivar combination were tested. The fraction of L1 that developed into the L2 stage is presented per treatment and cultivar. Black dots represent the individual observations. Boxplots show the distribution of the observations: the box represents the interquartile range, the horizontal line inside the box represents the median, and the whiskers indicate the range of data. The two cultivars significantly differed for larval development, but the thrips-induction treatment did not have a significant effect (ANOVA, genotype $p < 0.001$, treatment $p > 0.05$).

Table 1. Metabolites (mass cluster ID) that showed a significant difference in relative abundance between samples of thrips-induced and mock-treated leaves of the thrips-resistant Penny Lane and the thrips-susceptible Super Pink Pompon. Significance of differences was determined after correction for multiple comparison (FDR α = 0.10). Columns indicate the cultivar, the clusterID, whether the abundance was higher in the thrips-infested or in the mock-treated samples, the detected mass (mass SIM) and retention time in minutes (RT). Moreover, per cluster ID, the expected calculated mass ([M-H]-), elemental formula (EF), and putative ID are presented.

Cultivar	Cluster ID	higher in	mass SIM	RT (min)	[M-H]-	EF	putative ID
Super Pink Pompon	595	infested	607.2029	16.84	607.2032	C29H36O14	2-(3,4-Dihydroxyphenyl) ethanol; 1-O-[?-L-Rhamnopyranosyl-(1?3)-[4-hydroxy-E-cinnamoyl-(?6)]-?-D-glucopyranoside]
	1187	infested	667.2755	29.64	667.2760	C36H44O12	
	487	mock	449.1451	14.75	449.1453	C22H26O10	
Penny Lane	1692	infested	787.4493	47.97	741.4431	C39H66O13	16,22,26-Trihydroxycholestan-3-one, 16-O-[?-L-Rhamnopyranosyl-(1?3)-?-D-galactopyranoside]
	981	infested	587.3071	24.39	587.3073	C29H48O12	7(14)-Bisabolene-1,2,3,4,5,8,10,11-octol; 2,5,8-Tris-(2-methylpropanoyl), 4-Ac
	312	infested	491.1404	10.93	491.1406	C20H28O14	3,4-Dihydroxybenzoic acid; 3-Me ether, 4-O-?-D-glucopyranoside, ?-D-glucopyranosyl ester
	1257	infested	577.3224	31.21	unknown	unknown	
	129	infested	447.1137	6.69	447.1144	C18H24O13	2,5-Dihydroxybenzoic acid; 5-O-[?-D-Apiofuranosyl-(1?2)-?-D-glucopyranoside]
	993	infested	553.2287	24.66	553.2290	C27H38O12	3,3',4,4',5,5',9,9'-Octahydroxylignan, 3,3',5,5'-Tetra-Me ether, 9-O-xylopyranoside
	1100	infested	571.3119	27.44	unknown	unknown	
	1098	infested	753.3545	27.19	707.3494	unknown	
	20	mock	650.2147	2.02	unknown	unknown	

Metabolites induced by thrips infestation

Metabolites were extracted from leaves of plants of the two cultivars either exposed to adult thrips or mock treatment. A total of 258,821 individual mass signals were detected using Metalign software, of which 87,859 signals were present in at least three samples; these could be assembled into 1207 putative metabolites, based on similarity between signals in their LC-retention time and relative abundance across samples. The PCA plot based on the variation in relative intensities of leaf metabolites shows two distinct clusters due to a clear separation of the two *C. x morifolium* cultivars, Penny Lane and Super Pink Pompon (Figure S1). No clear separation was observed between mock-treated and thrips-infested samples. Univariate analysis indicated that the intensities of 697 metabolites differed significantly between Penny Lane and Super Pink Pompon for both treatments (t-test; $n = 3$, FDR $\alpha = 0.10$, $p < 0.05$). When considering the effect of thrips induction, within the susceptible Super Pink Pompon three metabolites were significantly different between mock-treated and thrips-infested leaves, while within the resistant Penny Lane nine metabolites were significantly different (Table 1). Six of these differential metabolites were putatively annotated, based on their MS information.

Larval development on the Super Pink Pompon x Penny Lane F1 population

Significant differences were found in development from L1 into L2 stage among the 48 F1 genotypes and the parents (ANOVA, $p < 0.001$) (Figure 2). Larval development significantly differed between the resistant parent, Penny Lane, and the susceptible parent, Super Pink Pompon. The nine most resistant, and nine most susceptible F1 genotypes were selected for validation. Again, significant differences for larval development were found (ANOVA, $p < 0.01$) (Figure 3).

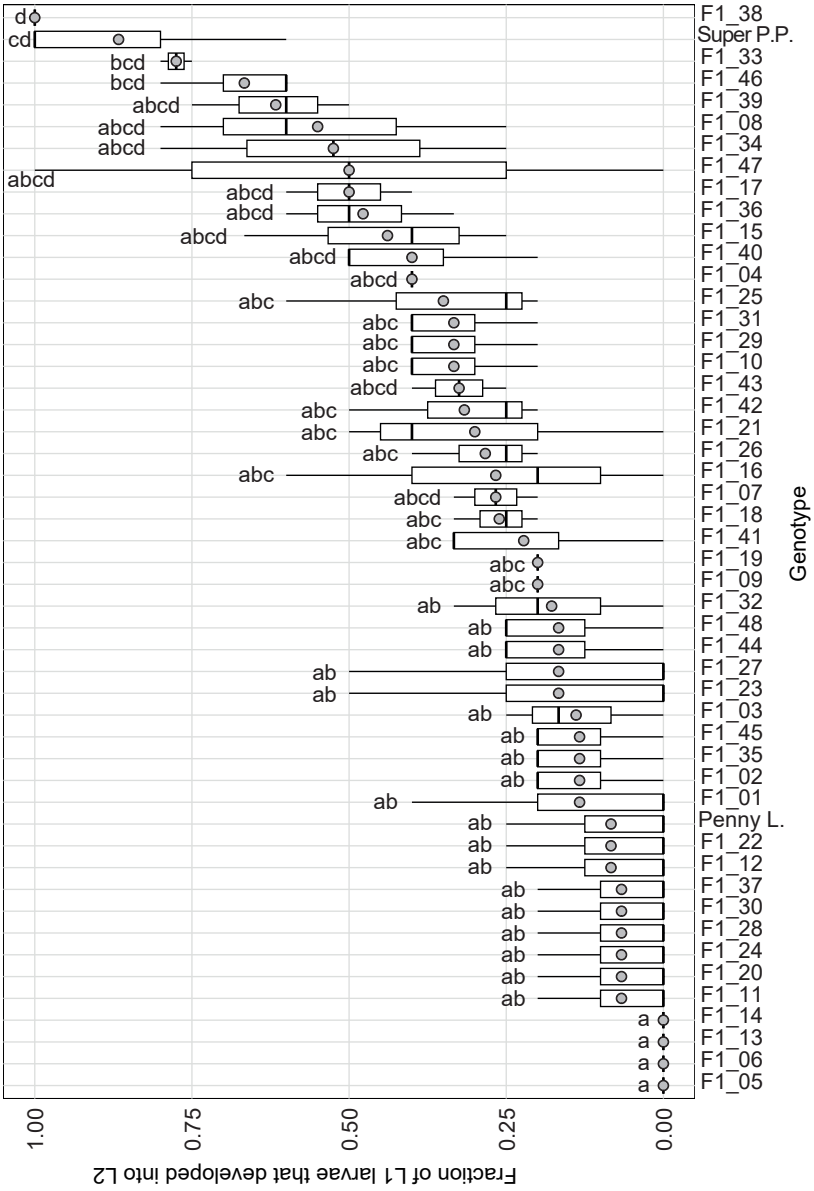

Figure 2. Larval development in Penny Lane, Super Pink Pompon (Super P.P.) and in 48 F1 individuals derived from their cross. Larval development was measured as the fraction of larvae that developed from L1 to L2 stage, and it significantly differed between genotypes (ANOVA, $p < 0.001$). Boxplots show the distribution of the observations: the box represents the interquartile range, the horizontal line inside the box represents the median, and the whiskers indicate the range of data. Grey dots represent the mean fraction of larval development into the L2 stage per genotype. Genotypes that have no letters in common significantly differ according to Tukey's HSD post-hoc test ($p < 0.05$).

