Tomato’s best thrips shield: acylsugars, sesquiterpenes, methylketones or phenolics?

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Abstract
To determine on which class of compound to concentrate breeding efforts the relative efficiency of Solanum trichomal defenses was assessed against the generalist western flower thrips, Frankliniella occidentalis. Wild and domesticated tomatoes were compared on both the level of thrips resistance and the amount of biomass, as trichome density and exudate yield, devoted to each defense mechanism. Low levels of herbivory were associated with high glandular trichome densities and high exudate yields. Thrips resistance was also influenced by the exudate chemistry. All targeted trichomal metabolites -acylsugars, sesquiterpenes, methylketones and phenolics- were identified and quantified through NMR and GC analyses of crude and partially purified exudates. Acylsugars and sesquiterpenic carboxylic acids played a minor role, providing protection against thrips only at high concentrations and in combination with high trichome densities. 7-Epizingiberene did not have any important effect on thrips whereas methylketones showed a clear concentration-dependent effect on mortality. Thrips also died through immobilization by rutin polymers on a yet unidentified domesticated line with high densities of lobed type-6 glands. In contrast to the latter, all other defense mechanisms allowed the insect to escape, resume probing and potentially spread viruses. Considering the levels of thrips damage and the amount of biomass involved in the different defense mechanisms, rutin-mediated immobilization was not only the most effective but also the most efficient thrips shield in tomato. It is important to emphasize that gland densities needed for protection against thrips are already present in the germplasm of domesticated tomatoes and ready to be exploited by breeders.

Introduction
After rapid dissemination across the world, the western flower thrips (WFT), Frankliniella occidentalis (Pergande; Thysanoptera: Thripidae), has become one of the most serious insect pests (Kirk and Terry, 2003). This thrips is a highly polyfagous herbivore, ca. 1.5 mm in length, with strong negative effects on a wide range of economically important crops and ornamentals. As a whole-cell sucking insect WFT feeds on epidermis and mesophyll leaving silvery scars on leaves, flower petals and fruits. Moreover, thrips is a vector for plant viruses, of which tomato spotted wilt virus (TSWV) is economically the most important one (German et al., 1992). As yet control of thrips relies mainly on pesticides, but very few are actually suitable for most integrated pest management programs. In addition, thrips high reproductive ratio and cryptic habits demand rather large and repeated doses of insecticides, which in turn leads to an accelerated development of resistance and increased health risks posed to humans. Therefore, host-plant resistance remains as a crucial alternative to lessen the negative impact of thrips and TSWV.

The Solanum genus harbors in its wild species a diverse collection of constitutive secondary metabolites proven to be effective against many biotic and abiotic stress factors. A lot of those defenses are trichome-borne and act only in combination with high densities of glandular trichomes. In tomato there are two major types of glandular trichomes, type 4 and type 6, which account for the production of most surface chemical defenses (Fig.1) (Simmons and Gurr, 2005). Type-4 trichomes are absent in domesticated tomatoes but rather abundant in the wild species S. habrochaites and S. pennellii, particularly in some accessions of the latter. This type of trichome has a short multicellular stalk whose monocellular glandular tip secretes mucilaginous glycolipids (sugar alkyl esters), commonly referred to as acylsugars (Fig 2). Acylsugars are a resistance factor in tomato and
potato as they cause entrapment in between the trichomes to a wide range of arthropods, such as potato aphid, *Macrosiphum euphorbiae* (Gentile and Stoner, 1968), silverleaf whitefly, *Bemisia argentifolii* (Liedl et al., 2003), green peach aphid, *Myzus persicae* (Simmons et al., 2003), cotton bollworm, *Helicoverpa armigera* (Simmons et al., 2004) and *F. occidentalis* (Mirnezhad et al., 2010). Type-6 trichomes on the other hand are more versatile chemical factories, which can have round or lobed tetra-celled prominent heads (Fig. 1C, E) on short multicellular stalks (Snyder and Carter, 1985). The round type-6 glandular trichomes are present in much fewer *Solanum* species but are especially dense in *S. habrochaites* (formerly *Lycopersicon hirsutum* f. *typicum* and f. *glabratum*). These round glands synthesize a diverse set of compounds, such as sesquiterpenes (e.g. zingiberene), sesquiterpenic carboxylic acids and long-chain methylketones. Zingiberene, a well-known reactive sesquiterpene from ginger roots, is produced by tomato in one of its epimeric forms, 7-epizingiberene (Fig. 2) (Breeden and Coates, 1994). This sesquiterpene has shown negative effects against different herbivores including Colorado potato beetle, *Leptinotarsa decemlineata* (Carter et al., 1989), two-spotted spider mite, *Tetranychus urticae* (Weston et al., 1989) and beet armyworm, *Spodoptera exigua* (Eigenbrode et al., 1994). Santalenoic acid and bergamotenoic acid (Fig. 2), are the main sesquiterpenic carboxylic acids present in *Solanum*, exclusively produced by the highly resistant accession *S. pennellii*.
**habrochaites** former f. *typicum* LA1777. Although these sesquiterpenic carboxylic acids proved to be oviposition stimulants to moths of tomato fruitworm, *Heliothis zea*, (Coates et al., 1988), Frelichowski Jr and Juvik (2001) demonstrated that the very same compounds deterred feeding and reduced survival of *H. zea*, and *S. exigua* larvae. Methylketones are broad-spectrum natural insecticides responsible for varying degrees of resistance in tomato to tobacco hornworm, *Manduca sexta* (Kennedy and Henderson, 1978), *H. zea* (Dimock et al., 1982), *L. decemlineata* (Kennedy and Sorenson, 1985), *T. urticae* (Chatzivasileiadis et al., 1999), *M. persicae* (Antonious et al., 2003), and sweetpotato whitefly, *Bemisia tabaci* (Antonious et al., 2005).

