A metabolomic approach to thrips resistance in tomato

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by Roman R. Romero González

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Main picture: young leaf of *Solanum lycopersicum* with adult and larval thrips. Framed pictures: main types of glandular trichomes in *Solanum*.

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Chapter 1

General introduction

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What is the objective of the project?

In this project a holistic approach will be used to unveil the morphological and/or chemical features in tomato plants that modulate its interaction with the pernicious herbivore western flower thrips (WFT). Tomato plants will be scrutinized searching for traits that resist WFT. The most effective and stable defenses would eventually be transferred onto commercial tomatoes in a later stage by means of conventional or modern breeding methods.

Why tomato?

With an annual trading volume of over one billion euros (€) tomato is unquestionably the main commodity in the international market of fruits and vegetables. China, USA, India, Turkey, EU and Egypt alone produced in 2008 more than 87% of the total world tomato fruit yield (Fig. 1) (FAO, 2008). Within the EU, Italy, Spain and Portugal are the top providers during the autumn-winter period while The Netherlands takes the lead along the aestival seasons. Tomato fruit is a rich and convenient source of basic nutrients like vitamin C, A and K, potassium, molybdenum and manganese, and valuable phytochemicals such as hydroxycinnamic acids, flavonoids and carotenoids. This latter class of secondary metabolites occupies a very important place in the nutritional value of tomatoes, of which lycopene is the most popular compound. Due to the presence of long-chain conjugated double bonds, lycopene is known to be the most potent natural lipophilic antioxidant (Lindshield et al., 2007). Since humans are unable to synthesize carotenoids *de novo*, we obtain them exclusively from our diet. At least 85% of our dietary lycopene comes from tomato fruit and tomato-based products, the remainder being obtained from watermelon, pink grapefruit, guava and papaya. Several epidemiological studies have shown an inverse correlation between tomato-rich diets and the incidence of several forms of cancer, especially that of prostate (Giovannucci et al., 1995). However, a more recent study based on the intake of tomato-based food stuffs offers statistical evidence that challenges the alleged health benefits of lycopene on prostate cancer (Kirsh et al., 2006).

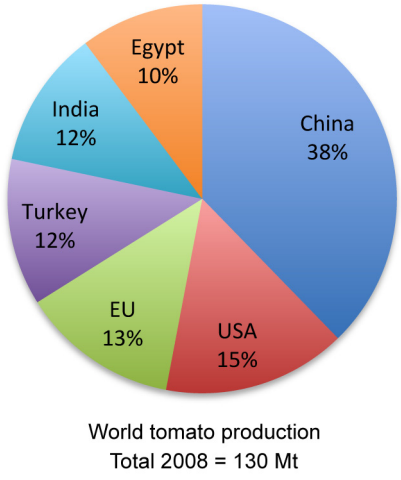

Figure 1. World distribution of the tomato production in 2008 according to the last update of the Food and Agriculture Organization (FAO). The world yield of tomato fruit in 2008 was 129,649,883 tons.

The commercial or domesticated tomato belongs to a large genus, the native South American *Solanum*. In addition to the scores of cultivars of domesticated tomato, *S. lycopersicum*, hundreds of accessions from a number of wild species carry the biggest part of the vast genetic diversity of this genus (Tanksley and McCouch, 1997). Wild tomatoes harbor a pool of phenotypic traits related to economically important aspects such as growth, yield, fruit size, fruit shape, composition and resistance to biotic and abiotic stress factors. During the long process of tomato domestication breeders gave preference to yield, aesthetic and organoleptic characters when selecting new generations. As a result of this bias many supposedly less important traits, such as those related to pest resistance, were partly left behind (Kennedy and Barbour, 1992). Some of these defenses excluded from the germplasm of the domesticated tomato are now required to increase its levels of resistance to different threats.

Pest control in tomato production still depends heavily on synthetic insecticides and herbicides. Due to their increasing accumulation in the environment these compounds represent a major health risk to humans. Directly through soil and water pollution, and indirectly through the disruption of entire organism networks, which sustain life in all ecosystems. Due to this far reaching and long lasting negative impact host-plant resistance is now more than ever a desirable alternative in pest control methods and a needed component in sustainable integrated pest management practices. Therefore, in this project *Solanum* will be screened for thrips resistance in order to identify suitable and potentially stable defense traits that could be brought back into the domesticated tomato.

Why thrips?

Although it was first described in 1895 from specimens collected in California, USA, the western flower thrips, *Frankliniella occidentalis* (Pergande; Thysanoptera: Thripidae), did not become a very serious problem until the late 1970s when a highly insecticide-resistant strain emerged as the result of intensive pesticide use in Californian greenhouses (Immaraju et al., 1992). Since then, and most likely due to an increase in horticultural and floricultural global trade, WTF experienced a rapid dissemination across the world (Kirk and Terry, 2003). In The Netherlands WFT was reported for the first time in 1983 (Mantel and van de Vrie, 1988) and it is nowadays the most common thrips in Dutch glasshouses. This thrips species is at present responsible for annual losses that amount to millions of euros in all continents. For tomato alone thrips causes in The Netherlands damages estimated in ca. €13 millions per year. Its increasing popularity in scientific literature represents an indirect proof of its significant economic impact. Out of over 5,000 thrips species WFT accounts for a third of all publications on Thysanoptera in the past 30 years (Reitz, 2009).

Western flower thrips was clearly designed by evolution to be a strong contender in the arms race between insects and plants. Several morphological, biological and physiological attributes explain its current pest status. This thrips is a highly polyphagous herbivore that feeds on more than 250 crop plants from ca. 60 different families (Lewis, 1997; Tommasini and Maini, 1995), including almost every single fruiting vegetable, leafy vegetable, ornamental, tree fruit and small fruit in addition to cotton (Lewis, 1997). However, it is important to note that not all food sources constitute a suitable breeding habitat for WFT (Mound, 2005). The range of feeding hosts is much greater than the range of hosts on which it reproduces (Paini et al., 2007). This polyphagy combined with a high fecundity and reproductive rate enable WFT to flock into crop fields from many different species, thus precluding a source control as a possible management strategy. Furthermore, due to its small size (max. 2.0 mm in length) and stereotactic behavior of both larvae and adults this thrips goes unnoticed very easily until infestation levels are reached. In addition, because eggs are deposited within plant tissue, these

are even less readily detected and less susceptible to fumigation than other life stages (Janmaat et al., 2002; Macdonald, 1993).

Adaptation to a broad range of host plants implies tolerance to an even bigger number of plant chemical defenses. Therefore, WFT must be able to metabolize a diverse range of allelochemicals, as well as produce inducible enzymes in response to specific compounds (Li et al., 2007). Metabolic detoxification enzymes such as cytochrome P450 monooxygenases, glutathione *S*-transferases, and esterases have been implicated in pesticide resistance in various western flower thrips populations (Jensen, 2000). These enzymes are known to confer resistance and cross-resistance to pyrethroids, organophosphates and carbamates.

Adults and larvae of WTF feed by piercing plant cells and sucking out the entire cell contents (Harrewijn et al., 1996; Hunter and Ullman, 1989). Both adults and larvae tend to feed in localized areas leaving visible silvery or necrotic patches on leaves, flowers and fruits. Feeding on buds leads to deformation of leaves and flowers (Childers, 1997). Foliar injury by thrips can have an adverse impact on the photosynthetic capacity of the plant and eventually can result in significant yield loss (Shipp et al., 2000; Welter et al., 1990).

The most significant damage caused by thrips is undoubtedly its ability to transmit *Tospoviruses*, such as the tomato spotted wilt virus (TSWV) (Allen and Broadbent, 1986) and impatiens necrotic spot virus (INSV) (Daughtrey et al., 1997). It has been reported that TSWV alone causes an estimate of $1 billion in annual losses worldwide (Goldbach and Peters, 1994). Over 1,000 species of plants in 84 families are susceptible to TSWV (Parrella et al., 2003), giving it one of the broadest host ranges of any plant pathogen. Virus transmission can occur quite rapidly, in as little as 5 min of feeding (Wijkamp et al., 1996), contributing significantly to the ineffectiveness of insecticides to limit the spread of TSWV. It has been suggested that TSWV epidemics may be greater in poor reproductive hosts (Reitz, 2005). In such cases, like tomato (Brodbeck et al., 2001; Reitz, 2002), viruliferous individuals are more likely to feed briefly but long enough to transmit the virus, infecting in this way a higher number of plants compared to a more preferred host.

Because of the severe economic implications of thrips damage producers have clung to insecticide control of WFT. However, in addition to the health risks mentioned above one of the most worrying aspects of pesticide use is thrips ability to develop resistance. For all these reasons host-plant resistance remains as the most promising alternative for thrips management.

Why metabolomics?

To defend themselves from all kinds of attackers and abiotic stress factors plants rely on a fascinating wealth of morphological and chemical defenses, either constitutive or induced. Despite such vital role plant allelochemicals, or chemical defenses, are still referred to as “secondary metabolites”, which account for most of the plant metabolome. Assuming that the number of metabolites in a single organism is approximately in the same order as the number of genes, it can be expected that a plant contains around 30,000 metabolites (Verpoorte et al., 2010). The total number of metabolites within the plant kingdom, including primary and “secondary”metabolism, is estimated to fall in the range between 100,000 and 200,000 (Oksman-Caldentey and Inzé, 2004).

The metabolome is a highly regulated and complex biochemical network that not only runs the most fundamental cellular functions but also controls the response of plants to all environmental stimuli, and mediates their interaction with other organisms in multiple trophic levels. Ideally, to understand the specific role and the relationship between those metabolites that modulate plant-herbivore

interactions the changes in the whole metabolome of the organisms involved should be examined in response to a conditional perturbation. Although not feasible that is precisely the aim of metabolomics: to obtain a snapshot of the complete metabolome of biological samples.

Metabolomics is the latest of "omics" and given that metabolites are the ultimate expression of genes it is expected for the metabolome to be more closely related to the phenotype of an organism. In addition, the metabolome is more sensitive than the proteome and the transcriptome to external factors that contribute significantly, and in some cases to a greater extent, to phenotypic differences between specimens. In this sense metabolomics can be a more informative and useful tool in functional genomics and systems biology compared to proteomics and transcriptomics. Since the pioneering works of Nicholson (1999) metabolomics has gained extraordinary popularity for phenotyping, diagnostic analysis and functional labeling of genes (Schauer and Fernie, 2006).

Table 1. Weaknesses and strengths of the main technological platforms for metabolomics.

	GC-MS	LC-MS	MS^n	NMR
Sample preparation	-	-	+	+++
Reproducibility	+	-	+	+++
Absolute quantification	-	-	-	+++
Relative quantification	++	+	++	+++
Identity	++	++	++	++
Compound number	ca. 30	ca. 200	ca. 1000	ca. 200
Sensitivity	++	++	+++	-

(Verpoorte et al., 2008)

Many different metabolomics technologies exist, namely NMR, GC-MS, LC-MS, FTIR, CE-MS, etc., and many different analytical approaches can be used. However, due to the astounding chemical diversity and the great range of concentrations in any organism no single satisfactory metabolomics platform has been devised yet. Each method has its own advantages and limitations (Table 1.) (Verpoorte et al., 2008). In fact, all metabolomics applications analyze only a fraction of the metabolome, which is usually referred to as metabolic profiling or metabolic fingerprinting depending on whether classes of metabolites are specifically targeted or not. Although the use of more than one technology as complementary analytical tools is encouraged to reduce the range bias, most metabolomics studies exclusively rely on a preferred platform. The choice is usually determined by practical limitations and/or special interest on specific classes of metabolites.

Because of its importance as a major commodity and as a model plant system numerous metabolomics studies have been conducted on tomato, most of which focused on fruit composition and its interdependence with agronomic, nutrition and organoleptic variables. NMR metabolic profiling, for instance, was used to detect in tomato potential unintended effects upon genetic modification for crop improvement purposes (Le Gall et al., 2003). More recently, Deborde et al. (2009) followed by NMR metabolic profiling the developmental changes of tomato fruit to assess its nutritional quality at harvest. Tikunov et al. (2005) developed a metabolomics method based on GC-MS and multivariate data analysis whereby 322 different volatile compounds could be distinguished in tomato fruit.

A GC-MS platform was also used to perform the metabolic profiling of a chromosomal substitution set of introgression lines, identifying 889 quantitative fruit metabolic loci and 326 loci that modify yield-associated traits (Schauer et al., 2006). Methods based on LC-MS have also been used as a complementary technique to GC-MS. Compounds with higher molecular weights and of intermediate polarity lie within the domain of liquid chromatography whereas those relatively smaller and more volatile are preferably separated by means of gas chromatography. Moco et al. (2006) used a LC-MS platform to developed a comprehensive open access metabolic database dedicated to tomato. The metabolic diversity in both leaves and fruit of domesticated and wild tomato species has only been surveyed by means of GC-MS (Schauer et al., 2005). The significant metabolic differences detected in this survey between tomato species in both kinds of tissue represent a source of potential traits for crop improvement.