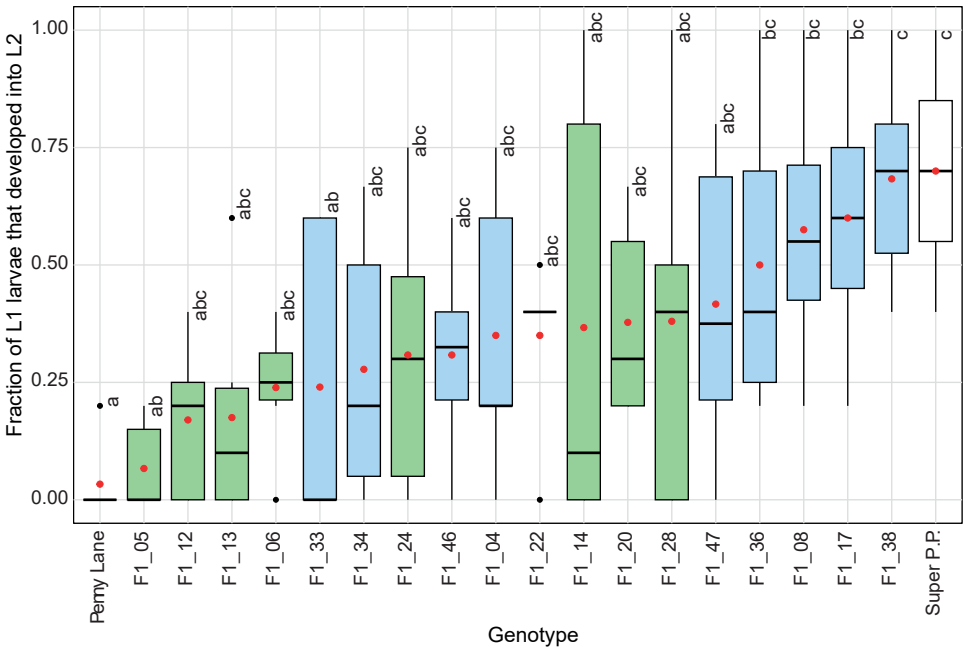

Figure 3. Validation of larval development in Penny Lane, Super Pink Pompon and 18 F1 individuals derived from their cross. Nine F1 genotypes were selected as resistant (F1_22 and those coloured in green) and nine genotypes were selected as susceptible in the previous assay (coloured in light blue). The fraction of L1 that developed into the L2 stage is presented. Boxplots show the distribution of the observations: the box represents the interquartile range, the horizontal line inside the box represents the median, and the whiskers indicate the range of data. Red dots represent the mean fraction larval development per genotype. Larval development significantly differed between genotypes (ANOVA, $p < 0.01$). Genotypes that have no letters in common significantly differ according to Tukey's HSD post-hoc test ($p < 0.05$).

Metabolomics of the selected F1 individuals

Metabolites were extracted from intact leaves of the nine selected resistant and nine susceptible F1 genotypes, as well as the resistant and susceptible parents that were simultaneously grown with the F1 population; all genotypes were grown in three biological replicates. In negative ionization mode 285,332 individual mass signals were detected, of which 52,041 were present in at least three random samples. These signals were then assembled into 1843 putative metabolites. The PCA plot based on metabolite profile of the tested samples, including both parents, showed a clear separation based on genotypes, where the three biological replicates of each genotype clustered together indicating a high reproducibility (Figure 4). A clear separation of the resistant Penny Lane and susceptible Super Pink Pompon parents was also observed. We did not observe a separation based on phenotype (thrips-resistance levels) among the F1 genotypes.

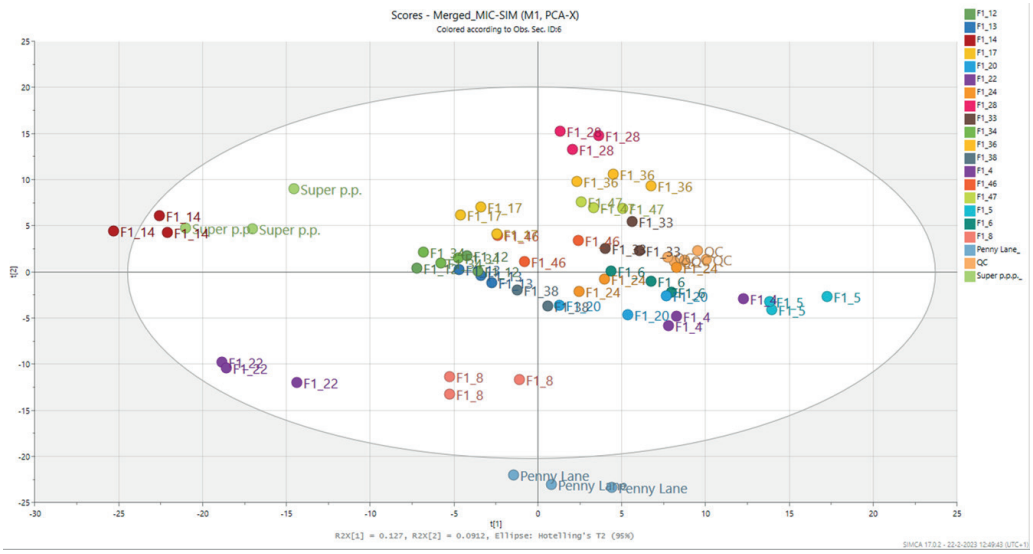

Figure 4. Principal Components Analysis (PCA) plot of the metabolite profiles found in leaf samples of a subset of F1 individuals from Super Pink Pompon x Penny Lane population, and the parental genotypes, determined by untargeted LC-MS. Coloured dots correspond to samples of individuals plant genotypes. "QC" dots correspond to the five technical quality control samples. Each sample was derived from the second youngest fully expanded leaf of three individual plants. Hotelling's T^2 ellipse is overlaid, with points outside the ellipses potentially indicating outliers identified at a significance level of $\alpha = 0.05$. Larger T^2 values suggest greater deviation from the multivariate mean.

After correcting for multiple comparisons (FDR, $\alpha = 0.10$), 59 metabolites significantly correlated with thrips larval development (Table S3). Of these, 44 metabolites showed a negative correlation with larval development (-0.50 <= r <= -0.38), i.e. their abundance correlated with resistance to thrips. Twenty-seven metabolites were more abundant in the resistant parent Penny Lane (Table 2). Seventeen metabolites significantly correlated with thrips resistance in the F1 population, but were more abundant in the susceptible parent Super Pink Pompon (Table S1). Fifteen metabolites positively correlated with faster larval development, i.e. their abundance correlated with susceptibility to thrips. Of these, 11 metabolites were more abundant in the susceptible parent Super Pink Pompon (Table 3), and four were more abundant in the resistant parent Penny Lane (Table S2). Forty of the 59 metabolites were putatively annotated. Of these, 12 metabolites were putatively annotated as phenols, 12 as terpenoids and 11 as flavonoids.

Table 2. Putative metabolites that significantly correlated with thrips resistance in the F1 population. Metabolites (cluster ID) abundance of which significantly correlated with thrips resistance (suppressed larval development) found in 18 individuals from the Super Pink Pompon x Penny Lane F1 population after correction for multiple comparison (FDR, α = 0.10). Metabolites of which abundance was higher in the resistant parent are listed here. Columns indicate clusterID, Pearson's coefficient r, the detected mass (putative molecular ion) and retention time in minutes (RT). Per cluster ID, the expected calculated mass ([M-H]-), elemental formula (EF), class and putative ID are presented. Significance of Pearson's r is presented as * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

Cluster ID	r	Putative molecular ion	RT (min)	[M-H]-	EF	class	putative ID
1935	-0.50***	479.2650	30.87		C26H40O8	terpenoid	Hydroxy-16-kauren-19-oic acid, D-Glucopyranosyl ester
1645	-0.49***	571.2415	27.33	571.2396	C27H40O13	terpene glycoside	Hastatoside; 8-Epimer, 6?-alcohol, 6-O-(8-hydroxy-2,6-dimethyl-2E,6E-octadienoyl)
2359	-0.48***	637.3037	40.10	unknown	unknown		
1902	-0.47**	181.0871	30.42	181.0870	C10H14O3	phenol	Dihydroconiferyl alcohol
1094	-0.47**	181.0870	21.80	181.0870	C10H14O3	phenol	Dihydroconiferyl alcohol
443	-0.46**	389.1817	15.17	unknown	unknown		
1390	-0.46**	285.0406	24.59	285.0405	C15H10O6	flavonoid	luteolin
734	-0.43**	295.1187	18.46	295.1187	C15H20O6	cinnamyl alcohol glycoside	3-Phenyl-2-propen-1-ol, Glucoside
1330	-0.43**	287.0562	24.06	289.0707	C15H12O6	flavonoid	dihydrokaempferol
1428	-0.42**	197.0820	25.05	197.0819	C10H14O4	phenol	2-(3,5-Dimethoxyphenoxy) ethanol
1635	-0.42**	405.1196	27.23	405.1191	C20H22O9	flavonoid	3,4',5,7-Tetrahydroxyflavan, 7-O-?-D-Apiofuranoside
2403	-0.42**	749.3172	41.87	749.3179	C41H50O13		

Table 2. continued.