![Chemical structures](image)

**Figure 2.** Defense metabolites accumulated in the glandular trichomes of *Solanum* species.
In contrast, the lobed type-6 glands, which are present in many wild species, as well as in domesticated tomatoes, *S. lycopersicum*, produce mostly phenolics like rutin (Fig. 2) and chlorogenic acid as the only chemical defense. These phenolics undergo enzymatic polymerization upon gland rupture immobilizing insects as the polymers solidify (Duffey, 1986).

During the domestication of tomato most of these defense traits were gradually lost in favor of marketable horticultural traits. Using classical plant breeding methods many unsuccessful attempts have been made in the past decades to bring these resistance features back into *S. lycopersicum*. Failure in achieving to do so results from the combination of sexual incompatibility, gene recessiveness, multigenic domain control, low pollination rates and/or linkage to undesirable horticultural traits like low fruit yield, small fruits and foliar size. Efforts in modern breeding techniques, including genetic engineering, are now required to increase host-plant resistance, some of which have already been independently undertaken. A marker-assisted selection breeding program was used by Lawson et al. (1997) in an attempt to transfer the ability to produce acylsugars from wild to domesticated tomato. Van der Hoeven et al. (2000) identified two *Sesquiterpene synthase* loci associated with the accumulation of different classes of sesquiterpenes. Ben-Israel et al. (2009) successfully transferred genes codifying for the production of methylketones into *Escherichia coli*. Considering the complexity of this kind of challenges it is surprising that no reports are known to date on the relative efficiency of these defenses against any pest as to determine on which compound to focus such efforts. The production of these defenses involves large amounts of biomass, mainly in the form of trichomes and exudates, compromising a share of photoassimilate and other resources that could otherwise be devoted to yield. It is, therefore, important not only to compare the relative efficacy of the chemical defense traits of *Solanum* but also the amount of biomass invested.

Using a leaf feeding bioassay Kumar et al. (1995) detected remarkable differences in resistance to thrips among wild and domesticated tomatoes. Mimezhad et al. (2010) not only reported similar differences but also a relationship between thrips resistance and acylsugars. However, the effect of specific trichome types and other chemical resistance traits were not explored in any depth. With the present study we want to provide further insight into the different trichome-based resistance mechanisms to thrips present in *Solanum* and compare their relative efficiency. Eleven accessions of different wild species and three cultivars were selected for this purpose according to their contrasting morphological and phytochemical features as reported in the literature. Thrips herbivory, measured as foliar silver damage, glandular and non-glandular trichome densities, exudate yield, exudate composition and acute toxicity of exudates were evaluated.

**Methods**

**Plants**

Wild and domesticated tomatoes were selected according to their contrasting trichomal morphology and chemistry as reported in the literature. A total of eleven accessions from four wild species together with three cultivars were used in the experiments. The set included *S. pennellii* (LA716), *S. pennellii* var. *puberulum* (LA1926), *S. habrochaites* former f. *typicum* (LA1777, LA1353, PI126445 and LA1033), *S. habrochaites* former f. *glabratum* (PI134417, LA1223 and PI251305), *S. chilense* (LA1029), *S. neo-rickii* (LA1326) and *S. lycopersicum* (cv Moneymaker, cv Yellow pear-shaped and a still unidentified
variety). Accessions LA and PI were respectively obtained from the Tomato Seedstock Center at the University of California (Davis, CA) and the USDA-Agricultural Research Service (Ithaca, NY), while the domesticated tomatoes were purchased from Dutch seed companies. Prior to sowing seeds of the wild tomatoes were scarified by soaking for 30 min in 2.7% sodium hypochlorite. Seeds were directly sown in 13 cm pots with potting soil and thinned to one plant per pot after one week. Five replicates of each accession and cultivar were grown in a randomized layout in a glasshouse during the months of July and August in 2009.