Ecology in general and plant-host interactions in particular have found in metabolomics a highway to deciphering the chemical mechanisms behind complex ecological phenomena and to establish chemical responsibilities for unexplained plant responses to biotic and abiotic stress factors. Metabolic profiling approaches have been successfully used in numerous cases for fundamental research in these fields (Allwood et al., 2008; 2006; Choi et al., 2004; Ferreres et al., 2007; Jansen et al., 2009; Leiss et al., 2010; Widarto et al., 2006). However, no reports are known to date on metabolomics of tomato in the context of resistance to any particular pest. In this project, NMR-based metabolic profiling will be applied on tomato foliage to identify in *Solanum* potential chemical traits related to host-plant resistance to WFT.

Chapter 2

Metabolomic analysis of host-plant resistance to thrips in wild and domesticated tomatoes

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Abstract
The western flower thrips, *Frankliniella occidentalis*, is one of the most serious crop pests worldwide. Its control depends mainly on pesticides whose excessive use leads to human health risks and environmental contamination. As an alternative, we study host-plant resistance to thrips in wild and domesticated tomatoes using nuclear magnetic resonance spectroscopy (NMR) metabolomics. Ten wild species and 10 domesticated tomato lines were compared. Five replicates of each species and lines were used for a thrips bioassay while another 5 replicates were used for the metabolomic analysis. The three most resistant and susceptible wild species and domesticated lines as identified by the thrips bioassay were selected for the metabolomics. Wild and domesticated tomatoes differed significantly in thrips resistance. Only wild tomatoes were thrips resistant, among which *Solanum pennellii* and *S. habrochaites* exhibited the lowest thrips damage. Principal component analysis showed that their ^{1}H NMR profiles were significantly different from those of thrips susceptible tomatoes. Thrips resistant tomatoes contained acylsugars, which are known for their negative effect on herbivores. The identification of acylsugars as a resistance factor for thrips in tomato proves NMR-based metabolomics an important tool to study plant defenses, providing fundamental information for the development and realization of herbivore resistance breeding programs in agricultural crops.

Introduction

Plants produce many metabolites that are important for their interaction with herbivores. Nuclear magnetic resonance spectroscopy (NMR)-based metabolic profiling may be a promising analytical tool for the detection of a wide range of compounds involved in host-plant resistance. Among diverse uses, NMR can identify and quantify metabolites of which no a priori knowledge is needed (Krishnan et al., 2005; Verpoorte et al., 2007; 2008). It provides a broad array of analytical information on the biomolecular composition of plants in a reproducible and constant manner, requiring comparatively little sample preparation (Verpoorte et al., 2007; 2008). The development of extensive databases and software packages have contributed to the advancement of NMR-based metabolomics, which has become a fast, convenient and effective tool to compare groups of samples despite its low intrinsic sensitivity (Verpoorte et al., 2007). Nevertheless, only a few studies have used a metabolomic approach to study the effect of herbivores on plants using NMR spectroscopy. In particular, the caterpillars *Plutella xylostella* and *Spodoptora exigua* in *Brassica rapa* (Widarto et al., 2006) and Arabidopsis (Arany et al., 2008) have been studied, as well as the western flower thrips, *Frankliniella occidentalis*, in *Senecio* (Leiss et al., 2009a) and chrysanthemum (Leiss et al., 2009b). In this study, we used a NMR-based metabolomic approach to investigate host-plant resistance to thrips in wild and domesticated tomatoes.

The western flower thrips (WFT), *Frankliniella occidentalis* (Pergande; Thysanoptera: Thripidae), is one if the most serious pests against agricultural and horticultural crops worldwide (Jensen, 2000). The WFT originated in the western part of North America from where it has spread rapidly across the world (Kirk and Terry, 2003). It is an excellent invader due to its small size, cryptic habits, and high reproduction rate (Lawton et al., 1986). WFT is highly polyphagous, invading a wide range of plants including about 200 wild and domesticated host species (Mantel and van de Vrie, 1988). Damage by WFT is estimated to cost millions of euros worldwide (Lewis, 1997). Thrips have piercing-sucking mouthparts, which allow them to feed on different types of plant cells (Hunter and Ullman, 1989). Feeding on actively growing tissue leads to distortion, reduction in plant growth and eventually yield loss. Feeding on expanded tissue results in the characteristic silvery foliar scars, which affect product

appearance and reduce market quality (de Jager et al., 1995). Moreover, WFT is the main vector of tospoviruses, of which tomato spotted wilt virus (TSWV) is the most economically important one (Riley and Pappu, 2004).

Up to now, control of thrips relies mainly on pesticides. Their efficacy, however, is limited as WFT feeds in the inner whorls of flowers and buds (Brodsgaard, 1994; Immaraju et al., 1992). Furthermore, most chemicals are effective only for a short time and repeated spraying is required (Lewis et al., 1997). Consequently, WFT has developed resistance to various insecticides (Jensen, 2000). Excessive use of pesticides may further pose risks to human health as well as toxicity towards non-target beneficial organisms and contamination of the environment. Host-plant resistance as part of an integrated pest management approach may thus be an important alternative to control WFT.

Host-plant resistance to WFT occurs, but little is known about the underlying mechanisms. Morphological plant characters such as hairiness, toughness, plant height, number of leaves and foliar surface area were not involved in WFT resistance neither in chrysanthemum (de Jager et al., 1995) nor in the wild plant *Senecio* (Leiss et al., 2009a). Instead, resistance was influenced by the chemical composition of the host plants. A new isobutylamide was suggested to be related to host-plant resistance to thrips in chrysanthemum (Tsao et al., 2005). Low concentrations of total aromatic amino acids in cucumber, pepper, lettuce, and tomato, compared to total foliar protein, were correlated with a decrease in damage by WFT (Mollema and Cole, 1996). While overexpression of cystein-protease inhibitors in transgenic chrysanthemums was not related to thrips resistance (Annadana et al., 2002), multi-domain cysteine-protease inhibitors in transgenic potato were affiliated with thrips resistance (Outchkourov et al., 2004). Potential interference of these multi-domain proteins with basic cell functions has hindered a practical function for pest management so far. Recently, two pyrrolizidine alkaloids, jaconine and jacobine, as well as the flavanoid kaempferol glycoside have been identified by NMR to be related to thrips resistance in the wild plant *Senecio* (Leiss et al., 2009a). A metabolomic approach to study WFT resistance in chrysanthemum identified chlorogenic and feruloylquinic acid as resistance factors (Leiss et al., 2009b).

Tomato is one of the major vegetable crops throughout the world. It is an exceptional source of nutrients, as well as folate, vitamin C, carotenoids and phytochemicals, such as polyphenols, which may be related to a lower risk of cancer (Campbell et al., 2004). Years of selection for yield and palatability traits have greatly reduced phenotypic and genetic diversity and may thus have led to loss of resistance (Kennedy and Barbour, 1992). Wild species are, therefore, an important source for host-plant resistance traits to herbivores.

In this study, we explored the natural variation in WFT resistance of the genus *Solanum* by performing bioassays and comparing thrips damage on some wild and domesticated tomatoes. We identified thrips-resistant and -susceptible tomatoes, on which subsequently NMR was performed to investigate the metabolic basis of resistance.

Methods

Plants

Ten wild tomato species, representing a large part of the variation in the *Solanum* complex [*Solanum peruvianum* (LA103), *S. chilense* (LA458), *S. pennellii* (LA716), *S. habrochaites* (formerly *Lycopersicon*

hirsutum f. *typicum* and f. *glabratum*) former f. *glabratum* (LA1223), *S. pimpinellifolium* (LA 1261), *S. lycopersicum* var. *cerasiforme* (LA1286), *S. peruvianum* f. *glandulosum* (LA1293), *S. chmielewskii* (LA1330), *S. habrochaites* former *typicum* (LA1353) and *S. neorickii* (LA2133)], were provided by the C. M. Rick Tomato Genetic Resource Center at the University of California Davis, USA. Ten lines of domesticated tomato, *S. lycopersicum*, deriving from genetically different parents, were made available by Rijk Zwaan (de Lier, Netherlands) and Enza Zaden (Enkhuizen, Netherlands). Seeds of the wild species were scarified soaking them in 2.7% sodium hypochlorite for 30 min. Seeds were directly sown in 13 cm diameter pots with potting soil. Seedlings were thinned to one plant per pot after one week. Ten replicates for each species and lines were grown in a randomized fashion in a climate chamber (16/8 hr photoperiod, 20 °C) for five weeks. Five replicates were used for the thrips bioassay while the other five replicates were used for the NMR metabolomics.

Whole plant bioassay

Five replicates of each species and lines were placed into individual thrips proof cages, consisting of Perspex cylinders (60 cm height, 20 cm diameter), closed on top with nylon gauze of 120 µm mesh size. The cages were placed in a complete randomized design in a climate chamber (16/8 hr photoperiod, 20 °C). Per plant, 20 (18 female and two male) adults of WFT reared on flowering chrysanthemum, were added and left for one week. Silver damage, expressed as the damaged foliar area in mm^2, was scored by eye for each leaf. Based on the whole plant bioassay the three most resistant tomatoes were tested along with two susceptible species in a second bioassay for repeatability.

At the time silver damage was measured, *i.e.* at a plant age of 6 weeks, also hairiness, toughness, and dry mass were measured to investigate morphological resistance on all wild and domesticated tomatoes. Trichomes per cm^2 were counted and toughness was measured with a penetrometer at two locations of each plant, a younger and an older leaf. A younger leaf was defined as the first fully extended leaf from the top of the plant with an area of at least 20 cm^2 and an older leaf as the first one from the bottom with a similar size. Averages per leaf were calculated. Plants were dried for three days in an oven at 50 °C whereupon dry mass was measured.

Differences in silver damage and morphological characters among wild species and domesticated lines were analyzed with a nested ANOVA using plant dry mass as co-variate. Species and lines were nested in wild and domesticated tomatoes respectively. To study the relationship between silver damage and hairiness as well as toughness with Pearson correlations were applied. Differences in silver damage between younger and older leaves were tested by a *T*-test. Data of silver damage and hairiness did not fit a normal distribution and were therefore log-transformed.

Sample collection and extraction procedure

Five plants of the three most susceptible and the three most resistant wild species and domesticated lines, as identified in the thrips bioassays, were used for NMR metabolomics giving a total of 60 ^{1}H NMR spectra. Immediately after collection the older and the younger leaves were kept in liquid nitrogen until subjected to freeze-drying. Samples were then ground to a fine powder in a mortar. Twenty mg of plant material was extracted under ultrasonication (15 min) with 1.5 mL of 80% methanol-d_4 in potassium phosphate buffer (90 mM, pH 6.0) containing 0.02% (w/v) trimethyl silyl-3-propionic acid sodium salt-d_4 (TMSP). After centrifugation (13 krpm, 15 min) an aliquot of 800 µL was taken for NMR analysis.

NMR measurements and data analysis

^{1}H NMR spectra of older leaves 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. Deuterated methanol 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: digital resolution (DR)=0.16 Hz per point, pulse width (PW30°)=11.3 µs, and relaxation delay (RD)=1.5 s. A pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. Free induction decay (FIDs) 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 at 0.0 ppm, using XWIN NMR (version 3.5, Bruker).

The optimized ^{1}H NMR spectra were then automatically binned by AMIX software (v. 3.7, Bruker Biospin). Spectral intensities were scaled to TMSP and reduced to integrated regions of equal width (0.04 ppm) from δ 0.3–10.0. The regions of δ 4.7–5.0 and δ 3.24–3.33 were excluded from the analysis because of the residual signals of water and methanol, respectively. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden) with scaling based on the Pareto method.

Results and discussion

Wild and domesticated tomatoes differed in their resistance to WFT. Wild tomatoes showed significantly less silver damage compared to the domesticated ones (F=8.539, df=18, p=0.009, Fig. 1). Silver damage of wild tomatoes (mean of 64.42 ± 9.325 mm²) was about two times less than in the domesticated lines (mean of 120.1 ± 9.194 mm²). Among the wild species there were also significant differences in thrips resistance (F=5.194, df=9, p<0.001). The most resistant species had a silver damage of 1.8 ± 2.05 mm² compared to 152.6 ± 25.94 mm² in the most sensitive wild species (Fig.1). The second bioassay, testing the three wild species with the least WFT damage along with two susceptible ones (*S. chilense* and *S. habrochaites* f. *glabratum*) also showed significant differences in silver damage (F=6.326, df=4, p=0.002). In both bioassays the most resistant species were the wild tomatoes *S. pennellii* (means of 1.8 ± 2.05 and 0 mm²) and *S. habrochaites* (means of 26.00 ± 32.81 and 5.33 ± 1.16 mm²). They showed 5 times less silver damage compared to the most susceptible wild species. While in the first bioassay *S. peruvianum* showed little silver damage, this was not true for the second bioassay, where *S. peruvianum* exhibited considerable damage. Susceptible species may sometimes by chance show little damage in a particular trial but a resistant species will never show high levels of damage, we therefore considered *S. peruvianum* as a susceptible species.