Cluster ID	r	Putative molecular ion	RT (min)	[M-H]-	EF	class	putative ID
2478	-0.42*	681.2513	44.75	unknown	unknown		
260	-0.41*	353.0877	12.49	353.0878	C16H18O9	phenol	Caffeoyl quinic acid
202	-0.41*	543.1718	10.92	unknown	unknown		
378	-0.41*	387.1660	14.45	387.1661	C18H28O9	jasmonic acids	Glucosyl-hydroxyjasmonic acid
565	-0.40*	277.1081	16.60	277.1081	C15H18O5	terpenoid	Chrysartemin B
634	-0.40*	555.2236	17.45	555.2236	C30H36O10	terpenoid	Sesquimarocanol A
2035	-0.39*	609.2551	32.45	609.2553	C30H42O13	terpenoid	4-Deacetylbaccatin IV
1860	-0.39*	213.1496	29.83	609.2553	C12H22O3	oxylipin	Hydroxy-dodecenoic acid
2430	-0.39*	635.2858	42.96	635.2862	C36H44O10	terpenoid	
643	-0.39*	277.1080	17.47	277.1081	C15H18O5	terpenoid	Dihydrolactucin
1068	-0.38*	549.1962	21.57	549.1977	C27H34O12	phenol	Tracheloside
936	-0.38*	197.0820	20.40	197.0819	C10H14O4		
2241	-0.38*	413.1736	36.42	unknown	unknown		
1055	-0.38*	561.1974	21.45	605.1876	C29H34O14	flavonoid	Dihydroxyflavanone, 7-O-[4-O-Acetyl-?-L-Rhamnopyranosyl-(1?6)-?-D-glucopyranoside]
252	-0.38*	382.1487	12.10	unknown	unknown		

Table 3. Putative metabolites that significantly correlated with thrips susceptibility. Metabolites (mass cluster ID) abundance of which significantly correlated with thrips susceptibility (high larval development) and abundance of which was higher in the susceptible parent found in 18 F1 individuals from the Super Pink Pompon x Penny Lane cross after correction for multiple comparison (FDR α = 0.10). Columns indicate clusterID, Pearson's correlation coefficient r, the detected mass (putative molecular ion) and retention time in minutes (RT). Per cluster ID, the expected calculated mass ([M-H]-), elemental formula (EF), class and putative ID are presented. Significance of Pearson's r is presented as * p < 0.01; ** p < 0.001; *** p < 0.0001.

Cluster ID	r	Putative molecular ion	RT (min)	[M-H]-	EF	class	putative ID
1486	0.50***	527.0807	25.59	unknown	unknown		
731	0.46**	671.1631	18.45	671.1618	C32H32O16	phenol	Vanilloyliridin
647	0.43**	637.1395	17.54	637.1410	C28H30O17	phenol	Ellagic acid; 2,3-Di-Me ether, 7-O-[?-L-rhamnopyranosyl-(1?6)-?-D-glucopyranoside]
1480	0.43**	459.0935	25.57	459.0933	C22H20O11	flavonoid	4',5,7-Trihydroxyflavone; 7-O-?-D-Glucuronopyranoside, Me ester
1682	0.43**	283.0611	27.82	487.1246	C24H24O11	flavonoid	Vitexin; 7-Me ether, 2''-Ac
1817	0.42**	519.1150	29.25	519.1144	C24H24O13	flavonoid	isorhamnetin-O-acetyl hexoside
284	0.41*	461.1670	12.93	461.1664	C20H30O12	phenol	2-(3,4-Dihydroxyphenyl)ethanol; 1-O-[?-L-Rhamnopyranosyl-(1?3)-?-D-glucopyranoside]_Verbasiode
1388	0.41*	601.2153	24.51	601.2138	C27H38O15	phenol	Rubricauloside
454	0.40*	471.1866	15.30	471.1872	C22H32O11	phenol	2-Methoxy-4-(2-propenyl)phenol; O-[?-L-Rhamnopyranosyl-(1?6)-?-D-glucopyranoside]
1652	0.39*	501.1044	27.40	501.1038	C24H22O12	flavonoid	4-',5,7-Trihydroxyflavone; 7-O-(2-O-Acetyl-6-O-methyl-?-D-glucuronopyranoside)
1631	0.38*	473.1097	27.15	473.1089	C23H22O11	flavonoid	Vitexin; 6''-Ac

Discussion

Previous thrips infestation has no effect on thrips-resistance level

In this study, we investigated the effect of thrips *F. occidentalis* infestation on the resistance level and metabolite profiles of two *C. x morifolium* cultivars, Penny Lane and Super Pink Pompon. While there were significant differences in the baseline metabolite profiles of the two cultivars, thrips infestation only had a limited effect on the global metabolite composition. Only two metabolites in Super Pink Pompon and eight in Penny Lane were present at higher levels in thrips-infested leaves. This study is the first untargeted LC-MS study done on thrips-resistance in *Chrysanthemum*. None of the annotated thrips-induced metabolites (Table 1) was previously reported in thrips resistance studies, suggesting we identified ten candidates as putatively novel bioactive compounds. Previous research on thrips resistance in *Chrysanthemum* may have missed these compounds because of the use of nuclear magnetic resonance (NMR)-based metabolomics (Leiss et al., 2009b), which allows for the detection of various metabolites but is less sensitive than LC-MS (Lei et al., 2011). In contrast, (Leiss et al., 2009b) found that chlorogenic acid was induced by thrips infestation in *Chrysanthemum*: in our study we detected three isomers of chlorogenic acid (m/z [M-H]- = 353.087 at retention times 6.3, 6.8 and 8.8 minutes) but none of them was significantly altered upon thrips infestation in either the susceptible or resistant cultivar. In other plant species, thrips infestation induced the production of additional phenolic compounds (Leiss et al., 2013; Maharijaya et al., 2012; Papadaki et al., 2008). We found no significant differences in resistance levels of thrips-infested and mock-treated plants of either cultivar. The level of thrips resistance, measured as the fraction of larvae developing from the L1 into the L2 stage, of Penny Lane was already high in the mock-treated plants. This could have made it difficult to detect an even higher resistance level. In contrast, in other plant species, a previous thrips-infestation affected not only the metabolite production but also the subsequent level of thrips-resistance. In *A. thaliana* a pre-infestation with thrips reduced subsequent thrips feeding (Abe et al., 2009, 2008). This has also been reported for *Chrysanthemum*, in which a lower degree of thrips damage was found on thrips-induced plants (Chen et al., 2020a, 2020b). However, while the latter two studies assessed thrips resistance based on silver damage in a whole plant assay, our study determined larval development in leaf disc assays. Additionally, their conclusions were based on different plant materials, in one study a single cultivar (Baltica), and in the other 95 cultivars, and it is unclear whether the cultivars we evaluated were included.