**Thrips bioassays and trichome counts**

Thrips bioassays were carried out six weeks after seed germination. At this point most plants had between nine and thirteen fully expanded leaves. Leaves from the lower and upper halves of the shoot were respectively designated as older and younger leaves to evaluate the ontogenic variation of resistance traits. For the choice herbivory bioassay four leaves were randomly excised from each plant, always leaving out the two youngest and oldest leaves. Excised leaves were divested of lateral leaflets and the surface of all leaflets was measured with a planimeter. In order to maintain the turgor the apex leaflets were put in test tubes filled with sufficient water. Samples, totaling 70 leaflets (14 accessions x 5 plants x 4 leaves), were randomly placed in two thrips-proof cages in a climate chamber (16/8 hr photoperiod, 20 °C). The cages were constructed from clear polyester walls covered with nylon gauze of 120 µm mesh size. Per leaflet, 10 adult WFT, reared on flowering chrysanthemum, were added to the cages. After three days thrips herbivory and trichome densities were measured.

Thrips herbivory was estimated based on the extension of characteristic silver damage. The area of the silvery scars was measured by eye in each case and expressed in mm². To correct for possible landing surface effects each measurement was divided by the corresponding leaflet area. Glandular and non-glandular trichomes were identified and classified following the descriptions of (Luckwill, 1943). Only the main types (1, 4, 5 and 6) were considered. Trichomes type 3 and 7 were very sparse and small respectively, representing no obstacle to thrips. Selected trichome types were counted in an area of 7 mm² at a middle central point of the adaxial side of each leaflet using a stereomicroscope at 80x magnification. Trichome densities were reported per foliar surface unit (cm²). Pictures of trichome morphology were obtained with a Leica MZ126FA fluorescence microscope (Leica Microsystems, www.leica-microsystems.com) equipped with a Leica DFC 420 C digital color camera. Videos of the interaction between thrips and younger leaves of the different accessions were recorded with a Leica MZ12 stereo light microscope equipped with a Leica DC 500 digital color camera.

In order to assess the relative contact toxicity of the different glandular exudates an acute toxicity test was conducted. A total of 10 adults of WFT were individually kept on younger leaflets of each tomato accession and monitored under the microscope. To prevent thrips from escaping and to promote contact with the glands a glass plate was placed above the leaflet surface at a distance of ca. 3 mm. After 5 min thrips were transferred to leaflets of susceptible *S. lycopersicum* placed in a Petri dish. Thrips were watched for the following 60 min to register mortality.

**Exudate removal and chemical analysis**

Due to limited yields on older leaves, especially from the least hairy species, glandular exudates were isolated only from the younger leaves of each tomato. In order to maximize the amount of extract the remaining younger leaves of each accession were pooled and redistributed into three extraction replicates. Prior to extraction the weight and the surface area of the leaves were measured. Exudates were then selectively removed from the fresh leaves through two consecutive flash washouts (five-second
immersions) with dichloromethane and ethanol. Extracts were filtered with Whatman paper No. 1 before solvent evaporation. Exudate yields were determined and reported in micrograms per foliar surface unit and in percentage of foliar dry mass. The average water content of tomato leaves was previously determined as 89.0 ±1.1%.

The chemical composition of glandular exudates and the concentration of all targeted metabolites were determined through a combination of GC, GC-MS, 1H NMR and column fractionation. 7-epizingiberene, (R)-α-curcumene (7-epizingiberene’s aromatic oxidation product), α/β-bergamotenoic acids, α/β-santalenoic acids, 2-undecanone and 2-tridecanone were all directly quantified by GC-FID in the crude extracts. β-Caryophyllene and 2-tridecanone, obtained from Sigma-Aldrich (Steinheim, Germany), were used as standards to quantify terpenoids and methylketones respectively. Levels of acylsugars were determined through a stepwise gravimetric approach due to the high complexity of these polyester mixtures. About 15 mg of each exudate was partially purified by triplicate into four fractions of very distinctive polarity as they were eluted from a silica microcolumn (0.6 g of SiOx 40-63 µm of particle size) with the following progressive polarity series: hexane, hexane-diethyl ether (7:3, v/v), acetone and methanol-water. Hexane fractions almost exclusively contained cuticular waxes, mostly long chain alkanes, which were washed off of the foliar surface along with glandular exudates during chemical immersion. Exudate yields were then corrected for wax content by subtracting the weight of the hexane fractions. Only H45 and H53 showed some contamination with (R)-α-curcumene and 2-undecanone, respectively, which were then quantified by GC. The hexane-diethyl ether solvent mixture eluted most sesquiterpenes and methylketones, whereas acylsugars were entirely and almost exclusively collected in the acetone fraction. In this way the quantification of the acylsugars was possible. Due to polymerization and irreversible adsorption onto silica, phenolics were roughly estimated by the weight difference between crude exudate and the sum of all other fractions. This highly polar fraction may therefore include as well free sugars and amino acids.