In contrast to the wild tomato species there were no considerable differences in silver damage among the domesticated tomato lines (F=1.85, df=9, p=0.89) (Fig. 1). Older leaves showed significantly more silver damage than younger leaves for both domesticated (younger leaves mean of 0.56 ± 0.25 mm² and older leaves mean of 24.68 ± 4.26 mm², T=5.73, df=49, p≤0.001) and wild tomatoes (younger leaves mean of 2.24 ± 1.04 mm², older leaves mean of 9.41± 2.51 mm², T=2.54, df=43, p=0.015). This is in accordance with earlier findings reporting a higher susceptibility of WFT on older leaves in chrysanthemum (de Jager et al., 1995) and in the wild plant *Senecio* (Leiss et al., 2009a). Since most of the thrips damage was attributable to the older leaves in the whole plant bioassays, we focused the metabolomic experiments on the older leaves.

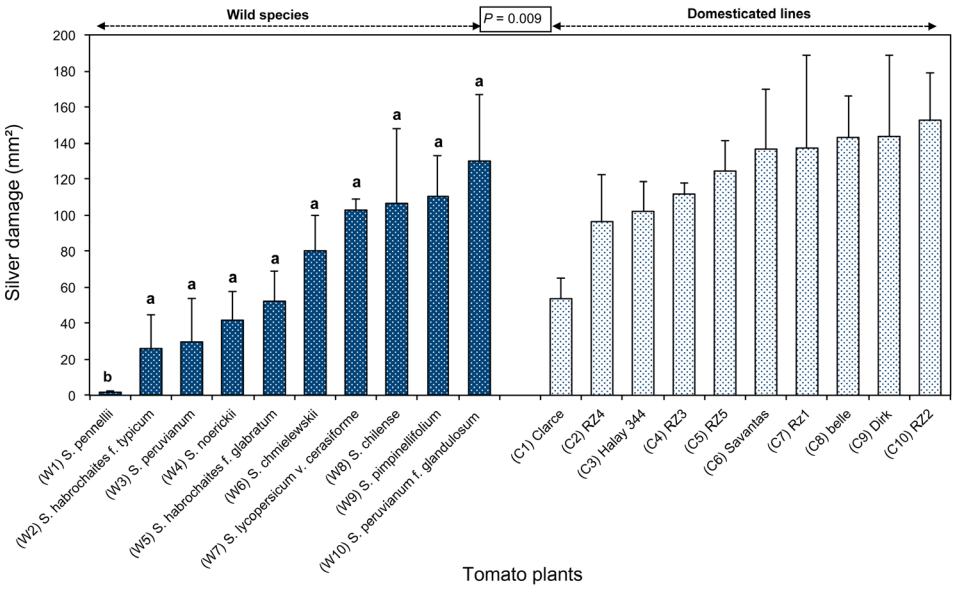

Figure 1. Thrips herbivory, silver damage, on older leaves of wild and domesticated tomatoes. Data represents means and standard errors of five replicates each. *P*= 0.009 refers to significant differences between wild and domesticated tomatoes, whereas letters refer to significant differences at the 0.05 level within the wild tomatoes.

Both bioassays showed that *S. pennellii* and *S. habrochaites* were most resistant to thrips. In the previous study of Kumar et al. (1995) feeding damage by adult WFT varied significantly among leaves of wild and domesticated tomatoes. The least amount of feeding occurred on *S. habrochaites*, *S. pennellii* and *S. chilense*. Regarding resistance to other piercing-sucking insects the present results are similar to those of Rossi et al. (1998), in which *S. pennellii*, *S., peruvianum* and *S. habrochaites* have been used as sources of genetic resistance to aphids. Significant differences in resistance to the green peach aphids, *Myzus persicae*, between wild and domesticated tomatoes were shown by Goffreda and Mutschler (1987), Kohler and St. Clair (2005) and Simmons et al. (2003). *S. pennellii* has been described to be resistant to various piercing-sucking insects such as the potato aphid, *Macrosiphum euphorbiae* (Kohler and St Clair, 2005), the two-spotted spider mite, *Tetranychus urticae* (Saeidi et al., 2007), the tomato red spider mite, *Tetranychus evensi* (de Resendel et al., 2008) and the sweetpotato whitefly, *Bemisia tabaci* (Baldin et al., 2005; Silva et al., 2008). Baldin et al. (2005) showed that genotypes of *S. pennellii*, *S. habrochaites* and *S. habrochaites* f. *glabratum* were highly non-preferable for *B. tabaci*, and in *S. peruvianum* the period of whitefly development was significantly delayed. Resistance to chewing insects in *S. pennellii* has been reported for the South American tomato pinworm, *Tuta absoluta* (de Resende et al., 2006; Pereira et al., 2008), the serpentine leafminer, *Liriomyza trifolii* (Hawthorne et al., 1992) and the cotton bollworm, *Helicoverpa armiger* (Simmons et al., 2004).

Wild tomato plants yielded a significantly lower average dry mass (1.37 ± 0.18 g) than domesticated tomatoes, with an average dry mass of 4.67 ± 0.14 g, (F=6.567, df=18, p<0.001). Within the wild plants *S. chilense* (0.3 ± 0.18 g) and *S. pennellii* (0.67 ± 0.43 g) were the smallest species (F=4.78, df=9, p< 0.001). Wild tomatoes also had significantly more trichomes on both younger (F=8.383, df=18, p<0.001) and older leaves (F=5.3, df=18, p<0.001) compared to domesticated tomatoes. In the younger leaves wild tomatoes, with an average of 306 trichomes per cm^2, had

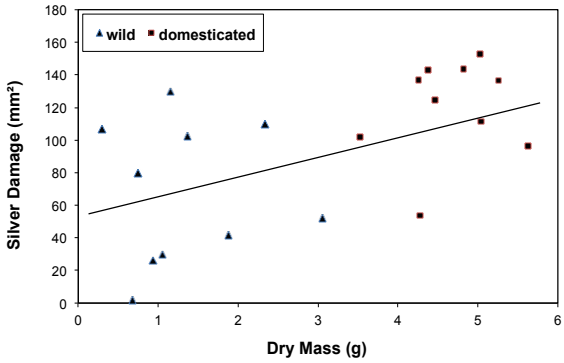

Figure 2. Correlation between silver damage and dry mass of ten wild species and ten domesticated lines of tomato; (R=0.615, N=20, p=0.004). Data represent means of five replicates.

double as many trichomes as domesticated tomatoes, with an average of 158 trichomes per cm^2. In the older leaves wild tomatoes, with an average of 164 trichomes per cm^2, had 4 times as many trichomes as domesticated tomatoes, with an average of 43 trichomes per cm^2 (F=5.3, df=18, p<0.001). Within the wild tomatoes the two most resistant species, *S. pennellii* and *S. habrochaites*, had the highest density of trichomes. Yet, no significant overall correlation was detected between silver damage and hairiness. We did not detect either any significant difference in toughness between wild and domesticated tomatoes. Dry mass was the only morphological trait significantly correlated with thrips damage (R=0.615, N=18, p=0.004, Fig. 2). Interestingly, smaller plants were more resistant to WFT. Wild tomato species were smaller compared to the domesticated lines and showed a higher resistance to WFT. Resistance to WFT may therefore be costly. The resource availability theory indeed predicts that growth rate varies with investment in resistant traits (Coley et al., 1985).

When multivariate data analysis methods, specifically principal component analysis, were applied to the ^{1}H NMR spectra of the selected species, the results exhibited a behavior similar to that of the silver damage test. PCA scores, mostly for component 1, clearly segregated the samples of *S. peruvianum* and *S. habrochaites* and to a lesser extent those of *S. pennellii* from the other samples (Fig. 3A). This concurrence of both thrips and metabolomic data suggested that the observed WFT resistance of these wild species might have a chemical origin.

A column loading plot for PC1 (Fig. 3B) exposed the signals that had the highest influence on this component. Two major groups stand out in this plot. The first corresponds to the typical chemical shifts of malic acid, *i.e.* δ 2.6, 2.8 and 4.3, contributing positively and negatively to PC1. The second, in the hydrocarbon region between δ 0.8 and 2.5, has a negative effect on PC1 and was assigned to fatty acids, mostly in the form α-linolenic acid as evidence by the triplet on δ 0.96 (J=7.5 Hz) typical of methyls β-removed from a vinyl functionality.

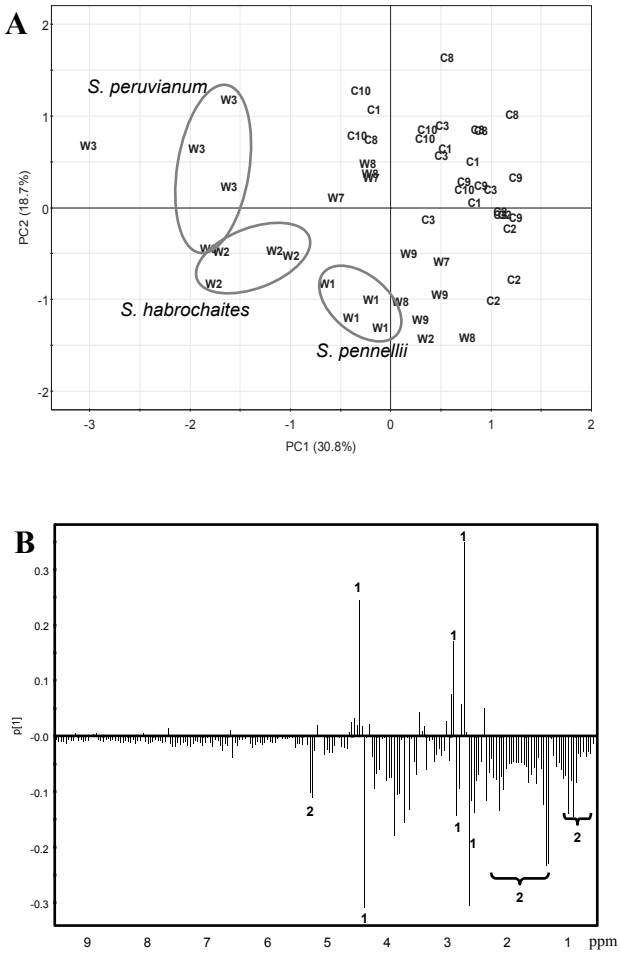

Figure 3. Multivariate analysis performed on the ^{1}H NMR spectra of older leaves from selected wild and domesticated tomatoes. (A) PCA score plot; (B) Column loading plot for PCA component 1. (**1**) malic acid, (**2**) fatty acids. For label meaning see Figure 1.

A closer inspection of the ^{1}H NMR spectra revealed that malic acid contributed to the PCA clustering by a significant shift of its signals to higher field (F=76.815, df=3, p<0.001) in the samples of *S. peruvianum* and *S. habrochaites*, as shown in Figs. 4A and 4B. This shift may evidence a pH variation greater than the buffering capacity of the solvent system. Considering how strongly pH can modulate the physiology of both plants and insects it is tempting to speculate on possible connections between this tissue acidity change and anti-herbivory. However, foliar intumescences commonly developed by certain wild tomato species, especially by *habrochaites* accessions, may instead account for the actual explanation. This physiological disorder, observed on all replicates of *S. peruvianum* and *S. habrochaites*, manifests as foliar galls, which are also referred to as plant tumors. These tumors result, among other possible factors, from an incapability of the plant to take up excess of water under high humidity conditions (Lang and Tibbitts, 1983).

To identify the possible metabolites exclusively related to WFT resistance a partial least square discriminant analysis (PLS-DA) was subsequently performed on the ^{1}H NMR dataset supervised by

the silver damage results. Clearer sample segregation was obtained in this case, mainly by PLS-DA component 1, for the resistant species, *S. pennellii* and *S. habrochaites*, (Fig. 5A). In addition to the contributions of malic acid and α-linolenic acid already observed in the PCA, a prominent and exclusive new group of signals arose in the column loading plot for PLS-DA component 1 (Fig. 5B, **3**). Based on existing phytochemical and spectroscopic data these multiple doublets (*J*=7.0 Hz), located around δ 1.1 and 1.04 for *S. pennellii* and *S. habrochaites* respectively (Fig. 4A), were identified as typical isoalkyl signals of glycolipids (Fig. 5C). Both wild species, in particular these accessions (Burke et al., 1987; King et al., 1990), are known to synthesize abundant amounts of sugar alkyl esters, commonly referred to as acylsugars. *S. pennellii* secretes a mixture of glucose and sucrose esters (Shapiro et al., 1994), which can account for up to 20% of the foliar dry mass (Fobes et al., 1985), while *S. habrochaites* produces only sucrose esters (King et al., 1990).