Metabolites associated with thrips-resistance in an F1 population of *Chrysanthemum x morifolium*

We also analysed a selection of 18 plants from the F1 population derived from Super Pink Pompon x Penny Lane, in which we observed genotype-dependent differences in thrips larval development from L1 to L2 larval stage. The resistant genotypes suppressed the larval development of thrips, whereas the susceptible genotypes did not. The thrips-resistance level of the F1 genotypes was validated in a second thrips assay, during which we also collected leaf samples for metabolomics analysis. We identified 59 metabolites significantly associated with thrips larval development, of which 44 were associated with resistance (Table 2, S1), and 15 with susceptibility (Table 3, S2). We were able to annotate 40 of the 59 metabolites, thirty-five of which were putatively annotated as belonging to the classes of phenols, terpenoids, and flavonoids, which are often associated with pest resistance (War et al., 2012). To identify candidate metabolites conferring resistance against thrips, from the 44 metabolites correlating with resistance we selected 27 metabolites, of which the abundance was higher in the resistant parent Penny Lane. Of the putatively annotated metabolites correlated with resistance (Table 2), caffeoyl quinic acid (chlorogenic acid) has been previously associated with thrips resistance in *C. x morifolium*. High doses of chlorogenic acid were reported to reduce thrips larval survival (Leiss et al., 2009b). Chlorogenic acid also affected growth and development at sub-lethal doses, and survival at higher doses on both the whitefly *Bemisia tabaci* and the armyworm *Mythimna separata* (Lin et al., 2022; Wang et al., 2023). Chlorogenic acid is toxic to herbivorous insects, particularly when it is oxidized to chlorogenoquinones. Upon their formation, chlorogenoquinones can rapidly react with amino acids and proteins, which then become less bioavailable and digestible to insects (Felton et al., 1992, 1989). On the other hand, in eggplant chlorogenic acid concentration was higher in thrips-susceptible than in thrips-resistant plants, and no significantly higher chlorogenic acid level was found in thrips-resistant tomato plants (Liu et al., 2022; Papadaki et al., 2008). Likewise, in the present study we did not detect significant differences in abundance of the three chlorogenic acid isomers neither between the susceptible and resistant *Chrysanthemum* cultivars, nor between mock- and thrips-treated plants. However, one chlorogenic isomer (caffeoyl quinic acid, cluster ID 260, Table 2) significantly correlated with resistance in the F1 population. Therefore, the relevance of chlorogenic acid in thrips-resistance in *Chrysanthemum* is not fully resolved. Furthermore, among the differential metabolites we found the flavonoid luteolin, which has previously also been linked to thrips resistance: in carrots, luteolin, sinapic acid and β-alanine were present in higher concentrations in resistant plants as compared to thrips-susceptible ones (Leiss et al., 2013). Luteolin was also found to affect the pea aphid *Acyrthosiphon*

pisum aphids by reducing passive ingestion (Goławska and Łukasik, 2012), and it was associated with resistance to the green peach aphid *Myzus persicae* in the tree *Robinia pseudoacacia* (Madanat et al., 2018). Moreover, two luteolin-derived compounds, apigenin-glycoside and luteolin methyl ether, were linked to thrips resistance in pepper (Macel et al., 2019). However, how luteolin is affecting thrips and aphids is still unknown.

In this comprehensive metabolomics study, involving >1000 compounds, we detected many novel metabolites significantly correlating with thrips-resistance (Table 2) and some of these, in particular the flavonoids, have previously been associated with insect resistance in other plant species or have shown deterrence of insect feeding. For example, vitexin reduced the survival of duckweed weevil larvae (*Tanysphyrus lemnae*) in *Lemna minor* (Lee et al., 2022). Moreover, dihydroxyflavanone and tracheloside were found to act as feeding deterrent of respectively, the bertha armyworm *Mamestra configurata* and *Tribolium confusum* (Harmatha and Nawrot, 2002; Onyilagha et al., 2004). Lastly, the terpenoid furanopetasine confers resistance to the land snail *Arianta arbustorum* (Hagele et al., 1998). Given the demonstrated involvement of these metabolites in pest resistance and deterrence, one or a combination of these metabolites may confer thrips-resistance in *Chrysanthemum*, but further validation of their putative annotations and direct involvement in resistance, for example with *in vitro* assays, is necessary.

Acknowledgements

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Supplementary materials

Table S1. Putative metabolites that significantly correlated with thrips resistance in the F1 population but the abundance of which was higher in the susceptible parent. LC-MS analysis done on 18 F1 individuals from the Super Pink Pompon x Penny Lane cross after correction for multiple comparison (FDR α = 0.10). Columns indicate clusterID, Pearson's r, the detected mass (putative molecular ion) and retention time in minutes (RT). Per cluster ID, the expected calculated mass ([M-H]-), elemental formula (EF), class and putative ID are presented. Significance of Pearson's r is presented as * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

Cluster ID	r	Putative molecular ion	RT (min)	[M-H]-	EF	Class	Putative ID
1658	-0.49***	535.2766	27.52		C25H44O12	terpenoid	Megastig-mene-diol, di-glucoside
2166	-0.47**	595.2543	34.75		C33H40O10		
2274	-0.45**	589.1716	37.48	589.1715	C32H30O11		
1915	-0.45**	231.1027	30.67	231.1027	C14H16O3		
2066	-0.43**	561.1537	32.97	561.1533	C28H31ClO10		physalin H
2142	-0.42**	535.1979	34.37	535.1974	C30H32O9	terpenoid	
2330	-0.42*	505.2823	39.05	505.282	C29H38N4O4	cyclo-peptide alkaloid	Xylopyrine-F
61	-0.41*	159.0299	2.38	159.0299	C6H8O5	amino acid	2-Oxoadipate
2339	-0.41*	585.2280	39.35	unknown	unknown		
2219	-0.41*	331.1914	35.89	331.1915	C20H28O4	terpenoid	Furanopetasine
2324	-0.41*	589.1718	38.88	589.1715	C32H30O11	coumarin	Rivulobirin E
2451	-0.41*	695.3083	43.62	695.3073	C38H48O12		Zaragozic acid C; Deacetoxy
1073	-0.40*	297.1344	21.65	297.1344	C15H22O6	terpenoid	
365	-0.40*	619.1672	14.30	619.1668	C29H32O15	flavonoid	Vitexin; 6''-Ac, 2''-O-?-L-rham-nopyranosyl
720	-0.39*	927.1830	18.28	927.1837	C42H40O24	flavonoid	Salvianin
2124	-0.38*	611.2490	34.01	611.2498	C33H40O11	terpenoid	orthosiphol J
55	-0.38*	317.0547	2.30	unknown	unknown		

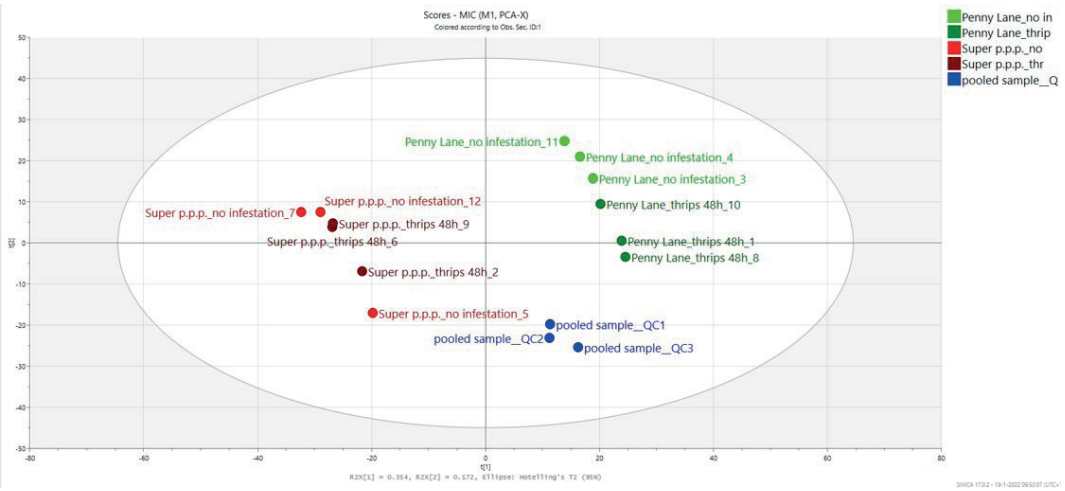

Figure S1. Principal components analysis (PCA) plot of the thrips-infested and mock-treated leaf samples of two *Chrysanthemum x morifolium* cultivars based on their metabolite profiles determined by untargeted LC-MS. Green dots correspond to the thrips-resistant Penny Lane samples, light green dots are for the mock-treated and dark green dots for the thrips infested samples. Red dots correspond to the thrips-susceptible Super Pink Pompon samples, lighter red dots are for the mock-treated and dark red dots for the thrips-infested samples. Each sample was derived from the second youngest fully expanded leaf of three individual plants. Blue dots correspond to the three technical quality control samples and consisted of pooled samples. The ellipse represents Hotelling's T^2 with a 95% confidence level.