Instrumental parameters
Crude exudates and semi-purified fractions were dissolved in toluene at concentrations of 1 to 4 mg mL⁻¹ and analysed with an Agilent 6890 series gas chromatograph (Agilent Technologies; www.agilent.com) equipped with a 7683 autosampler and an FID. Injections of 4 µL (split ratio 1:20) were made onto a DB-5 column (30 m x 250 m i.d. x 0.25 µm; Agilent J&W) initially held at 100 °C whereby temperature was gradually increased as follows: to 140 °C at 10 °C min⁻¹, to 215 °C at 3 °C min⁻¹ and finally to 275 °C at 18 °C min⁻¹. The carrier gas was nitrogen at a constant flow rate of 0.7 mL min⁻¹. The inlet and the detector temperatures were set at 265 °C and 285 °C respectively.

GC-MS analysis was performed on an Agilent 7890A series gas chromatograph equipped with a 7693 autosampler and a single quadrupole mass spectrometer 5975C. The column type and the oven program were as in GC-FID. The MS source was set to 230 °C, the single quad temperature was 150 °C, and the transfer line temperature was set to 280 °C. The GC-column was linked to the MS via a quickswap (Agilent Technologies) and restrictor (0.11 mm internal diameter, Agilent Technologies). The injector temperature was 230 °C with an injection volume of 2 or 4 µL (split ratio 1:20) and a carrier gas (He) flow rate of 0.63 ml min⁻¹. The mass range analyzed by the mass spectrometer was 50-500 amu. The GC-MS was controlled by Enhanced Chemstation software version E.02.00.493 (Agilent Technologies). The NIST library version 2.0f (Standard Reference Data Program of the National Institute of Standards and Technology, Distributed by Agilent Technologies) was used to assist metabolite identification.

Samples for NMR analysis of crude exudates and semi-purified fractions were respectively
prepared in DMSO-$d_6$-TMSP-$d_4$ 0.005% (w/v) and CDCl$_3$-TMS 0.03% (v/v) at a concentration of about 14 mg ml$^{-1}$. $^1$H NMR spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. The corresponding deuterated solvent was used as the internal lock. Each $^1$H NMR spectrum consisted of 128 scans requiring 10 min and 26 s acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW30°)=11.3 µs, and relaxation delay (RD)=1.5 s. Free induction decays were Fourier transformed with a line broadening (LB)=0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to the internal standard TMSP-$d_4$ or TMS at 0.0 ppm, using XWIN NMR (version 3.5, Bruker).

**Statistical analysis**

Silver damage and trichome-related variables were respectively square-root and natural-logarithm transformed to approach a normal distribution of data. The significance of differences in all variables was investigated with ANOVA followed by a post-hoc Duncan test. Correlations were verified using Pearson's $R$ coefficient. Multiple linear regression analysis was performed on the transformed variables as these fitted a linear model best. All statistical tests were performed using SPSS v. 17.0 (SPSS Inc., Chicago, IL, USA).

![Figure 3](image-url)  
**Figure 3.** Thrips herbivory as silver damage (A) and glandular trichome density (B) on selected *Solanum* accessions. Leaflets of two age groups, older and younger, were excised and exposed to thrips for three days in a choice assay. Bars represent the average (± SE) of ten biological replicates.

Results

Trichome densities and thrips damage in Solanum

Glandular trichome density (G: types 1, 4 and 6 combined) and silver damage (D) varied significantly in both leaf ages, older ($F_{G}=94.4$, $F_{D}=13.9$, df=13, $p=0.0001$) and younger ($F_{G}=129.2$, $F_{D}=27.6$, df=13, $p=0.0001$), across the set of wild and domesticated tomatoes, (Fig. 3, Table I). A negative correlation between glandular trichome density and thrips damage was detected across all accessions (Pearson, $R=-0.549$, $N=14$, $p=0.042$). Similar correlation values were obtained within several accessions. But more importantly, a significant change in silver damage between older and younger leaves was observed only if there was a concomitant opposite change in glandular trichome density. This confirmed that glandular trichomes are the primary defense shield against WFT in tomato. Such significant ontogenic changes in silver damage occurred in S. habrochaites former f. typicum LA1777 (H77), LA1353 (H53), PI126445 (H45) and LA1033 (H33), S. habrochaites former f. glabratum PI134417 (G17) and a still unidentified variety of S. lycopersicum (LXX). Solanum chilense LA1029 (Chil) was the only accession in which a significant difference in silver damage between older and younger leaves ($T=2.5$, df=18, $p=0.021$) was not associated with an equivalent change in glandular trichome density. Instead, insect damage was correlated with the density of type-5 non-glandular trichomes (NG, Pearson, $R=-0.665$, $N=20$, $p=0.001$). This wild species exhibited the lowest density of glandular and the highest density of non-glandular trichomes. Type-5 non-glandular trichomes were observed only in Chil, Neo and the domesticated tomatoes, all of which had the lobed type-6 trichomes as the only relevant glandular trichomes. A multiple linear regression analysis performed on this subset showed that the influence of the glandular trichomes on silver damage as the dependent variable is greater than that of the non-glandular ones ($F_{2,97}=25.3$, $p<0.001$, $N=100$; adjusted $R^2=0.33$, $\beta_c=-0.466$, $p<0.001$, $\beta_{NG}=-0.373$, $p<0.001$). This indicates that non-glandular trichomes play at most a supporting role in tomato defense against thrips.