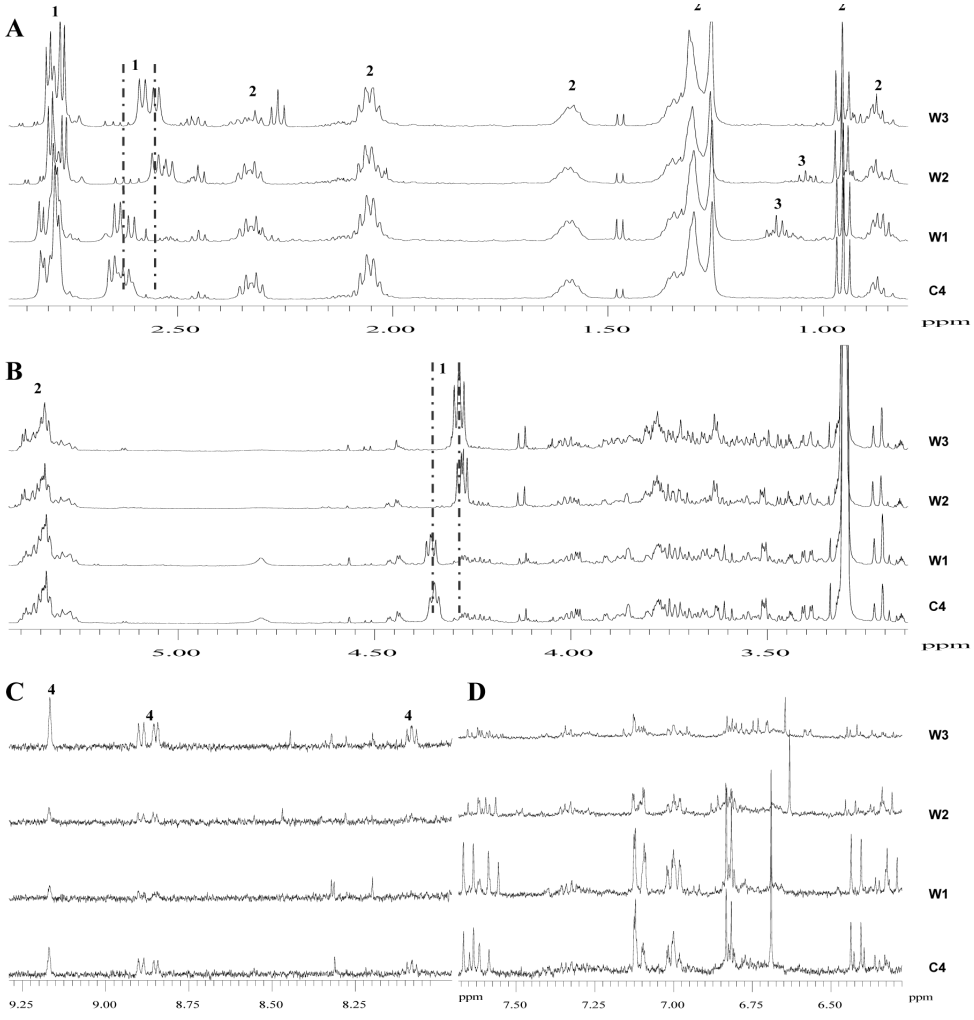

Figure 4. ^{1}H NMR spectra of 80% deuterated methanol extracts of older leaves from selected wild and domesticated tomatoes in the range of δ 0.8 – 3.0 (A), δ 3.0 – 5.5 (B), δ 8.0 – 9.3 (C) and δ 6.0 – 8.0 (D). (**1**) malic acid, (**2**) fatty acids, (**3**) acylsugars, (**4**) trigonelline. For label meaning see Figure 1.

Acylsugars have been reported as natural insecticides present in *S. pennellii* (Walters and Steffens, 1990). There was a significant negative relationship between the foliar concentration of sugar esters and the level of potato aphid infestation in a segregating *S. lycopersicum* and *S. pennellii* F2 population (Goffreda et al., 1990). High amounts of foliar acylsugars in *S. pennellii* were related to repellency of *T. evansi* (de Resende et al., 2008; Pereira et al., 2008). F1 and F2 populations of crosses between *S. lycopersicum* and *S. pennellii* revealed that recessive genes were responsible for the high concentrations of acylsugars causing resistance to *T. urticae* (Saeidi et al. 2007).

Fobes et al. (1985) have described significant differences in the yield of acylsugars between *S. pennellii* and *S. lycopersicum*. Selection of F2 genotypes of interspecific crosses between these species resulted in a highly negative correlation between levels of acylsugars and damage by *T. absoluta* (de Resende 2006; Pereira et al., 2008). The level of acylsugars in *S. pennellii* was 2.25 times higher compared to that in *S. lycopersicum*. Acylsugars of wild tomato artificially applied onto domesticated tomato deterred feeding and oviposition of *L. trifolii* (Hawthorne et al., 1992). Increased amounts of acylsugars from *S. pennellii* reduced the settling of the adult as well as oviposition of the silverleaf whiteflies, *Bemisia argentifolii*, (Liedl et al., 1995). High concentrations of acylsugars also caused a reduction in egg laying of *B. tabaci* in tomato plants (Silva et al., 2008).

Acylsugars are produced in type-4 glandular trichomes of *Solanum* spp. (Burke at al., 1987). These specific glandular trichomes are reported as abundant in the wild tomatoes here identified to be thrips resistant, *S. pennellii* and *S. habrochaites*. Other wild species as well as *S. lycopersicum* do not possess type-4 trichomes at all (Simmons and Gurr, 2005). The negative effect of these glandular trichomes, like entanglement or entrapment, is thought to be conferred by the chemical exudates rather than by the physical effect (Simmons and Gurr, 2005). In fact, removal of glandular trichome exudates significantly reduced negative effects on insects (see references in Simmons and Gurr, 2005). This is confirmed by our results showing that the most resistant wild tomatoes, *S. pennellii* and *S. habrochaites,* had the highest overall number of foliar trichomes. In contrast, sugar esters produced in *Datura wrightii* did not correlate with densities of glandular trichomes, suggesting that other factors such as environmental conditions and different plant populations, may play a role in the production of sugar esters for plant defense (Forkner and Hare, 2000).

Our NMR-based metabolomics approach to study host-plant resistance in wild and domesticated tomatoes indicated that acylsugars are a resistance factor against WFT. Using the same approach we identified pyrrolizidine alkaloids and a kaempferol glycoside in the wild plant *Senecio* (Leiss, et al., 2009a), as well as chlorogenic and feruloylquinic acids in the ornamental plant chrysanthemum (Leiss et al., 2009b), to be involved in resistance to WFT. Combining these substances for defense against WFT may constitute a very promising prospect in tomato breeding strategies. The amount of acylsugars have indeed increased in breeding programs crossing wild and domesticated tomatoes (de Resende 2006; Pereira et al., 2008). Furthermore, tomatoes with increased amounts of chlorogenic acid (Niggeweg et al., 2004) and flavonoids, including kaempferol (Le Gall et al., 2003), have already been engineered for dietary purposes.

Another differentiating metabolite that stood out in the column loading plot for PLS-DA component 1 was trigonelline (Fig. 5B, **4**). A quantitative analysis of its integrals revealed that the resistant species contain significantly lower amounts of this compound (F=14.253, df=3, $p< 0.001$), as shown in Fig. 4C. In the absence of any reports on the direct involvement of trigonelline in herbivory modulation, we hypothesize that this observation may be the result of a metabolic trade-off favoring the production of acylsugars. Trigonelline is an alkaloid with multiple regulatory functions

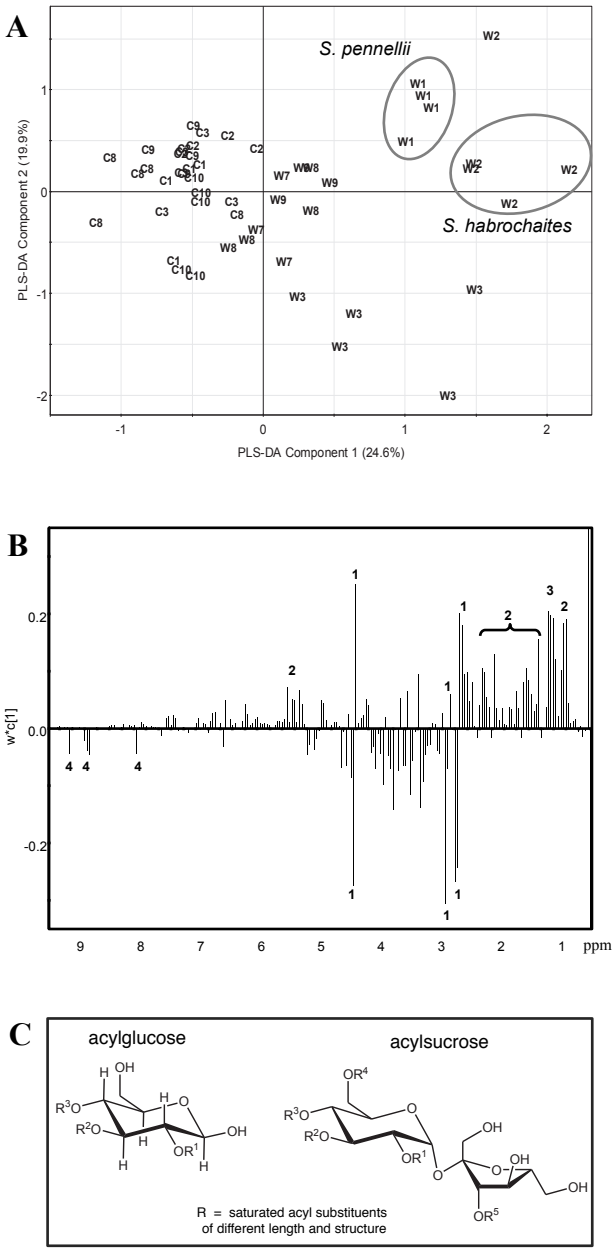

Figure 5. Multivariate analysis performed on the ^{1}H NMR spectra of older leaves from selected wild and domesticated tomatoes. (A) PLS-DA score plot supervised by WFT damage data; (B) Column loading plot for PLS-DA component 1. (**1**) malic acid, (**2**) fatty acids, (**3**) acylsugars, (**4**) trigonelline; (C) General structure of the glycolipids produced by *Solanum* spp. For label meaning see Figure 1.

in plants, such as cell cycle, nodulation, oxidative, UV and salt stress response, DNA methylation and nyctinasty (Minorsky, 2002).

It is also worth pointing out the great diversity of hydroxycinnamic esters observed across this

set of species both qualitatively and quantitatively, as evidenced by the numerous signals present in the phenylpropanoid region between δ 6 and 8 (Fig. 4D). Although these compounds, along with other phenolic metabolites, represent a major group of plant defenses, their profile was inconsistent across the studied tomatoes and hence unrelated to thrips resistance.

NMR-based metabolomics proved to be a successful tool to study host-plant resistance to thrips in *Solanum*. It allowed the simultaneous detection of different compounds involved and thus contributed to a deeper holistic approach. As such, NMR provides fundamental information for the development and realization of herbivore resistance breeding programs in agricultural and horticultural crops.

Acknowledgements

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Chapter 3

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

Submitted for publication

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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

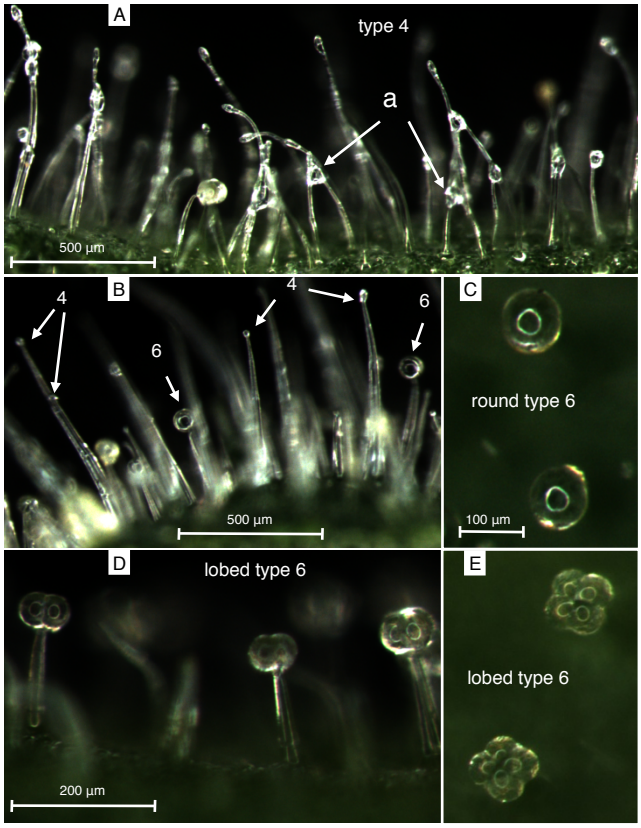

Figure 1. Light microscopy of the main glandular trichomes in *Solanum*. A) Trichomes type 4 on *S. pennellii* LA716 bearing droplets of secreted acylsugars (a). B) Trichomes type 4 and round type 6 on *S. habrochaites* LA1777. C) Aerial view of round type-6 glands. D) Lobed type-6 trichomes on *S. lycopersicum*. E) Aerial view of lobed type-6 glands.

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., 1995; Muigai et al., 2002), 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, *Tetranycus 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.*

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).