Table S2. Putative metabolites of which the abundance significantly correlated with thrips susceptibility in the F1 population but was higher in the resistant parent. LC-MS analysis done on 18 F1 individuals from the Super Pink Pompon x Penny Lane cross after correction for multiple comparison (FDR α = 0.10). Columns indicate clusterID, Pearson's r, the detected mass (putative molecular ion) and retention time in minutes (RT). Per cluster ID, the expected calculated mass ([M-H]-), elemental formula (EF), class and putative ID are presented. Significance of Pearson's r is presented as * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

Cluster ID	r	Putative molecular ion	RT (min)	[M-H]-	EF	class	putative ID
2321	0.43**	388.1130	38.68	388.1125	C22H19N3O2S		
270	0.40*	325.0928	12.78	325.0929	C15H18O8	phenol	6-O-p-Coumaroyl-glucose
1945	0.40*	532.2184	31.13	532.2188	C27H35NO10		
697	0.39*	683.2191	18.06	683.2193	C31H40O17	terpe-noid	Catalpol; 6-O-[(4-Hydroxy-3-meth-oxy-E-cinnamoyl)-(?3)-?-L-rham-nopyranoside]

Table S3. List of 59 metabolites significantly correlating with resistance and susceptibility (expressed as fraction of larval development) in the F1. Per metabolite, cluster ID, significant Pearson's r, p-value and relative abundance (log10 transformed) in the two parents, Penny Lane and Super Pink Pompon, are indicated. Significant metabolites have been selected with FDR α = 0.10. * p < 0.01; ** p < 0.001; *** p < 0.0001.

Cluster ID	Pearson's r	Penny Lane	Super Pink Pompon	Cluster ID	Pearson's r	Penny Lane	Super Pink Pompon
1935	-0.50***	5.84	5.15	**1388**	0.41*	3.08	4.10
1486	0.50***	5.53	5.66	**2219**	-0.41*	2.71	2.82
1658	-0.49***	5.68	5.76	**260**	-0.41*	8.64	8.56
1645	-0.49***	4.26	3.02	**2324**	-0.41*	4.46	4.96
2359	-0.48***	3.04	2.63	**202**	-0.41*	5.21	4.80
2166	-0.47**	5.69	5.80	**2451**	-0.41*	3.44	5.47
1902	-0.47**	6.56	2.76	**378**	-0.41*	7.96	7.85
1094	-0.47**	8.13	5.52	**1073**	-0.40*	4.04	4.29
443	-0.46**	6.64	6.37	**565**	-0.40*	6.34	6.23
1390	-0.46**	7.34	6.48	**634**	-0.40*	5.79	5.73
731	0.46**	3.54	4.37	**454**	0.40*	3.77	5.71
2274	-0.45**	5.06	5.48	**1945**	0.40*	4.24	3.17
1915	-0.45**	2.67	3.37	**365**	-0.40*	3.77	3.89
734	-0.43**	5.32	4.12	**270**	0.40*	3.16	2.83
647	0.43**	2.66	5.04	**1652**	0.39*	4.04	5.14
1480	0.43**	7.39	8.13	**2035**	-0.39*	4.39	2.67
1330	-0.43**	7.45	7.00	**1860**	-0.39*	6.88	6.38
2321	0.43**	5.09	2.77	**697**	0.39*	4.21	2.74
2066	-0.43**	2.76	2.81	**720**	-0.39*	7.69	7.70
1682	0.43**	2.78	2.81	**2430**	-0.39*	5.90	5.40
1428	-0.42**	5.76	3.50	**643**	-0.39*	7.36	7.34
1635	-0.42**	3.14	2.60	**1068**	-0.38*	5.08	2.63
2142	-0.42**	2.78	3.55	**1631**	0.38*	3.54	5.11
2403	-0.42**	3.31	3.25	**936**	-0.38*	5.93	5.12
1817	0.42**	2.59	5.22	**2241**	-0.38*	3.26	2.69
2330	-0.42*	3.55	4.50	**1055**	-0.38*	6.71	5.92
2478	-0.42*	3.16	2.61	**252**	-0.38*	2.74	2.67
61	-0.41*	6.50	6.94	**2124**	-0.38*	3.94	4.16
284	0.41*	5.60	5.98	**55**	-0.38*	5.76	6.48
2339	-0.41*	2.82	3.69				

Chapter 6

General discussion

Chrysanthemum plants and flowers can be heavily damaged by thrips feeding (Reitz, 2009; Riley et al., 2011; Rotenberg et al., 2015). This results in unmarketable material and thus, large economic losses. To prevent thrips from damaging chrysanthemums, an Integrated Pest Management approach (IPM) can be used, combining agronomic practices, biocontrol agents, and insecticides (Kivett et al., 2015; Mouden and Leiss, 2021; Rodríguez and Coy-Barrera, 2023). However, insecticides being a key component of thrips control has its downsides. First, intense insecticide use causes the fast development of insecticide resistance by the thrips, making them ineffective (Bielza, 2008; Jensen, 2000). Second, insecticides are more and more raising concerns because of their effect on the environment, other beneficial animals, and humans, which results in the banning of dangerous compounds (European Union, 2018). Alternative strategies to control thrips are therefore needed and host plant resistance (HPR) could be a promising one to integrate in an IPM approach (Mouden and Leiss, 2021).

The aims of this thesis were 1) to investigate the natural occurrence of thrips resistance within *Chrysanthemum* accessions, both cultivated material (Chapter 2) and wild relatives (Chapter 3), 2) to investigate if these resistance(s) acted against different thrips populations and species (Chapter 4), and 3) to unravel the underlying mechanism of resistance (Chapter 5). In this Chapter 6, the main findings are discussed in the light of improving thrips resistance breeding in *Chrysanthemum*.

The definition of resistance and the importance of a phenotyping method

In insect-plant interaction studies, resistance is defined as “the relative amount of heritable qualities possessed by the plant which influence the ultimate degree of damage done by the insect” (Painter, 1951), or also as “the sum of the constitutive, genetically inherited qualities that result in a plant of one cultivar or species being less damaged than a susceptible plant lacking these qualities” (Smith, 2005). Painter (1951) defined three mechanisms of resistance: non-preference, antibiosis and tolerance. Nowadays, these terms can be used to define categories or types of resistance than resistance mechanisms (Smith, 2005; Stout, 2013) because it is clear that several mechanisms are involved in each of these three categories (War et al., 2012). Mechanisms of resistance are preferably classified into constitutive/inducible and direct/indirect (Stout, 2013).

Despite the critical comments received, the categories defined by Painter are still widely used and therefore, they are worth being defined. Non-preference or antixenosis refers to chemical or physical characteristics of the plant that affect

insect behaviour, which results in lower preference for this plant: feeding and oviposition are deterred (Kogan and Ortman, 1978; Painter, 1951). On the other hand, antibiosis refers to plant characteristics that negatively affect the life history traits of the insect: for example by reducing the survival and reproduction, or by affecting its development (Painter, 1951). Lastly, tolerance is a characteristic of plants that do not affect the insect but results in yield and reproduction being less affected or unaffected by the insect attack, for example in plants showing less damage, as tolerant plants "better compensate for injury" (Painter, 1951; Peterson et al., 2017). Depending on the set-up of the experiment and phenotyping method, one or most likely a combination of these categories are assessed. Typically, choice assays, in which the insects have freedom to move between plants or plant parts, are performed to test for antixenosis, whereas no-choice assays, in which the insects have no choice over substrate, are performed to test for antibiosis.