The most resistant tomatoes were: 1) P16, with no thrips damage at all, 2) H77, with almost no damage on younger leaves and, 3) H53 and G17, with very low scores of thrips damage on younger leaves. In addition to these wild accessions one of the S. lycopersicum varieties, LXX, unexpectedly exhibited herbivory scores comparable to those of the above-mentioned wild species. In this cultivar the overall density of lobed type-6 glands was more than four times significantly higher compared to the average of the more susceptible cultivars, LPS and LMM ($F=21.1$, df=2, $p=0.0001$), while the non-glandular trichome density did not differ significantly.

Exudate yield

Yield values varied significantly throughout the set of tomatoes ($F=115.8$, df=13, $p=0.0001$) and strongly resembled the pattern of glandular trichome density (Figs. 4 and 3B, Table I). This resemblance suggests that the amount of exudates was influenced to a greater extent by the trichome density than by the gland yield. A strong negative correlation between averages of exudate yield and thrips damage on the younger leaves was obtained across all accessions (Pearson, $R=-0.832$, $N=14$, $p=0.0002$).

Some wild species produced very high amounts of exudate, up to 12% of the plant’s dry mass in H77 and 30% in P16. Similar exceptional yields have been previously reported for both H77 (Coates et al., 1988; Frelichowski Jr and Juvik, 2001) and P16 (Fobes et al., 1985). In the domesticated tomatoes and other highly susceptible accessions the amount of glandular exudates...
did not exceed 3% of the plan’s dry weight. Exudate yields, however, are in general very sensitive to plant ontogeny and other abiotic factors (Fobes et al., 1985).

Within the group of least susceptible accessions LXX produced the lowest amount of exudate with an average of 7.5±0.6 mg cm⁻², which represents a significant difference of about four times compared to the average value of the other two cultivars, LPS and LMM (F=66.8, df=2, p=0.0001). Accessions H53 and G17 produced comparable amounts of exudates with 14.3±0.3 and 12.5±0.2 mg cm⁻² respectively. H77 and P16 produced 30.9±1.1 and 435±40 mg cm⁻² of exudate respectively, which are about 20 and 250 times the average value of the susceptible cultivars, LPS and LMM.

Chemical analysis of exudates
A comprehensive analysis by GC-MS, NMR and column fractionation allowed the identification and quantification of every targeted chemical defense metabolite in all accessions (Fig. 5). In the Pennellii accessions acylsugars were the only relevant phytochemicals observed. Although in a previous study no acylsugars were detected in the Puberulum variety (P26) (Shapiro et al., 1994), in our experiment measurable amounts of these polar lipids were isolated from P26 (2.30 mg cm⁻²) as verified by NMR (Figs. 6 and 7). All major signals in the ¹H NMR spectra of P16 and P26 were characteristic for acylsugars. The levels of these glycolipids in P26 were on average about 150 times lower than in P16, which is known to produce the highest amount of glycolipids among all Pennellii accessions studied so far (Shapiro et al., 1994).

The chemical diversity of round type-6 glands was confirmed in the Habrochaites accessions. Sesquiterpenic carboxylic acids were the main component in the exudate of H77, while in the rest of the accessions acylsugars exceeded any other targeted chemical defense. Relatively high levels of methylketones (2-undecanone and 2-tridecanone) were detected in H53 and G17, with 0.85 and 3.49 mg cm⁻² respectively. Whereas in G23 and G05 concentrations of methylketones were much lower than expected, with 0.006 mg cm⁻² in the crude exudate of G23 and below detection limits in the exudate of G05. In H53 2-undecanone constituted 83% of the binary mixture while in G17 the opposite distribution was observed with 2-tridecanone as the major component at a relative concentration of 89%. Yield of 7-epizingiberene in H45, expressed as the sum of its oxidized
form \((R)-\alpha\)-curcumene) and the remaining of its native form, was 0.3 mg cm\(^{-2}\). Although low compared to the other chemical defenses it was in agreement with previous reports (Carter et al., 1989; Weston et al., 1989). Several other sesquiterpenes detected by GC-MS, possibly cyclic and epoxydic derivatives of 7-epizingiberene, account for the rest of H45 exudate.