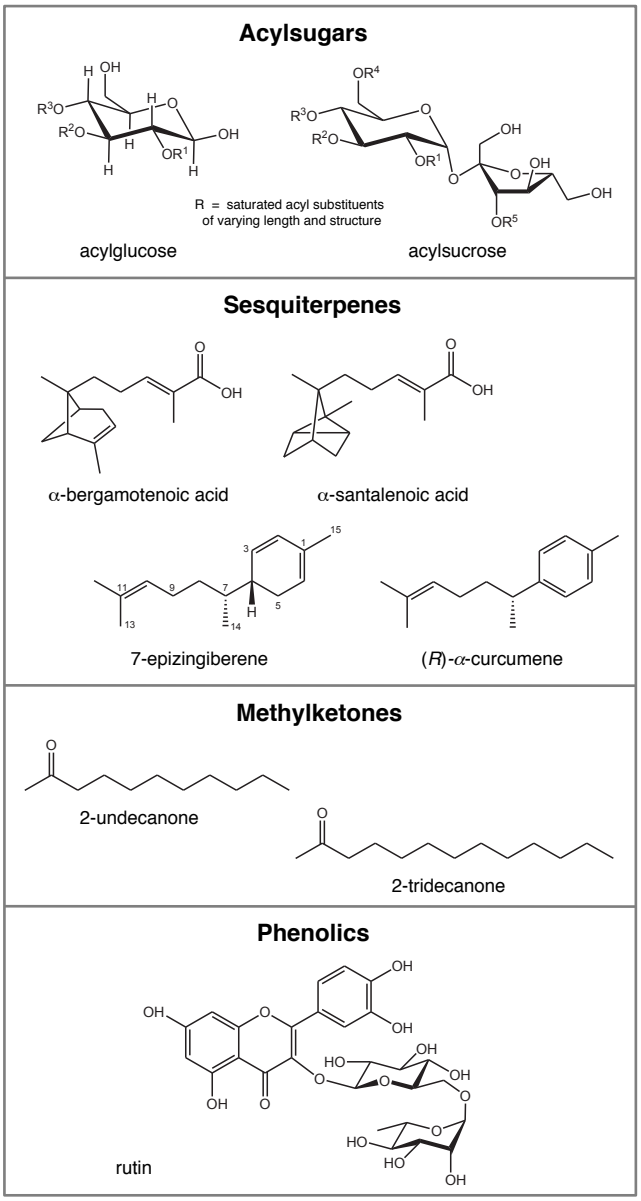

Figure 2. Defense metabolites accumulated in the glandular trichomes of *Solanum* species.

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. Mirnezhad 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. neorickii* (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 mm^2. 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 mm^2 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 (cm^2). 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, ^{1}H 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 substracting 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 quadropole 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}. ^1H 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 ^1H 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 Pearsons *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).

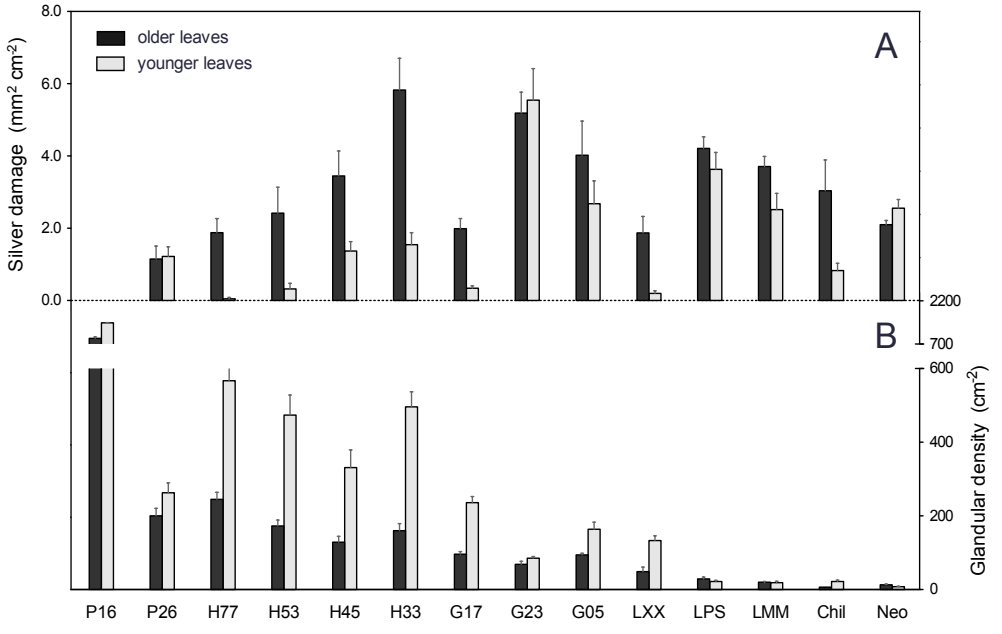

Figure 3. Thrips herbivory as silver damage (A) and glandular trichome density (B) on selected *Solanum* accessions. Leaflets of two age groups, older and younger, were excised and exposed to thrips for three days in a choice assay. Bars represent the average (± *SE*) of ten biological replicates.
Accessions: **P**, *S. pennellii;* **H**, *S. habrochaites* former f. *typicum;* **G**, *S. habrochaites* former f. *glabratum;* **L**, *S. lycopersicum;* **Chil**, *S.chilense;* **Neo**, *S.neorickii*. **XX**, unidentified variety; **PS**, v. Pear-shaped; **MM**, v. Moneymaker. Numbers correspond to the last two digits of the LA or PI codes, see materials and methods for full accession identification.

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, β_G=-0.466, p<0.001, β_{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

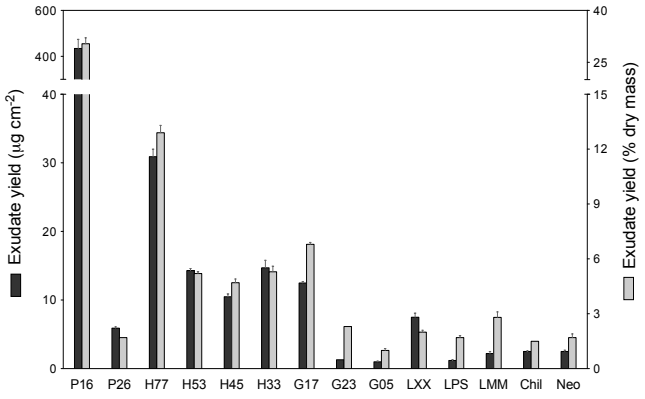

Figure 4. Exudate yield of younger leaves from selected *Solanum* accessions. Foliar exudates were removed through consecutive flash washouts with dichloromethane and ethanol. Bars represent the average (± *SE*) of three biological replicates.

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 ^{1}H 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

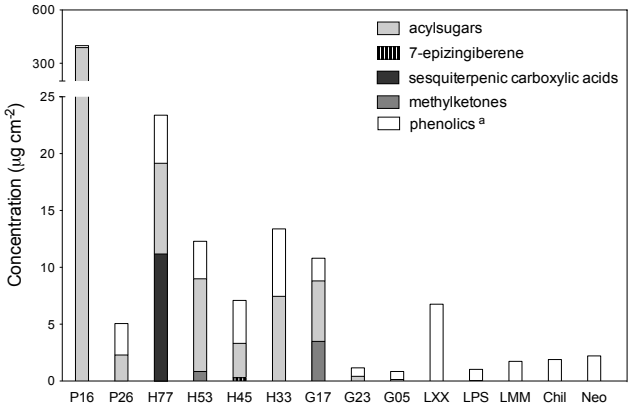

Figure 5. Concentration of targeted secondary metabolites produced by glandular trichomes in younger leaves of selected *Solanum* accessions. Values for sesquiterpenes and methylketones were determined by GC while those for acylsugars and phenolics were determined gravimetrically after column fractionation. Bars represent the average (± *SE*) of three technical replicates. [a] This fraction may contain free sugars, amino acids and inorganic salts.

form ((*R*)-α-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, *Solanun berthaultii* (Duffey, 1986). It has been recently demonstrated, however, that chlorogenic acid is a lamellar secondary metabolite present in the exudate of *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, *i.e.* P16 to G05, contained mostly very polar metabolites such as free sugars and amino acids, rather than major phenolics.

Within the *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; adjusted R^2=0.37, β_{G-4}=-0.257, p=0.001, β_{G-6}=-0.475, p<0.001). Although only 37% of the variation in the

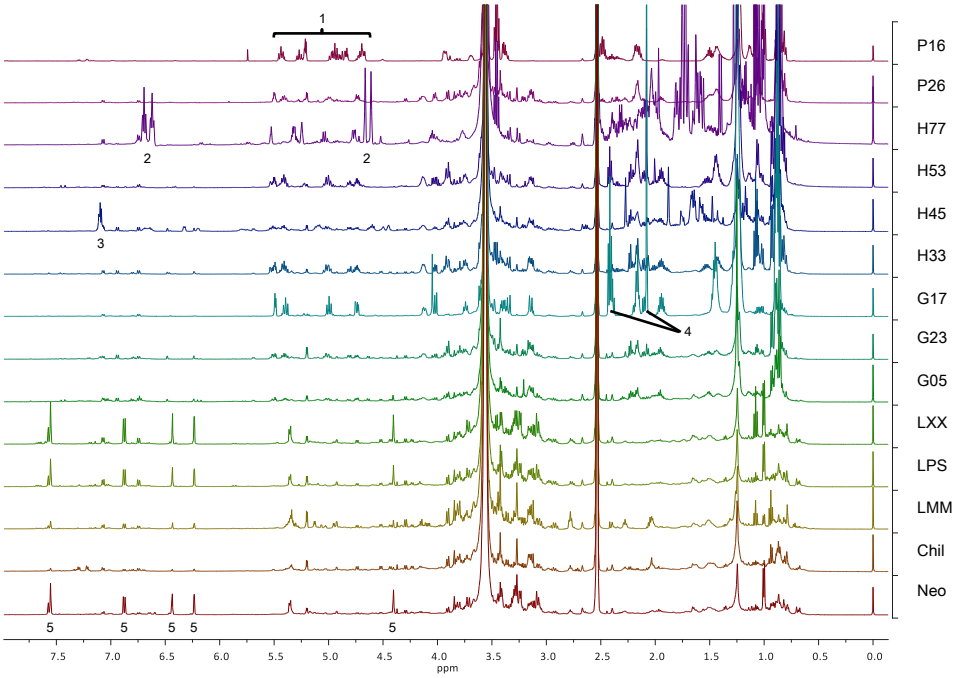

Figure 6. ^{1}H NMR spectra (500 MHz) of crude glandular trichome exudates from younger leaves of selected *Solanum* accessions. Exudates were totally or partially dissolved in deuterated dimethylsulfoxide containing 0.005% (w/v) trimethylsilyl-3-propionic acid sodium salt-d_4 as internal standard. **1,** acylsugars; **2**, sesquiterpenic carboxylic acids; **3**, (*R*)-α-curcumene; **4**, methylketones; **5**, rutin.

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-epizingiberene (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, 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.

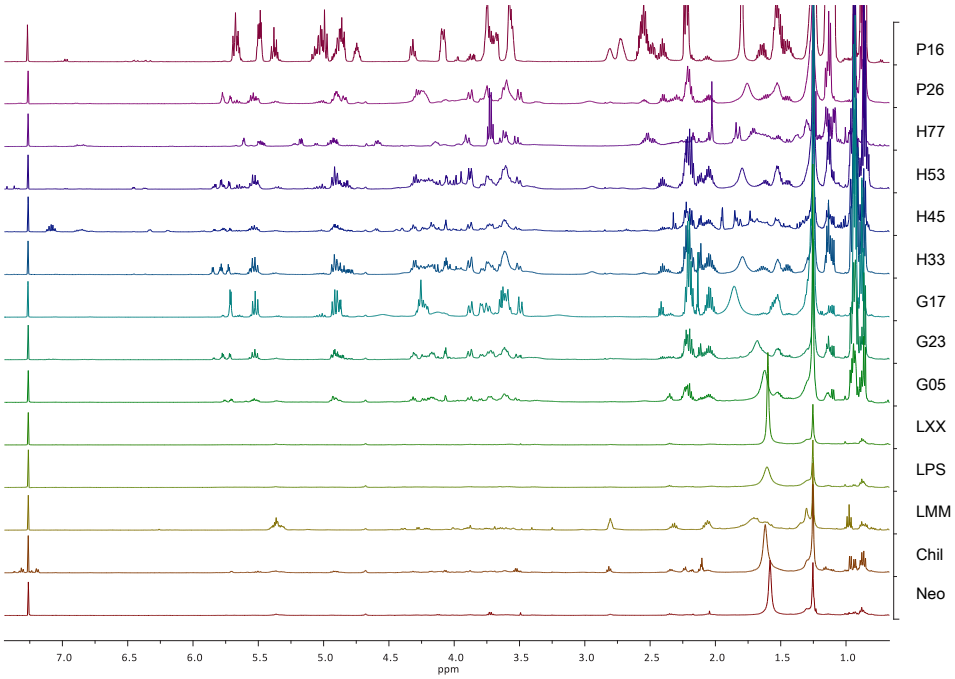

Figure 7. ^{1}H NMR spectra (500 MHz) of the acetone fraction of trichomal exudates from younger leaves of selected *Solanum* accessions. Exudates were totally or partially dissolved in deuterated chlorofom containing 0.03% (v/v) tetramethylsilane TMS as internal standard. Most signals on the spectra of accessions P16 to G05 correspond to acylsugars. These compounds are absent in the domesticated tomatoes (LXX to LMM), Chil and Neo. Signals on the spectra of accessions LXX to Neo correspond to traces of fatty acids and other hydrocarbons.