In the past 30 years several papers that investigated resistance to thrips in *Chrysanthemum* have been published. In this thesis, the focus is on antibiosis and optimization of phenotyping methods that could be used to screen for or measure life history traits (Chapter 2 and Chapter 3). No-choice whole plant assays were performed to assess the population build-up of thrips over three weeks. Then, leaf disc assays were performed to observe the life cycle of the thrips and how each stage was affected (Chapter 2 and Chapter 3) and detached leaf assays were performed to observe larval development from the L1 to the L2 stage (Chapter 2). In previous research on thrips resistance in chrysanthemum choice assays have been performed, valuating antixenosis (De Jager et al., 1995a). Antixenosis is unlikely to reduce the pest pressure in the case of monoculture. Moreover, previous research assessing both antixenosis as well as antibiosis often evaluated silver damage and growth damage inflicted by thrips (Chen et al., 2020b; Kos et al., 2014; Leiss et al., 2009b; Rogge and Meyhöfer, 2021). In a few cases, the evaluation of damage was combined with thrips population growth and development studies (De Jager et al., 1993; De Kogel et al., 1998; Ohta, 2002). However, by evaluating damage only, tolerance cannot be distinguished from antixenosis or antibiosis because the plant might be showing less the thrips damage. The tolerance type of resistance is thought to have lower chances of being overcome by the insect because it depends strictly on the plant and not the insect (Peterson et al., 2017). Because tolerance does not affect the reproduction or survival of the pest, it may lead to the thrips population going unnoticed until the pest pressure exceeds the economic threshold. If the aim is to reduce pest numbers in greenhouses, assessing antibiosis in no-choice assays by directly evaluating insect life history parameters, such as survival and reproduction, may be more informative than evaluating damage. Reducing pest numbers should reduce direct damage (silver and growth damage), as well as thrips

indirect damage (virus transmission). At the same time, even when performing no-choice whole plant assays like the ones we performed in Chapter 2 and 3, a combination of antibiosis and antixenosis is likely evaluated. These categories might be inseparable because the causal mechanism of these two types of resistance may overlap (Stout, 2013). Plant exudates and metabolites may have deterrent and toxic properties at the same time (Mithöfer and Boland, 2012).

Especially in the light of the overlap between the types of resistances and definitions in insect-plant resistance studies, it is important to clearly define the study objectives and consequently select proper phenotyping methods. This would also facilitate literature reviews and comparisons. In this thesis, we refer to antibiosis type of resistance because we aimed to identify factors that reduce thrips population build-up in chrysanthemum cultivation.

Identifying sources of thrips resistance

Once the type of resistance to investigate is defined, the next step in breeding for resistance is identifying sources of resistance with an effective phenotyping method. In this thesis, the focus was on antibiosis type of resistance against the thrips *Frankliniella occidentalis*, which was identified in a panel of 70 *Chrysanthemum x morifolium* accessions (Chapter 2) and of 47 wild relatives of *Chrysanthemum* (Chapter 3). In a smaller subset of accessions, also antibiosis against *Thrips tabaci* and *Thrips parvispinus* was identified (Chapter 4). Accessions that showed a reduced population build-up and suppressed larval development were classified as resistant. Among these is the cultivar Penny Lane, which has been identified as resistant in the past (Leiss et al., 2009b), but several other resistance sources were identified. Similar no-choice assays were performed in the past to identify resistant chrysanthemum cultivars that suppress larval development (De Kogel et al., 1998; Ohta, 2002). Antibiosis type of resistance against thrips that suppressed larval development was identified also in pepper (Maharijaya et al., 2012; Van Haperen et al., 2019). Other instances of antibiosis resistance were found in some genotypes of *Cucumis sativus*, cultivars of *Arachis hypogaea* and wild peanut *Arachis diogoi*. In these cases thrips developmental time was prolonged and larval mortality was higher (Soria and Mollema, 1995; Srinivasan et al., 2018). Resistance that suppress larval development hampers the life cycle, thereby contributing to the reduction of thrips numbers in chrysanthemum cultivation in greenhouses and fields. This identified resistance should also reduce silver and growth damage, as well as virus transmission by thrips. Because the larvae are the only thrips stage capable

of acquiring tospoviruses (Moritz et al., 2004; Whitfield et al., 2005), a resistance affecting the development into later stages also interrupts the virus transmission cycle.

Thrips can affect plants of different ages, stages and also different organs. In *Chrysanthemum*, they can feed on leaves and flowers (Rhainds and Shipp, 2003; Van Dijken et al., 1994), and flowers are usually preferred because of the presence of pollen, a high-quality feed source that speeds up the life cycle (Hulshof et al., 2003). Moreover, due to the ornamental nature of this crop, flowers are particularly important, and thus, it would be most useful to have flowers resistant to thrips. However, in this thesis we focused mainly on resistance in non-flowering plants. I believe that breeding for thrips-resistant plants in an early plant growth stage will help to reduce waste of resources that occurs in the first cultivation stages, when thrips cause growth damage to chrysanthemum leaves and stems, and transmit tospoviruses. Keeping the thrips number low in the early cultivation stages should also contribute to a lower number when the flowers appear. Furthermore, young chrysanthemum cuttings and flowers are shipped internationally, with a zero tolerance for the presence of thrips in transported material. This makes this antibiosis type of resistance particularly crucial for chrysanthemum cultivation.

Crop wild relatives as an additional resource for resistance traits

Crop wild relatives may act as a pool for a variety of traits, including resistances to pathogens and pests (Dempewolf et al., 2017; Warschefsky et al., 2014; Zhang et al., 2017), which have been lost during the domestication and breeding processes (Dempewolf et al., 2017). Therefore, screening for resistance among crop wild relatives is a good approach to identify novel sources and novel resistance mechanisms against thrips that are not present in the cultivated germplasm. In Chapter 3, the resistance level of 47 wild relatives of chrysanthemum was evaluated. These accessions belong to several species from the *Chrysanthemum* and *Artemisia* genera. Resistance against thrips *F. occidentalis* was found across the genus *Chrysanthemum*, and in a few instances resistant and susceptible accessions were identified within the same species, e.g. *Chrysanthemum seticuspe* and *Chrysanthemum indicum*. These resistant accessions, like the selected resistant cultivars, showed antibiosis type of resistance, suppressing the population build-up and the larval development. Prior to this study, nothing was known about thrips resistance in the *Chrysanthemum* genus or related *Artemisia* species except on *C. x morifolium*. Resistance to thrips has been identified in wild relatives of other

crops, for example, in wild pepper accessions (Maharijaya et al., 2011), as well as in wild tomato relatives (Vosman et al., 2018), and efforts have been made to identify resistance QTLs and introgress these into cultivated material.

Challenges in breeding for thrips-resistant chrysanthemums

Once the resistance source is identified, if markers assisted breeding is the objective, the next step for breeding for resistance is selecting resistant and susceptible parents to cross to produce a segregating population for QTL mapping. In this thesis, with the optimized phenotyping method and identified resistant and susceptible accessions we laid the foundations to perform QTL mapping and identify thrips resistance QTLs in *Chrysanthemum*. Unfortunately, no genetic studies were conducted in this thesis, but a larger F1 population resulting from the cross between Super Pink Pompon and Penny Lane was obtained compared to the one screened in Chapter 5. This population could be used for QTL mapping of resistance against thrips *F. occidentalis* in *C. x morifolium*.

Genetic analysis may be performed in the cultivated *C. x morifolium* as well as in the wild relatives, and each approach poses different challenges. In fact, *C. x morifolium* is hexaploid and outcrossing, which results in large genetic diversity (Drewlow et al., 1973; Van Geest et al., 2017). Moreover, costs for sequencing and marker analysis in polyploids are high. On the other hand, the wild relatives' ploidy ranges from diploid to decaploid and can be exploited to our advantage, and after trait discovery, genetic analysis and subsequent identification of genes can be done in populations derived from a cross between diploid parents. Diploid resistant wild accessions have been identified in Chapter 3, and crosses between resistant accessions, *C. seticuspe* PB-MB133, *Artemisia keiskeana* PB-MB102, *Artemisia feddei* PB-MB101, and susceptible accession *C. seticuspe* PB-MB132 have been attempted. When populations are obtained, the next step would consist of phenotyping these populations, and if segregation of the trait is observed, QTL mapping could be performed. QTL mapping in such populations may be easier and cheaper than in (hexaploid) polyploid chrysanthemums.