Rutin was the single relevant metabolite exclusively detected in the exudate of species and cultivars bearing lobed type-6 trichomes. The typical signals for the aromatic protons of this flavonoid can be easily distinguished in the \(^1\)H NMR spectra of LXX, LMM, LPS, Chil and Neo (Fig. 6, 8.0-6.0 ppm). Other catechol-like phenolics such as chlorogenic acid and caffeoylglucaric acid have been allegedly found in type-6 trichomes of tomato and potato, \textit{Solanum berthaultii} (Duffey, 1986). It has been recently demonstrated, however, that chlorogenic acid is a lamellar secondary metabolite present in the exudate of \textit{S. lycopersicum} as an extraction artifact of the immersion method (Kang et al., 2010). Caffeoylglucaric acid was not detected either in the lobed type-6 glands. Instead, kaempferol, quercetin and myricetin derivatives were detected in some exudates at very low relative concentrations compared to rutin. Yet, these phenolics may also be extraction artifacts. Therefore, the phenolic fraction of accessions bearing round type-6 glands, \textit{i.e.} P16 to G05, contained mostly very polar metabolites such as free sugars and amino acids, rather than major phenolics.

Within the \textit{Habrochaites} group it was possible to estimate the relative contribution of sesquiterpenic carboxylic acids, methylketones and 7-epizingiberene to thrips resistance by comparing all accessions to H33, whose type-6 glands do not produce any major anti-insect metabolite besides acylsugars. Differences in susceptibility within this group seemed to depend on both the amount of biomass (trichomes and exudate) and, more importantly, its composition. In general, thrips resistance improved with the presence of sesquiterpenic carboxylic acids, methylketones or 7-epizingiberene in addition to acylsugars, suggesting that this latter class of defense metabolites play a supporting role. A multiple regression analysis for insect damage versus type-4 and type-6 trichome density supports this statement \((F_{2,137}=42.3, p<0.001, N=140; \text{adjusted } R^2=0.37, \beta_{G-4}=-0.257, p=0.001, \beta_{G-6}=-0.475, p<0.001)\). Although only 37% of the variation in the
dependent variable was explained by glandular trichome densities, a much higher influence on SD was obtained from glands that produce sesquiterpenic carboxylic acids, methylketones or 7-epiizin-giberene (type 6) than from those that produce acylsugars (type 4). However, this regression analysis overlooks the fact that Habrochaites accessions synthesize different glandular defenses. Fortunately, accessions H53 and H33 are very similar phenotypes, providing in this way further evidence on the relative importance of the different trichome types. These accessions not only had comparable glandular trichome densities and total acylsugar concentrations (Figs. 3-5 and Table 1) but also had the same profile of acylsugars (Fig. 7). Yet, H53 was significantly less susceptible than H33 (\( T=3.316, \text{df}=18, p=0.004 \)), most likely because of toxic methylketones.

**Toxicity of exudates**

Microscopy observations of the direct interaction between thrips and the different accessions revealed that particularly on P16 and H77 thrips get entrapped between the abundant mucilaginous trichomes. This prevents the insect from reaching the leaf surface to feed on and consequently the entrapped thrips struggles to fly off the trichome net (Videos S1 and S2). These observations and the absence of both acute toxicity and mortality suggest that sesquiterpenic carboxylic acids in H77 act in a similar fashion to acylsugars, *i.e.* entangling small insects in between the sticky trichomes. Our data, nevertheless, do not rule out repellency as another negative effect of sesquiterpenes on thrips.
Mortality was observed in the methylketone-producing accessions, H53 and G17 (Video S3), and in the domesticated tomato LXX. However, the physiological impact of methylketones is concentration-dependent. More than 50% of thrips recovered within 30 minutes once removed from the leaf surface. At the end of the herbivory choice bioassay, on the other hand, dead thrips were consistently observed on the younger leaves of LXX. In this particular case a different defense mechanism takes place (Video S4). Mechanical rupture of lobed type-6 glands allows cytoplasmic polyphenol oxidases to polymerize rutin, which is initially confined to the periplasm (Duffey, 1986). These polymers in turn literally act as “super glue” accumulating around thrips legs and eventually welding the insect to the leaf, ultimately causing its death.