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 hervibory, trichome densities, exudate yield and exudate composition for the younger leaves of selected *Solanum* accessions.

	[1] Damage (mm^2 cm^{-2})	[1] Trichome densities (cm^{-2})			[2] Yield (μg cm^{-2})	[2] Exudate composition (μg cm^{-2})				
		[3] Type 4	[3] Type 6	[4] Type 5		AS	EZ	SCA	MK	[5] Ph
P16	0.00^{a}	1429^{e}	13^{ab}	n.d.	434.8^{h}	388.32^{f}	n.d.	n.d.	n.d.	11.23^{g}
P26	1.22^{d}	257^{d}	6^{a}	n.d.	5.9^{b}	2.30^{b}	n.d.	n.d.	n.d.	2.76^{cd}
H77	0.05^{a}	324^{d}	169^{d}	n.d.	30.9^{g}	7.98^{e}	n.d.	11.18	n.d.	4.22^{e}
H53	0.32^{ab}	347^{d}	99^{d}	n.d.	14.3^{f}	8.14^{e}	n.d.	n.d.	0.85^{b}	3.31^{d}
H45	1.37^{de}	187^{c}	101^{d}	n.d.	10.5^{d}	3.03^{c}	0.29	n.d.	n.d.	3.77^{e}
H33	1.55^{de}	321^{d}	129^{d}	n.d.	14.7^{f}	7.46^{e}	n.d.	n.d.	n.d.	5.92^{f}
G17	0.34^{bc}	34^{a}	164^{d}	n.d.	12.5^{e}	5.32^{d}	n.d.	n.d.	3.49^{c}	2.00^{bc}
G23	5.55^{g}	24^{a}	19^{c}	n.d.	1.3^{a}	0.41^{a}	n.d.	n.d.	0.01^{a}	0.74^{a}
G05	2.68^{ef}	74^{b}	30^{bc}	n.d.	1.0^{a}	0.14^{a}	n.d.	n.d.	n.d.	0.70^{a}
LXX	0.20^{ab}	0	133^{d}	109^{a}	7.5^{c}	0.00^{a}	n.d.	n.d.	n.d.	6.76^{f}
LPS	3.63^{f}	0	22^{c}	160^{ab}	1.2^{a}	0.04^{a}	n.d.	n.d.	n.d.	0.99^{ab}
LMM	2.52^{f}	0	19^{c}	200^{bc}	2.2^{a}	0.00^{a}	n.d.	n.d.	n.d.	1.74^{bc}
Chil	0.83^{cd}	0	22^{c}	242^{c}	2.5^{a}	0.00^{a}	n.d.	n.d.	n.d.	1.90^{bc}
Neo	2.56^{f}	0	8^{bc}	120^{ab}	2.5^{a}	0.00^{a}	n.d.	n.d.	n.d.	2.22^{c}

AS: Acylsugars, EZ: 7-Epizingiberene, SCA: Sesquiterpenic carboxylic acids, MK: Methylketones, Ph: Phenolics, n.d. not detected.

[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.

Accessions: **P**, *S. pennellii;* **H**, *S. habrochaites* former f. *typicum;* **G**, *S. habrochaites* former f. *glabratum;* **L**, *S. lycopersicum*; **Chil**, *S.chilense;* **Neo**, *S.neorickii*. **XX**, unidentified variety, **PS**, v. Pear-shaped, **MM**, v. Moneymaker. Numbers correspond to the last two digits of the LA or PI codes, see materials and methods for full accession identification.

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 non-glandular 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 (Antonious 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

The Tomato Seedstock Center at the University of California (Davis, CA) and the USDA-Agricultural Research Service (Ithaca, NY) are kindly acknowledged for proving the seeds of wild tomatoes.

Chapter 4

Thrips resistance and NMR-based metabolic profiling of a *Solanum pennellii* x *lycopersicum* introgression population

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Abstract

Interspecific chromosomal-substitution lines, also referred to as introgression lines (ILs), represent an excellent platform to explore the genetics of multiple biological and chemical traits of agronomical importance. Using a NMR metabolomics approach we have chemotyped a *Solanum pennelii* x *lycopersicum* introgression population to investigate the genetic and chemical basis of thrips resistance in tomato. ILs were screened for thrips resistance in a choice bioassay and subjected to ^{1}H NMR profiling. A total of 24 primary and secondary foliar metabolites were identified. Both the concentration of the metabolites and thrips damage varied significantly throughout the 76 ILs. Nine quantitative trait loci (QTL) were identified for thrips resistance and 268 for the metabolic traits. Neither multivariate data analysis nor correlation tests evidenced an association between thrips resistance and any of the metabolites. Trait mapping did not show either consistent colocalization of thrips resistance and the profiled metabolites. Metabolic correlations were detected mostly within classes of compounds, *e.g.* lipids, amino acids and phenolics. Interesting QTL were identified for valuable phytochemicals such as chlorogenic acid and rutin. Particularly important were IL10-1 and 10-1-1, which respectively showed a 2.6 and 4.4 fold increase in the chlorogenic acid content compared to the recurrent parent *S. lycopersicum* M82.

Introduction

A long period of selection in tomato for yield and taste-related traits has greatly reduced phenotypic and genetic diversity in domesticated tomatoes. Compared with the rich reservoir in wild species, domesticated tomatoes contain only a very small part of the genetic variation that is accessible in related wild species (Tanksley and McCouch, 1997). Thus, tomato breeding may have led to loss of resistance traits (Kennedy and Barbour, 1992). Indeed, there is a great source of resistance traits available in the wild tomato species (Bai and Lindhout, 2007). It has, therefore, become important to screen wild genetic resources for valuable traits, including resistance, that could be introduced into commercial varieties (Zamir, 2001). Having identified the valuable agricultural traits of wild species these can be transferred by introgression breeding (Zamir, 2001). To introgress the favorable wild allele into domesticated tomato, marker-assisted selection plays an important role, and the map positions and markers linked to the QTL provide a basis for breeders to design optimal breeding strategies (Bai and Lindhout, 2007).

In tomato domestication traits have been studied for growth and fruit characteristics, and the underlying qualitative genes and quantitative trait loci (QTL) have been identified. A QTL is a polymorphic location of a chromosome containing alleles that differentially control the expression of a phenotypic trait. Many important tomato traits are genetically controlled by a combined action of QTL with favorable alleles often present in the wild species (Bai and Lindhout, 2007). This includes resistance to pathogens and insects. Regarding pathogens, QTL for resistance to the tomato powdery mildew, *Oidium lycopersici* in *Lycopersicon parviflorum* (Bai et al., 2003), to the gray mold, *Botrytis cinerea* in *S. lycopersicoides* (Davis et al., 2009), to the bacterial wilt, *Pseudomonas solanacearum*, in a cross of *L. esculentum* and *L. pimpinellifolium* (Thoquet et al., 1996) and to the bacterial spot, *Xanthomonas* spp. in *S. lycopersicun* var. *cerasiforme* (Hutton et al., 2010) have been identified. In regard to insects QTL for resistance to the sweetpotato whitefly, *Bemisia tabaci*, in a cross of *S. lycopersicum* and *S. habrochaites* (Momotaz et al., 2010) were reported. The molecular genetics of thrips

resistance is not well understood. Only a few studies have been reported in various crops. QTL for resistance to the flower bud thrips, *Megalurothrips sjostedti* (Omo-Ikerodah et al., 2008) and for resistance to *Thrips tabaci* and *Frankliniella schultzei* in cowpea (Muchero et al., 2010), for resistance to *Thrips palmi* in common bean (Frei et al., 2005) and for resistance to *T. palmi* and *Megalurothrips usitatus* in potato (Galvez et al., 2005) have been identified.

Interspecific chromosomal-substitution lines or introgression lines (ILs), are more powerful compared with interspecific crosses in QTL identification. These lines carry a single introgressed genomic region, and are otherwise identical for the rest of their genome. As a result, the phenotypic variation in these lines can be associated with individual introgression segments (Bai and Lindhout, 2007). In tomato, several sets of ILs have been developed for wild relatives of tomato. These include introgressions with *S. lycopersicodes* and *S. sitiens* (Canady et al., 2006), *S. lycopersicum* and *S. hirsutum* (Monforte and Tanksley, 2000) and *S. lycopersicum* and *S. pennellii* (Eshed and Zamir, 1995). The set of ILs with *S. pennellii* (accession LA716) comprises 76 lines. In these ILs a marker-defined genomic region of the domesticated variety *S. lycopersicum* M82 was replaced with its homologous interval in the wild species. Over a series of field studies on these lines a number of phenotypic traits were quantified and QTL identified (Eshed and Zamir, 1995; Gur et al., 2004). Metabolic profiles of *S. esculentum* and *S. pennellii* as well as of six ILs were compared by Overy *et al.* (2005). Schauer *et al.* (2006) established a metabolic profile of the *S. pennellii* introgression population to identify loci associated with fruit metabolism and yield. Kamenetzky *et al.* (2010) determined the genetic basis of metabolic regulation in tomato fruit by constructing a detailed physical map of genomic regions spanning previously described metabolic QTL of the *S. pennellii* introgression population.

In this study we are interested in the genetics of secondary metabolite markers related to thrips resistance. We thus used the *S. pennellii* x *lycopersicum* introgression population to detect QTL for WFT resistance and secondary metabolites, and their co-localization.

Methods

Plants and thrips bioassay

Seeds of the 76 ILs and the recurrent parent *S. lycopersicum* M82 were provided by the C. M. Rick Tomato Genetic Resource Center at the University of California Davis, USA. Seeds were directly sown in 13 cm diameter pots with potting soil. Seedlings were thinned to one plant per pot after one week. Six replicates for each line were grown in a randomized fashion in a glass house during the months of June and July in 2008. All windows on the glass house were covered with nylon gauze of 120 µm mesh size to make it thrips-proof. The thrips herbivory bioassay was started when the plants had in average six fully expanded leaves. Three replicates of each IL were kept in the glass house for the thrips bioassay while the other three replicates were taken away for the NMR metabolomics. Per plant, 15 adult thrips, reared on flowering chrysanthemum, were released into the glass house. Three weeks later thrips herbivory, expressed as mm^2 of silver damage, was scored by eye for each plant.

Sample collection and extraction procedure

For the ^{1}H NMR metabolomic analysis the third oldest leaf was taken from each plant at the beginning of the thrips bioassay. Immediately after collection leaves were flash frozen with liquid nitrogen and kept at -80 °C until freeze-dried. Samples were ground to a fine powder with a Retsch ball-mill (Retsch GmbH, Haan, Germany). Fifty mg of each sample, weighed in 2 mL Eppendorf tubes, was extracted under ultrasonication (15 min) with 1.5 mL of 70% methanol-d_4 in potassium phosphate buffer (90 mM, pH 6.0) containing 0.02% (w/v) 3-trimethylsilyl propionic acid sodium salt-d_4 (TMSP). After centrifugation (13 krpm, 15 min) an aliquot of 800 µL was taken for NMR analysis.

NMR measurements and data analysis

^{1}H NMR spectra were recorded at 25 °C on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz. Deuterated methanol 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: digital resolution (DR)=0.16 Hz per point, pulse width (PW30°)=11.3 µs, and relaxation delay (RD)=1.5 s. A pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. Free induction decay (FIDs) were Fourier transformed with a line broadening (LB)=0.3 Hz. The resulting spectra were manually phased, baseline corrected and calibrated to the internal standard TMSP at 0.0 ppm using XWIN NMR (version 3.5, Bruker).

For the multivariate data analysis the optimized ^{1}H NMR spectra were automatically binned by AMIX software (v. 3.7, Bruker Biospin). Spectral intensities were scaled to TMSP and reduced to integrated regions of equal width (0.04 ppm) from δ 0.3–10.0. The regions of δ 4.7–5.0 and δ 3.24–3.33 were excluded from the analysis due to the residual signals of water and methanol, respectively.

Metabolites were quantified by integrating an optimum signal of each compound, or class of compounds, selected according to minimal crowdedness and signal overlapping. These signals are shown in bold characters in Table 1. Peak integration was performed with MestReNova software (v. 6.1.1, Mestrelab Research SL, Santiago de Compostela, Spain). Relative concentrations of the metabolites were determined in each sample dividing the peak areas by that of the internal reference, TMSP, in each replicate.