Introgressing the resistance from wild *Chrysanthemum* relatives may be more challenging than from cultivated chrysanthemum because it would involve intergeneric or interspecific hybridization. There are examples of successful hybridization of cultivated chrysanthemum (*C. x morifolium*) with *Ajania*, *Artemisia,* and other *Chrysanthemum* spp. (Cheng et al., 2011; Deng et al., 2011, 2010). For example, intergeneric hybrids between *Artemisia vulgaris 'Variegata'* and cultivated chrysanthemum have been successfully obtained and showed enhanced resistance

to the aphid *Macrosiphoniella sanbourni* (Deng et al., 2010). In both cases, either using cultivated or wild relatives as resistance source, hybridization may be followed by backcrossing (Su et al., 2019).

Thrips resistance mechanisms in *Chrysanthemum*

Insect resistance is caused by physical and/or chemical plant characteristics that may act as defenses against insect attacks (Fürstenberg-Hägg et al., 2013; War et al., 2012). As to physical leaf traits, no correlation was found between thrips resistance and trichome density and leaf toughness in cultivated chrysanthemum (Chen et al., 2020b; De Jager et al., 1995b). In this thesis, we showed that trichome density did not play a role in thrips resistance in wild *Chrysanthemum* spp. (Chapter 3), even though wild relatives showed a large variation in trichome density. *Chrysanthemum* spp. are also known to produce many different metabolites (Hao et al., 2022; Steenbergen et al., 2018). Thrips might encounter these metabolites while feeding. In a small F1 population derived from a resistant cultivar, Penny Lane, and a susceptible cultivar, Super Pink Pompon, a set of metabolites associated with suppression of larval development was obtained, which may be linked to resistance (Chapter 5). Many metabolites putatively involved in insect resistance were found and annotated, among which some were already described to be associated with thrips resistance, such as chlorogenic acid (Leiss et al., 2009b) and luteolin (Leiss et al., 2013); others were reported to be associated with resistance to other pests, and yet others were either not associated with resistance or not annotated. Because this was a correlative study, validation studies are needed to determine which metabolite or combination of metabolites is causing thrips resistance. Moreover, combining mechanism (involved metabolites) and QTL information can help to further reduce the region of interest to introgress and to identify candidate genes. In addition, understanding of the mechanism may also facilitate the transfer of knowledge to identify thrips resistance in other crops.

Breeding for durable resistance: virulence of *F. occidentalis* populations

Overcoming of resistance by virulent populations is a serious problem in plant resistance breeding and pest control. Therefore, to evaluate the effectiveness of the selected resistances against diverse thrips populations, five populations of *F. occidentalis* were collected in the Netherlands. Both Penny Lane and the wild

C. seticuspe PB-MB133 were resistant to all five populations tested (Chapter 4), which indicates that these are sources of resistance against *F. occidentalis* to be used in chrysanthemum breeding. At the same time, we saw different responses of the *F. occidentalis* populations to the partially resistant accession *C. makinoi* PB-MB119. Introducing resistance in cultivated material, when not effective against all or most of pest populations in the area of interest, not only fails to contribute to pest control and damage reduction but also increases the chances of the virulent population to become prominent, accelerating the overcoming of resistance by the pest. Among insect pests with high reproduction rates, with parthenogenic reproduction and with many generations per year, the chances of advantageous adaptation, such overcoming a resistance and thus being able to survival and reproduce, of spreading and becoming common are large (Smith, 2005; Smith, 2021; Taggar and Arora, 2017). Aphids are notorious for overcoming resistance (Smith and Chuang, 2014; Sun et al., 2020), but reports of virulent populations of thrips have also been made. *Frankliniella occidentalis* populations performed differently on resistant cucumber, chrysanthemum, leek, lettuce and tomato (De Kogel et al., 1998, 1997; Mirnezhad et al., 2012). What differentiates and make the five populations we tested respond differently to chrysanthemum accessions is unknown.

While characterizing the *F. occidentalis* populations for genetic diversity we found that some individuals had the *CO1* (Cytochrome Oxidase 1) gene haplotype associated with the lupin or wet/cold *F. occidentalis* strain (Nielsen et al., 2010). *Frankliniella occidentalis* is now considered a species complex in which two strains/lineages are found, the glasshouse and the lupin strains (Rugman-Jones et al., 2010). These strains thrive in different environments and the lupin strain has been associated with colder and more humid climates, and shows a slower development and lower reproduction (Nielsen et al., 2010), but the two strains are morphologically indistinguishable. Our study was the first reporting the presence of this lupin strain in the Netherlands, and in Europe, but we do not know what the implications of this are. This strain may have influenced the results of phenotyping experiments because of its longer developmental time. The lupin strain may differently affect chrysanthemum cultivation compared with the more common glasshouse strain, and its ability of damaging chrysanthemums may be relevant to investigate. Especially in the light of differences in interaction with chrysanthemum, identifying and monitoring over time the strain to which the thrips population used for screening belongs is also necessary.

Future-proof thrips-resistant chrysanthemums

Management of thrips in chrysanthemum cultivation is important, not only to reduce their numbers and the feeding damage they cause, but also to reduce thrips-transmitted tospoviruses. However, several considerations are relevant to avoid problems in thrips control, such as the development of insecticide resistance by thrips (Jensen, 2000), or the emergence of more virulent thrips populations that could overcome plant resistance. For example, growing cultivars with different resistance mechanisms in the same area might reduce the probability of emergence of virulent populations, but also stacking different resistance genes might have the same effect (Mundt, 2014; Seifi et al., 2013). To do this, more than one mechanism and QTLs need to be identified to allow stacking.

In Chapter 2 and 3 we identified accessions resistant to *F. occidentalis* that were subsequently tested with *T. tabaci* and *T. parvispinus* (Chapter 4). A significant interaction between genotype and thrips species was found. For example, Penny Lane was found resistant against *F. occidentalis* but not against *T. tabaci* and *T. parvispinus*, hence the resistance against *F. occidentalis* found in Penny Lane could either be based on a different mechanism than the resistance found in *C. seticuspe* PB-MB133, which showed resistance to all three thrips species, or the latter could be a source of more than one resistance mechanism against thrips larvae. In this thesis only larval development was investigated, but resistances affecting other life stages might be available and worth investigating. The genetics of the resistance(s) against *F. occidentalis* and *T. tabaci* in this wild relative may be studied in an intraspecific *C. seticuspe* F1 or F2 population because we identified also a susceptible accession of the same species. On the other hand, the genetics of resistance against *T. parvispinus* cannot be studied in this population because both accessions were resistant.

Over the years, with the increasing globalization and transport of (plant) material from place to place, many pests have been transported with plants. Non-native species can have a great impact on the biodiversity in the newly invaded area, therefore changing the evolutionary course of native species by, for example, predation, hybridization, competition, niche displacement, and even extinction. Moreover, the evolution of the invasive species is not only influenced by the native species (not only the ones they might displace but also the possible natural enemies), but also by the environment they encounter (Mooney and Cleland, 2001). Harmful non-native species are often referred to as invasive. Some native species might be displaced by the new species, but the natural enemies of the native pests might not be effective against the newly introduced species. Characteristics, such as

the emergence of insecticide resistance, such as the one occurring in *F. occidentalis* in the 1970s, may increase the invasive potential of certain populations (Morse and Hoddle, 2006). The current climate warming (increasing mean temperature) we are experiencing in temperate regions is allowing pests from tropical zones to expand their distribution range and, thus, increases the probability of establishment of these invasive species (Hulagappa et al., 2022; Skendžić et al., 2021). For example, *T. parvispinus* is native to Southeast Asia, and in recent years spread to other areas with favourable climatic conditions and host plants, such as India where it is now considered invasive (Sridhar et al., 2021). Also, an increase in mean temperature generally favors higher developmental rates, shorter generation time, increased population size, and higher outbreak frequencies of pests (Schneider et al., 2022). If the invasive species comes in contact with resistant plants but it is not affected by the resistance, then the invasive species further proliferates and spreads. Therefore, it is of extreme importance not only to explore resistance variation within the plant material available with the thrips species that are already established (Chapter 2 and 3), but also to be ready for incoming threats and to include possibly 'new' invasive thrips species, such as *T. parvispinus*, in resistance screenings (Chapter 4).