**Discussion**

**Glandular trichomes form the main defense against thrips**

Our results demonstrate that glandular trichomes, in combination with their metabolic products, are the main defensive trait against WFT in *Solanum*. Evidence in the literature supporting a partial or total contribution of glandular trichomes to resistance against several classes of pests is extensive. Simmons and Gurr (Simmons and Gurr, 2005) present in their review a comprehensive analysis of the available
Table 1. Averages of herbivory, trichome densities, exudate yield and exudate composition for the younger leaves of selected *Solanum* accessions.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Damage (mm² cm⁻²)</th>
<th>Trichome densities (cm⁻²)</th>
<th>Yield (µg cm⁻²)</th>
<th>Exudate composition (µg cm⁻²)</th>
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<td>n.d.</td>
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<td>H77</td>
<td>0.05e</td>
<td>324d</td>
<td>169d</td>
<td>n.d.</td>
</tr>
<tr>
<td>H53</td>
<td>0.32de</td>
<td>347d</td>
<td>99d</td>
<td>n.d.</td>
</tr>
<tr>
<td>H45</td>
<td>1.37de</td>
<td>187c</td>
<td>101d</td>
<td>n.d.</td>
</tr>
<tr>
<td>H33</td>
<td>1.55de</td>
<td>321d</td>
<td>129d</td>
<td>n.d.</td>
</tr>
<tr>
<td>G17</td>
<td>0.34bc</td>
<td>34a</td>
<td>164d</td>
<td>n.d.</td>
</tr>
<tr>
<td>G23</td>
<td>5.55e</td>
<td>24b</td>
<td>19e</td>
<td>n.d.</td>
</tr>
<tr>
<td>G05</td>
<td>2.68de</td>
<td>74b</td>
<td>30bc</td>
<td>n.d.</td>
</tr>
<tr>
<td>LXX</td>
<td>0.20ab</td>
<td>0</td>
<td>133d</td>
<td>109e</td>
</tr>
<tr>
<td>LPS</td>
<td>3.63f</td>
<td>0</td>
<td>22c</td>
<td>160ab</td>
</tr>
<tr>
<td>LMM</td>
<td>2.52f</td>
<td>0</td>
<td>19c</td>
<td>200bc</td>
</tr>
<tr>
<td>Chil</td>
<td>0.83cd</td>
<td>0</td>
<td>22c</td>
<td>242c</td>
</tr>
<tr>
<td>Neo</td>
<td>2.56f</td>
<td>0</td>
<td>8bc</td>
<td>120ab</td>
</tr>
</tbody>
</table>


¹ Average of ten biological replicates, ² Average of three technical replicates, ³ Glandular, ⁴ Non-glandular, ⁵ This fraction may contain free sugars, aminoacids and inorganic salts.


Letters refer to significant differences at the 0.05 level as analyzed with ANOVA.

Information on the role of trichomes in tomato host-plant resistance against both pests and natural enemies. Antixenotic and antibiotic effects are reported for numerous lepidopterans and a few important species from the orders Hemiptera, Acarina, Coleoptera and Diptera, but none from Thysanoptera. Two approaches are commonly used to establish the defensive role of trichomes: removal of glandular exudates with organic solvents and correlation analysis. As opposed to the latter with the exudate removal method any change in ecological variables such as herbivore survival, oviposition, mortality, growth, etc., is proof enough of a causal relationship between glandular trichomes and host-plant resistance. Exudate washouts, however, are aggressive treatments that drastically alter the properties of plant tissues. Due to the relatively long duration of thrips feeding bioassays, usually between three and eight days, this approach was not an option in our study. Even water-methanol mixtures cause withering of tomato leaves within a few hours, rendering the plant material unsuitable for thrips to feed on. Furthermore, the lack of controls for possible physiological effects of the solvent casts serious doubts on the results of studies based on this method.

The significant ontogenic variation of thrips damage and glandular trichome density suggest the existence of threshold density values below which the insect escapes the negative effects of the glandular exudates. In such cases the insect manages to move in between trichomes rupturing a minimal number of glands. This critical glandular trichome density will mainly be determined by the insect size.
However, other variables such as insect motility, exudate physical properties and gland surface tension may also play a decisive role. Differences in these features could determine the effectiveness of tomato trichome-based defense against other important pests such as two-spotted spider mite and whiteflies. Compared to WTF whiteflies have better flying skills, choosing almost exclusively the abaxial side of leaves to feed and oviposit (Simmons, 1994). This finer motion control may enable whiteflies to avoid rupturing glands, particularly those of type 6, and hence exposure to their exudates. This is different in tomatoes with high densities of type-4 trichomes. The abundant secretion of mucilaginous acylsugars by these glands literally transforms leaves into glue paper traps. Once entrapped small insects cannot escape regardless of how good fliers they are. In fact, accessions with high densities of type-4 glands from *S. habrochaites* and *S. pennellii*, including H77, H53 and P16, are the only ones consistently reported as resistant to whiteflies, *B. argentifolii* and *B. tabaci* (Baldin et al., 2005; Heinz and Zalom, 1995; Muigai et al., 2003; Muigai et al., 2002; Snyder et al., 1998). Our results showed that nonglandular trichomes alone do not represent an effective protection against WFT, not even at densities as high as 240 cm⁻². In combination with glandular trichomes, however, they may have a synergistic effect on thrips resistance.