Statistical analysis

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed with SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) with scaling based on the Pareto method. To determine the significance of differences in thrips damage and metabolite concentration between each IL and the recurrent parent, *S. lycopersicum* M82, *T*-tests were performed at a confidence level of 0.05. Correlations were verified through Spearman's ρ coefficient. All statistical tests were performed using SPSS v. 17.0 (SPSS Inc., Chicago, IL, USA). Concentration averages were determined for each metabolite in all ILs and transformed into folds of the corresponding value in M82. These ratios were centered to the unity and plotted in a color-scaled heat map using the public-domain open-source software Multi Experiment Viewer, MeV v 4.5 (Saeed et al., 2003). Euclidean distance and complete linkage were used for the hierarchical cluster analysis.

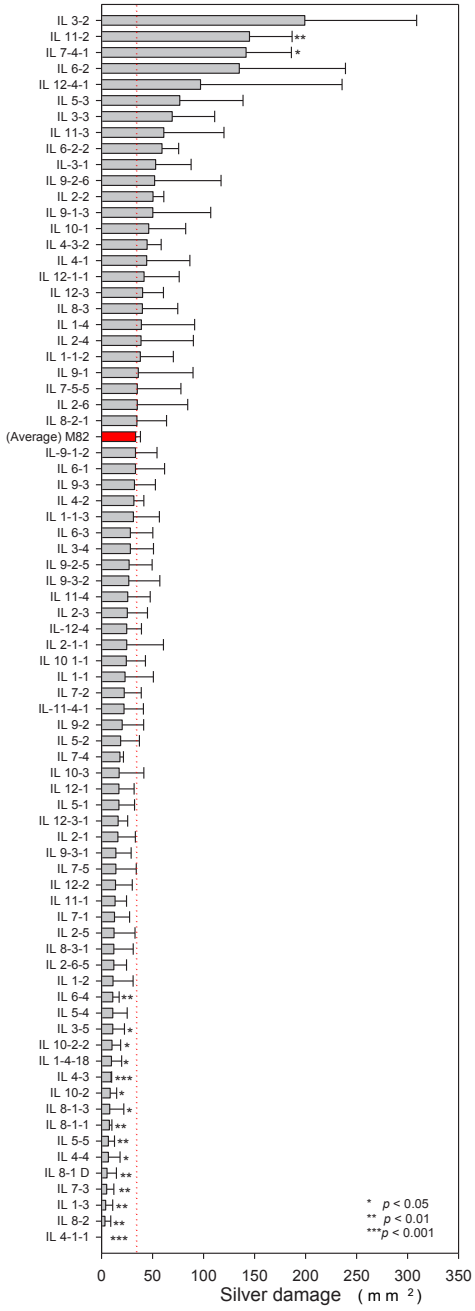

Figure 1. Thrips herbivory as silver damage on the *Solanum pennellii* x *lycopersicum* introgression population. ANOVA for silver damage was performed against the parent *S. lycopersicum* M82.

Results and discussion

Quantitative trait loci for thrips resistance

In the whole plant choice bioassay thrips herbivory varied significantly throughout the introgression lines, ranging from zero to a maximum of 200 mm^2 of silver damage per plant (Fig. 1). However, the variability in silver damage between replicates was high in most cases, which contributes to obscuring performance differences in the IL population against WFT. Lines were designated resistant (R) or susceptible (S) as their silver damage levels were respectively lower or higher than that of *S. lycopersicum* M82 at $p \leq 0.05$. Insect damage on M82, with 33.6 mm^2, was on average 5.8 times higher than on the resistant lines, with 5.8 mm^2, (T=6.674 d.f.=35, p<0.0001) and 4.3 times lower than on the susceptible ones, with 143.3 mm^2, (T=7.573 d.f.=14, p<0.0001). Resistant lines must contain specific polymorphic genomic segments or QTL from the wild parent that are associated with an increased level of thrips resistance compared to *S. lycopersicum*. Susceptible lines, on the other hand, may have either lost genomic fragments related to thrips resistance in M82 or gained QTL from the donor parent associated with an increased level of susceptibility to WFT.

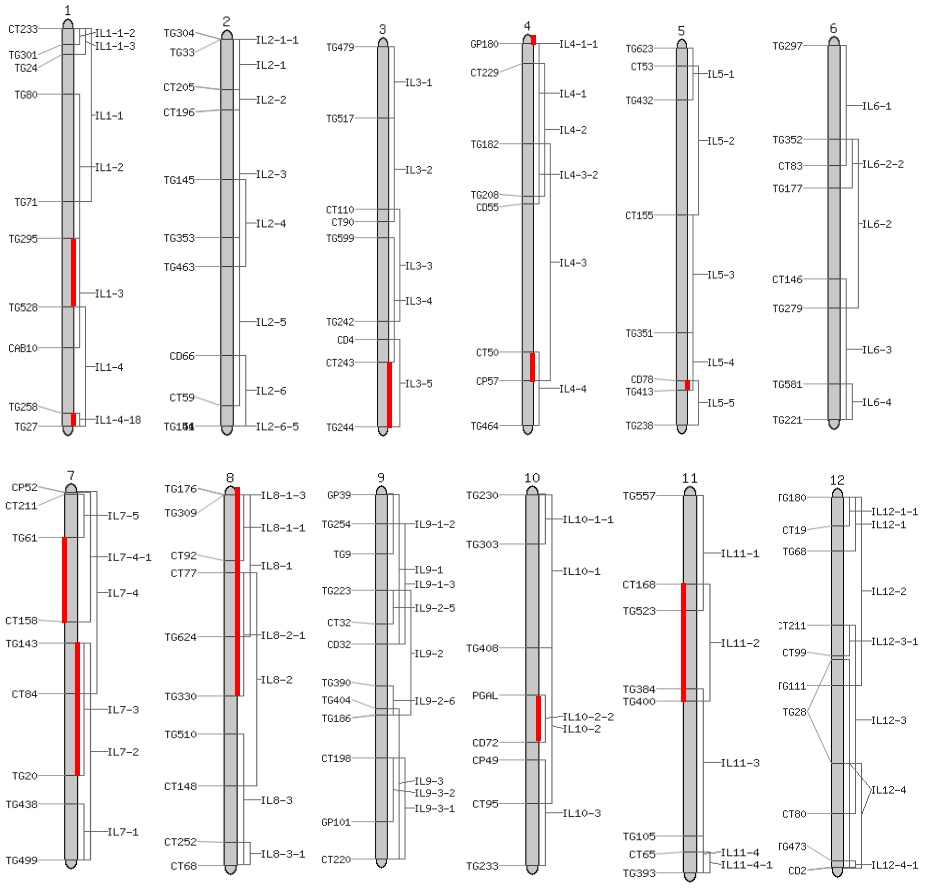

Figure 2. Genetic map of QTL for thrips performance on the *Solanum pennellii* x *lycopersicum* introgression population. Bars matching QTL for increased thrips damage are located on the left side of each chromosome while those on the right side correspond to QTL for decreased thrips damage relative to the parent *S. lycopersicum* M82.

Only two QTL were detected for increased susceptibility to WFT, IL7-4-1 and IL11-2 (Fig. 2). Interestingly, the number of resistant lines was much higher and most of them derived from a few chromosomes. Particularly relevant were ILs 1-3, 4-1-1, 4-3, 4-4, 5-5, 8-1-D, 8-1-3, 8-2, 10-2 and 10-2-2. Fine mapping for thrips resistance allowed the identification of several clearly defined shorter trait loci (Fig. 2). No previous reports are known to date on direct QTL analysis for pest resistance in the *pennellii* x *lycopersicum* introgression population. Indirect mapping, however, has been performed through QTL analysis for the accumulation of acylsugars. First on an interspecific cross F2 population (Mutschler et al., 1996) and second on the *pennellii* x *lycopersicum* introgression population (Schilmiller et al., 2010). Acylsugars are proven trichomal defense compounds of *Solanum* spp. (Mirnezhad et al., 2010; Simmons and Gurr, 2005). In the interspecific cross five genomic regions associated with different aspects of acylsugar production were identified. Two on chromosome 2 and one each on chromosomes 3, 4 and 11. While in the introgression population QTL for acylsugars were detected on four different chromosomes, 1, 5, 8 and 11. QTL overlapping occurred then only on chromosome 11, specifically at IL11-3, which was consistently reported in both studies to be associated with higher levels of acylsugars. In another interspecific cross F2 population between *S. habrochaites* (LA1777) and *S. lycopersicum* four QTL were identified for tomato resistance to *B. tabaci* (Momotaz et al., 2010). Based on this IL11-3 was expected to be thrips resistant. Surprisingly, this was not the case, suggesting either that IL11-3 did not yield amounts of acylsugars high enough to deter thrips or that other factors, morphological or chemical, play a more important role in tomato resistance to thrips.

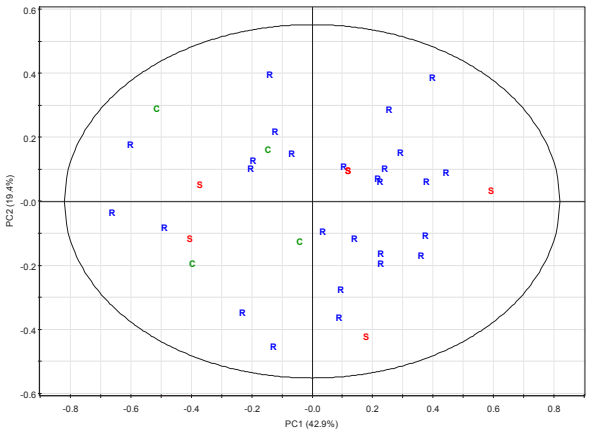

Figure 3. Principal component analysis performed on the ^{1}H NMR data of susceptible and resistant introgression lines. Points represent the scores of the first two principal components for lines with levels of thrips damage significantly higher (susceptible, S, red) and lower (resistant, R, blue) than the parent *Solanum lycopersicum* M82 (control, C, green). Each point represents the average of three (S and R) or five (C) biological replicates.

Multivariate analysis on the NMR data of the ILs

To identify the putative chemical bases for resistance to WFT in the IL system ^{1}H NMR spectra of all lines were obtained and subsequently analyzed with pattern recognition multivariate data analysis. Principal component analysis was applied to the ^{1}H NMR spectra of resistant and susceptible lines, along with M82 as control, to identify differences in the NMR fingerprint of these sample groups. The scatter plot for the first two components, accounting for 62% of the variance, unfortunately shows no apparent clustering of the classes (Fig. 3). Such overlapping of principal component scores indicates that the ^{1}H NMR spectra of *S. lycopersicum* and the resistant and susceptible lines are rather homogeneous. As a second approach to unveil the chemistry of thrips resistance partial least square discriminant analysis followed. The obtained model, however, failed the cross validation tests confirming the lack of consistent spectral differences between susceptible, resistant and control samples. According to these results the variation in resistance to WFT among the introgression lines may have its origin either in compounds present at concentrations below the NMR detection limit or in morphological traits such as hairiness and toughness. Unfortunately, none of these physical features were surveyed in our harvest. Glandular trichomes in particular have been extensively reported as effective defenses against many different pests (Simmons and Gurr, 2005), and their exudates usually go undetected in NMR analysis of whole leaf samples (see chapters 2 and 3).

Table 1. ^{1}H NMR data of the metabolites identified in the *Solanum pennellii* x *lycopersicum* introgression population. Signals in bold characters were used for relative quantification. Dried leaf samples were directly extracted with MeOD-D_2O 7:3 for ^{1}H NMR (600 MHz) analysis.

	Leaf metabolite	Chemical shift of main signals
1	Sterols	0.81 (s), **0.84** (s)
2	SFA	0.88 (brs), **1.26** (brs)
3	UFA	0.95 (t, *J* = 7.5 Hz), **1.30** (brs), 5.35 (brs)
4	Threonine	1.33 (d, *J* = 6.6 Hz)
5	Alanine	1.48 (d, *J* = 7.2 Hz)
6	Acetic acid	1.99 (s)
7	Aspartic acid	**2.65** (dd, *J* = 17.4, 9.3 Hz), 2.83 (dd, *J* = 17.4, 3.5 Hz)
8	GABA	1.91 (m), 2.35 (t, *J* = 7.2 Hz), **3.00** (t, *J* = 7.2 Hz)
9	Choline	3.21 (s)
10	Malic acid	2.54 (dd, *J* = 15.8, 8.3 Hz), 2.78 (dd, *J* = 15.8, 3.9 Hz), **4.29** (dd, *J* = 8.3, 3.9 Hz)
11	Glucose	5.16 (d, *J* = 3.7 Hz)
12	Sucrose	4.14 (d, *J* = 8.7 Hz), **5.40** (d, *J* = 3.8 Hz)
13	Rutin	**6.30** (d, *J* = 2.5 Hz), 6.51 (d, *J* = 2.5 Hz), 6.95 (d, *J* = 8.4 Hz), 7.67 (d, *J* = 2.5 Hz)
14	CQA	**6.34** (d, *J* = 15.9 Hz), 7.11 (d, *J* = 2.5 Hz), 7.59 (d, *J* = 15.9 Hz)
15	CGAa	6.36 (d, *J* = 15.9 Hz)
16	CGAb	**6.37** (d, *J* = 15.9 Hz), 7.61 (d, *J* = 15.9 Hz)
17	Fumaric acid	6.64 (s)
18	PAL	**7.32** (d, *J* = 8.0 Hz), 7.36 (d, *J* = 8.0 Hz)
19	CGAc	6.45 (d, *J* = 15.9 Hz), **7.63** (d, *J* = 15.9 Hz)
20	CGAd	6.45 (d, *J* = 15.9 Hz), **7.65** (d, *J* = 15.9 Hz)
21	UDPG	7.99 (d, *J* = 8.0 Hz)
22	Formic acid	8.45 (s)
23	AMP	8.23 (s), **8.55** (s)
24	Trigonelline	8.10 (dd, *J* = 8.2, 6.3 Hz), 8.85 (d, *J* = 6.2 Hz), 8.89 (d, *J* = 8.1 Hz), **9.16** (s)

SFA: saturated fatty acids, UFA: unsaturated fatty acids, GABA: γ-aminobutyric acid, CQA: caffeoylquinic acid (chlorogenic acid), CGAa-d: regioisomers of caffeoylglucaric acid, PAL: phenyalanine, UDPG: uridine diphosphoglucose, AMP: adenosine monophosphate.