Conclusion

In this thesis, several sources of thrips resistance were identified in *Chrysanthemum* spp. We studied population build-up of thrips on whole plants and larval development on chrysanthemum leaves to identify resistant accessions that hamper the development of thrips larvae in non-flowering plants. The resistant accessions were also phenotyped for resistance against multiple *F. occidentalis* populations and *T. tabaci* and *T. parvispinus*. The results suggest that wild relatives of *Chrysanthemum* may act as sources of several resistance mechanisms and broad-spectrum resistance to thrips species. Furthermore, leaf metabolites associated with resistance in the cultivar Penny Lane were putatively annotated.

Several questions remain unanswered regarding thrips resistance in *Chrysanthemum.* Further research is needed to develop mapping populations and to identify QTLs for resistance. Moreover, the metabolites associated with resistance remain to be validated, possibly both *in vitro* and *in planta*. I am hopeful that a follow-up project can contribute to unravelling the genetics of the resistance and, finally, lead to thrips resistant *Chrysanthemum* cultivars.

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Summary

Thrips are one of the major pests in *Chrysanthemum* cultivation, causing both direct damage through feeding and indirect damage through transmission of viruses. Damaged plants and flowers are unmarketable, and in this way, thrips infestations result in large economic losses. Control methods include insecticides, biocontrol agents and agronomic practices, which are often combined in an Integrated Pest Management approach (IPM). Despite the great efforts put into thrips control, it is often difficult and ineffective. This is due not only to the intrinsic biological characteristics of these insects i.e. high reproduction rate, short life cycle, vast host range and fast development of insecticide resistance, but also to limited effectiveness of biocontrol agents and the current banning of insecticides. Host plant resistance could be a promising addition to an IPM approach to control thrips.

Host plant resistance against thrips *Frankliniella occidentalis* in *Chrysanthemum x morifolium* cultivars has been investigated using various screening methods and evaluating diverse biological traits. Resistant material that showed reduced feeding damage and/or reduced thrips population build-up has been identified, and investigations into the resistance mechanisms have been made. However, no genetic studies on thrips resistance in *Chrysanthemum* have been conducted, and the identification of resistance sources did not lead to strong resistance being introgressed into cultivars.

In this thesis I aimed to optimize a phenotyping method for antibiosis resistance against thrips in early, non-flowering, cultivation stages of *Chrysanthemum*, to identify resistance sources among cultivated *Chrysanthemum* and wild relatives, to investigate whether the identified resistance is effective against diverse thrips *F. occidentalis* populations and different thrips species, and to characterize the resistance mechanism.

A comprehensive screening of 70 *C. x morifolium* accessions was conducted using two phenotyping methods, a no-choice whole plant assay for thrips population build-up and a detached-leaf assay for larval development. This screening revealed large variation in both thrips population build-up and larval development from L1 into L2 stage among the 70 accessions. Two cultivars were selected as references in this thesis, Penny Lane as thrips-resistant, and Super Pink Pompon as thrips-susceptible. Then, a subset of accessions was re-evaluated in a second whole plant assay, and the reduced population build-up of nine resistant accessions was validated. The subset of accessions was further characterized with two other phenotyping methods, leaf disc assays for larval survival and development and leaf disc assays for reproduction. The resistant accessions showed not only a slower and

reduced development from L1 to L2 stages but also a reduced larval survival. On the other hand, the reproduction rate of thrips did not significantly differ among accessions. The identified resistant accessions could serve as valuable resources for further genetic and metabolomics studies on thrips resistance.

To find even stronger or broader sources of resistance, thrips resistance in *Chrysanthemum* wild relatives was investigated. Forty-seven accessions from different *Chrysanthemum* species and related genera were screened for thrips population build-up and larval survival and development. In resistant accessions, larval development from L1 into L2 stage was suppressed. When comparing 4-week-old to 8-week-old plants, the resistance level increased with plant age. However, adult reproduction on leaves did not differ between accessions. Additionally, reproduction in wild accessions has also been studied in flowers for some accessions that showed resistance in leaves, and significant differences between accessions were found. The resistance identified in wild relatives of *Chrysanthemum* did not depend on trichome density. The identified resistant and susceptible wild accessions of *Chrysanthemum* can be used in further studies to unravel the genetics and mechanism of resistance.

Thrips populations can differ in response to resistant plants. Therefore, five *F. occidentalis* populations were collected and their larval development was assessed on a few wild and two cultivated *Chrysanthemum* accessions. Two resistant accessions, *Chrysanthemum seticuspe* PB-MB133 and Penny Lane, consistently exhibited resistance across different *F. occidentalis* populations, while one accession showed different levels of resistance depending on the thrips population. The five *F. occidentalis* populations were also characterized for the mitochondrial *CO1* gene variation and five distinct haplotypes were detected. Four *CO1* haplotypes belonged to the *F. occidentalis* glasshouse strain, and one belonged to the lupin strain, which was not reported before to occur in Europe. The accessions were also phenotyped for *Thrips tabaci* and *Thrips parvispinus* larval development. While the cultivated chrysanthemum proved to be susceptible to both, in the wild accessions the larval development of both species was suppressed. This suggests that diverse mechanisms against thrips are present in the *Chrysanthemum* genus.

The mechanism underlying thrips resistance found in the *C. x morifolium* cultivar Penny Lane was investigated. Plant resistance levels of a thrips-resistant (Penny Lane) and a thrips-susceptible (Super Pink Pompon) cultivar were determined and their leaf metabolite profiles were quantified by an untargeted LC-MS-based metabolomics approach, both prior to and after thrips infestation. While previous thrips infestation had no effect on thrips resistance level in both cultivars, ten metabolites were significantly induced by thrips infestation, two in Super Pink

Pompon and eight in Penny Lane. Furthermore, an F1 population of 48 genotypes derived from a cross between Super Pink Pompon and Penny Lane was examined for correlations between thrips larval development and leaf metabolites. Nine resistant and nine susceptible genotypes were selected for leaf metabolite profiling using an untargeted LC-MS approach. Twenty-seven metabolites significantly correlated with resistance and 11 correlated with susceptibility, among which various phenolic compounds, terpenoids, and flavonoids. Some of these, namely chlorogenic acid and luteolin, were known to be associated with thrips resistance, others were previously found in relation to resistance to other pests and still others are novel candidates for thrips resistance. While this study provides a promising foundation for identifying resistance-associated metabolites, further validation is necessary to confirm the functional role of these metabolites in conferring resistance to thrips.

Finally, in the general discussion the main findings of this thesis are summarized and discussed. I review the importance of defining resistance and the use of the correct phenotyping method for efficient screening and identification of host plant resistance against thrips. The role of crop wild relatives as sources of (resistance) traits and the possible resistance mechanisms against thrips in cultivated and wild relatives of *Chrysanthemum* are revised. Lastly, the importance of broad-spectrum and durable resistance against thrips is discussed.

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About the author

Marcella Bovio was born on the 6th of September 1994 in Borgomanero, Italy. At the age of six she moved with her family to Dagnente, where she learned to appreciate green spaces and to spend time alone in the garden. Always fascinated by plants and flowers, and chemistry thanks to nonna (her grandmother), she with her friends started to cook flowers and plants to create colours and “potions” to measure pH.

Following her interest in plants she enrolled in the BSc Management of cultivated plants and landscaping at Università Statale di Milano. During an entomology course she discovered a new passion for insects and started her entomological collection. In December 2016 she visited Wageningen for the first time, and despite the gloomy winter weather she was enchanted by the highly advanced university. In September 2017, she started her MSc in Plant Biotechnology and for her theses she decided to combine her interests in plants and insects researching first whitefly resistance in tomato and then thrips resistance in chrysanthemum. At the end of her MSc in September 2019, she got offered the PhD position, whose results are presented in this thesis.

In 2023, Marcella wrote a TKI proposal together with Dr Lotte Caarls to continue the research on thrips resistance in *Chrysanthemum*. The project was granted and thus, from April 2024, Marcella will continue working on this topic as a Post-Doc at the laboratory of Plant Breeding of Wageningen University and Research.

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