**Methylketones and rutin polymers are the most efficient defenses**

Based on the amount of biomass we showed that methylketones and rutin in tomato are more efficient defenses against WFT than acylsugars and sesquiterpenes. Due to the extraordinary thick layer of viscous exudates of acylsugars that cover the entire aboveground parts of P16 this accession is exceptionally resistant. P16 is almost immune to a wide range of pests, including pathogens (Nonomura et al., 2009). However, the cost of this protection, representing 30% of the plant’s dry mass, is high. According to the resource availability theory such a high allocation to defense is likely to compromise growth and fruit yield (Coley et al., 1985). In addition, the biosynthesis of acylsugars is known to be controlled by a multigenic domain distributed on multiple chromosomes (Mutschler et al., 1996), which makes the engineering of domesticated tomatoes for the production of sugar esters rather challenging. In contrast, methylketones are effective insecticides against various insect species (Antinous et al., 2005) at a much lower cost, below 5% of plant’s dry mass. Genes encoding for proteins involved in the biosynthesis of methylketones in type-6 glandular trichomes have already been isolated and successfully expressed in *Escherichia coli* (Ben-Israel et al., 2009).

The 7-epizingiberene-producing accession H45 was more susceptible to thrips than the other *Habrochaites* accessions H77 and H53. This could imply that 7-epizingiberene is less effective than sesquiterpenic carboxylic acids and methylketones. However, the low levels of 7-epizingiberene detected in H45 in combination with a lower glandular trichome density may instead account for the poor performance of this accession. Unfortunately, 7-epizingiberene is a labile compound that readily oxidizes into (R)-α-curcumene upon gland rupture. We confirmed this through GC-MS analysis of both H45 exudate and freshly collected type-6 glands, whereby the presence of 7-epizingiberene was verified only in the latter. In addition, both 7-epizingiberene and (R)-α-curcumene have been reported to show minor to moderate negative effects on different insects (Carter et al., 1989; Eigenbrode et al., 1994; Yano, 1987). Volatile-derived repellency to whitefly has been reported as part of the anti-insect effects of tomato trichome exudates, in particular from 7-epizingiberene-producing accessions (Bleeker et al., 2009; 2011). If there were any repellent volatiles in H45 modulating thrips behavior we would not have observed any thrips damage in most of its leaves. Therefore, their influence is most likely secondary compared to the non-volatile trichome barrier.
Additionally, specific underlying lamellar defense mechanisms, either constitutive or induced, may also play a role. Accessions H33 and P26 contain only acylsugars as main component of their exudates. P26 showed a lower trichome density and exudate yield compared to H33. However, silver damage in P26 is not significantly higher than in H33. Glycoalkaloids, as present in Solanum, could account for these differences in tomato resistance to thrips. WTF is known to be sensitive to pyrrolizidine alkaloids (Leiss et al., 2009a; Macel et al., 2005).

Solanum lycopersicum varieties are in general significantly more vulnerable to thrips than wild tomatoes (Kumar et al., 1995; Mirnezhad et al., 2010). Surprisingly, one of the domesticated varieties, LXX, possessed densities of lobed type-6 glands high enough to confer levels of thrips resistance comparable to those of the most resistant wild accessions, i.e. P16, H77, H53, and G17 (Fig. 3, Table I). Considering the level of thrips damage and the amount of trichome exudate involved in these defenses, LXX and its rutin polymers came out as the clear winner for the best thrips shield in tomato. In addition, the other defense mechanisms discussed here allow the insect to escape and eventually jump further onto less heavily protected leaves. Rutin-mediated immobilization, in contrast, causes mortality of the insect, minimizing therefore probing and potential virus spreading. Furthermore, as opposed to methylketones rutin polymers may not necessarily have a negative effect on higher trophic levels, including natural enemies of thrips, reinforcing in this way a sustainable pest resistance in tomato. For these reasons rutin-mediated immobilization is not only the most efficient but also the most effective defense mechanism against WFT in Solanum. Although lobed type-6 glandular trichomes may provide protection against a limited number of pests it is important to realize that such a convenient resistance trait against thrips is already present in S. lycopersicum and hence ready to be exploited by tomato breeders.

By combining the great morphological and chemical diversity of the genus Solanum with an integrative multivariate approach we were able to identify the basis for thrips resistance in tomato. Moreover, we have assessed the relative efficacy and efficiency of the main defense mechanisms against this particular herbivore. Interestingly, the mechanism used by the domesticated tomatoes, lobed type-6 gland-mediated immobilization, turned out to be the best protection against WFT. It is important to emphasize that gland densities needed for protection against thrips are already present in the germplasm of S. lycopersicum and ready to be exploited by breeders.
Supplemental data

The following material will be available online along with a separate publication of this chapter or upon request at r.romero@chem.leidenuniv.nl:

**Supplemental Video S1.** Footage of the interaction between adult thrips and leaves of *S. pennellii* LA716.

**Supplemental Video S2.** Footage of the interaction between adult thrips and leaves of *S. habrochaites* LA1777.

**Supplemental Video S3.** Footage of the interaction between adult thrips and leaves of *S. habrochaites* PI134417.

**Supplemental Video S4.** Footage of the interaction between adult thrips and leaves of *S. lycopersicum* cv unidentified.

Acknowledgments

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