Metabolic profiling of the ILs

In a further step the ^{1}H NMR spectra of the ILs were thoroughly scrutinized to identify and quantify the maximum possible number of metabolites extracted from the tomato leaves. Table 1 summarizes the spectral data of all compounds or classes of compounds that were unequivocally identified. The list comprises 24 major plant metabolites, primary and secondary, of very diverse structures and polarities. Most of these compounds can be classified into one of five main classes: lipids, amino acids, free sugars, organic acids or phenolics. Among the latter only catechol-like metabolites could be detected: chlorogenic acid (caffeoylquinic acid, CQA), the four regioisomers of caffeoylglucaric acid (CGA) and the flavonoid glycoside rutin. The exact position of the caffeoyl moiety on the CGA regioisomers was not determined.

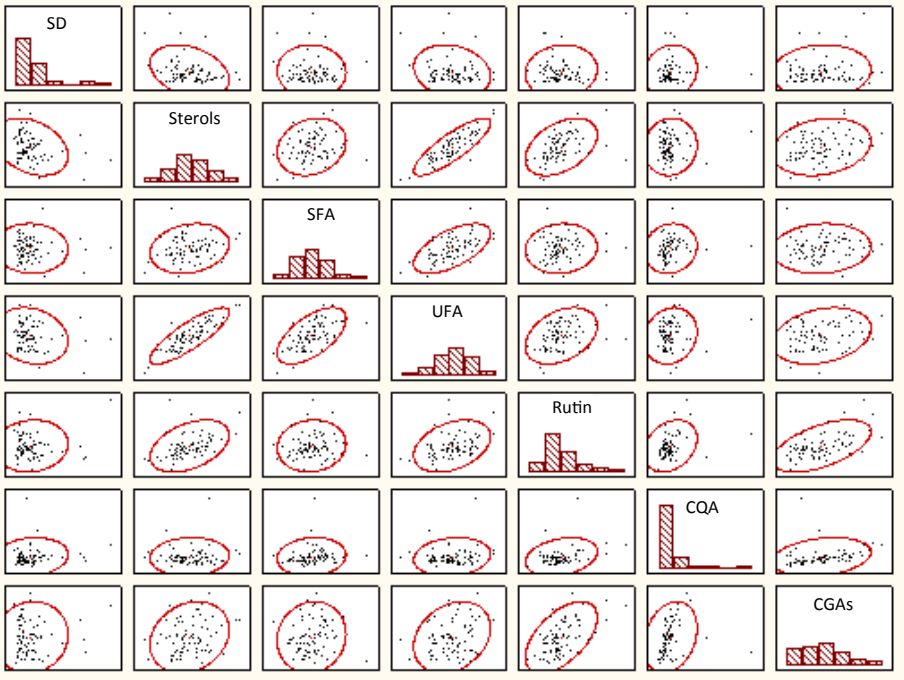

Figure 4. Metabolic correlation matrix. Each box contains a correlation plot between the relative concentrations, as determined by ^{1}H NMR, of relevant metabolites in the *Solanum pennellii* x *lycopersicum* introgression population. Thrips damage (SD) was also included as the single ecological variable. Dots in every scatter plot represent the mean of three replicates for each of the 76 lines. Ellipses represent the 90% confidence contour. Narrow ellipses indicate strong correlations. Histograms on the diagonal show the data distribution for each trait. SFA: saturated fatty acids, UFA: unsaturated fatty acids, CQA: caffeoylquinic acid (chlorogenic acid), CGA: caffeoylglucaric acid.

Correlation analysis was performed between all pair combinations of quantified metabolites. Thrips damage was included as well in order to verify the results from the multivariate data analysis. The minimum value of the Spearman correlation coefficient (ρ) to consider two variables as correlated was set at 0.5, for which probability values (*p*) were in all cases lower than 0.0001. Under this criterion no

single correlation was detected between silver damage and any of the listed metabolites. Strong correlations were mostly observed within classes of metabolites. Unsaturated fatty acids, for instance, were correlated with sterols (ρ=0.852) and saturated fatty acids, mainly as palmitic acid, (ρ=0.591). These correlations between lipids may result from their co-accumulation as membrane building blocks rather than from a metabolic network connection. Amino acids in general were also correlated. Coefficient values between phenylalanine and threonine, alanine and aspartic acid were 0.513, 0.653 and 0.457, respectively. A significant correlation was also detected between threonine and alanine (ρ= 0.552). Although these amino acids have diverging biosynthetic pathways and are the precursors of very different secondary metabolites their metabolism in plants is highly regulated (Galili and Hofgen, 2002), resulting in false correlations. Regarding the small organic acids, formic acid and acetic acid did not correlate with any other metabolite whereas malic acid was positively correlated with fumaric acid (ρ=0.664). Both fumaric and malic acid were negatively correlated with the signal of unsaturated fatty acids (ρ=-0.545 and -0.528, respectively) and sterols (ρ=-0.550 and -0.429, respectively). Fumaric acid was also negatively correlated with rutin and glucose (ρ=-0.698 and -0.510, respectively) but positively with alanine and phenylalanine (ρ=0.623 and 0.646, respectively). The highest coefficients were obtained for correlations between the regioisomers of CGA, ranging from 0.851 to 0.690. Such close structures are undoubtedly genetically linked, *i.e.* sharing the same set of QTL, and for that their expression levels are expected to be highly correlated. CGA isomers were in turn correlated with the other phenolics, rutin and CQA (ρ=0.511 and 0.530, respectively). All these phenolic compounds share the same catechol-like hydroxycinnamic moiety, which relates them all upstream in their metabolic biosynthetic pathway, explaining in this way their correlation. A matrix plot showing some of the most relevant correlations is presented in Figure 4. The results from the multivariate analysis were confirmed as no strong correlations were observed between thrips damage and any of the identified metabolites. The highest correlation coefficients obtained were -0.327 (p=0.004) and 0.380 (p=0.0007) for sterols and the nucleotide uridine-diphosphate glucose, respectively.

Correlation analysis, however, does not provide any information about the genetic regulation of metabolite concentrations. To close in on the genetic bases for increased or decreased content of relevant metabolites their relative concentrations must be compared throughout the entire IL population. A very simple visual method is the use of color-scaled expression maps or heat maps (Fig. 5), which allow a quick identification of ILs with lower or higher content of any metabolite relative to the control, M82. An optimized hierarchical clustering analysis has been additionally applied. In such analysis both metabolites and ILs have been organized according to maximum similarity in the level of expression. The grouping of all isomers of CGA somehow validates this type of clustering as a similarity analysis. Some false positive linkage can also be recognized with clustering analysis. In this case threonine and fatty acids have been placed next to each other due to a matrix interference in the ^{1}H NMR spectra. The proton integration signals of these metabolites overlap with that of a very broad band corresponding to all non-reactive methylenes, at around 1.3 ppm, which obscures the concentration differences of these metabolites throughout the IL population.

Genetic mapping of QTL for phenolics content

To verify whether the differences detected in metabolic expression levels were significant *T*-tests between each line and M82 were conducted. A total of 268 QTL for all the quantified metabolites

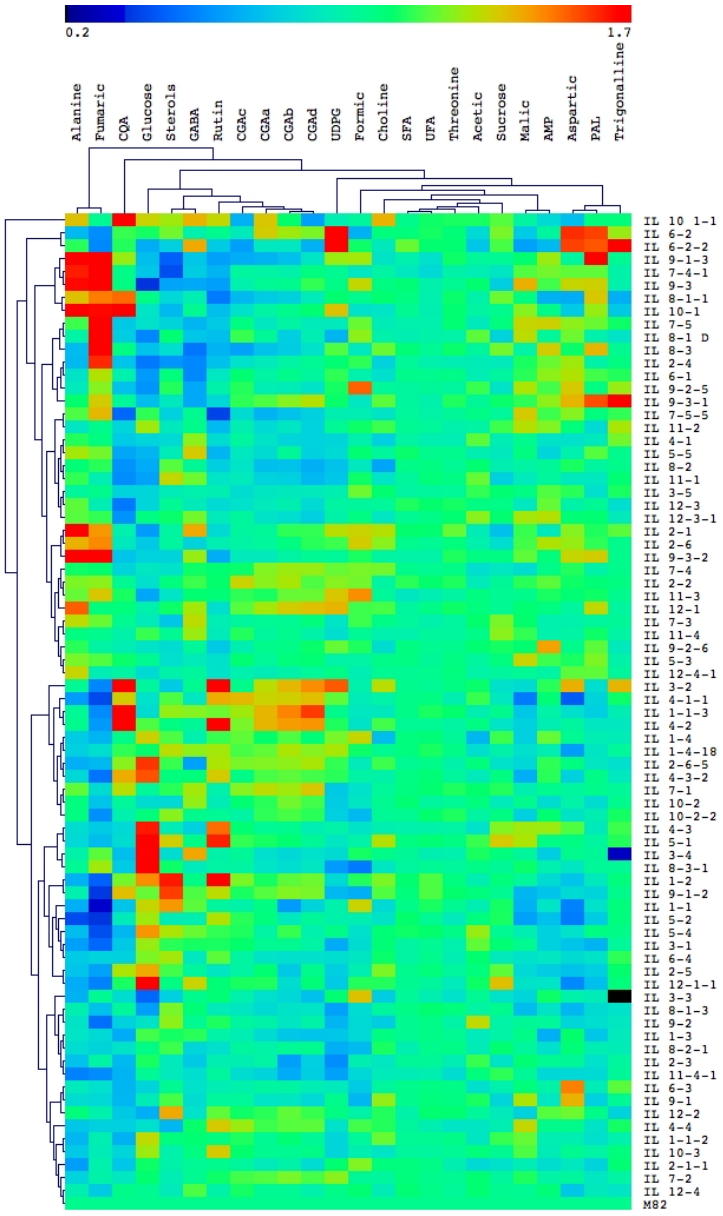

Figure 5. Metabolic heat map of the *Solanum pennellii* x *lycopersicum* introgression population. Relative concentrations of each metabolite, as determined by ^{1}H NMR, were expressed as folds of the corresponding mean in the parent *S. lycopersicum* M82 and plotted in a true color scale. Red and blue respectively denote increase and decrease in the content of a metabolite after a genomic region of *S. lycopersicum* M82 has been replaced by its homologous from *S. pennellii.* Complete linkage was used for the hierarchical cluster analysis. SFA: saturated fatty acids, UFA: unsaturated fatty acids, GABA: γ-aminobutyric acid, CQA: caffeoylquinic acid (chlorogenic acid), CGA: caffeoylglucaric acid, PAL: phenyalanine, UDPG: uridine diphosphoglucose, AMP: adenosine monophosphate.

Table 2. Foliar metabolic QTL in tomato identified through the *Solanum pennellii* x *lycopersicum* introgression population. X-fold values represent the concentration ratio between each line and the recurrent parent *S. lycopersicum* M82, determined at a significance level of 0.05.

Metabolite	Locus	x-Fold	Metabolite	Locus	x-Fold	Metabolite	Locus	x-Fold
Acetic acid	IL 1-4	0.69 ± 0.04	CGAd	IL 1-1	0.79 ± 0.02	PAL	IL 1-1	0.85 ± 0.04
	IL 6-2-2	0.74 ± 0.05		IL 1-1-3	1.59 ± 0.17		IL 1-1-3	0.77 ± 0.00
	IL 9-2	1.31 ± 0.03		IL 1-3	0.73 ± 0.09		IL 1-3	0.74 ± 0.02
Alanine	IL 1-1	0.67 ± 0.04		IL 2-3	0.77 ± 0.04		IL 1-4-18	0.87 ± 0.00
	IL 1-1-2	0.69 ± 0.01		IL 2-6-5	1.27 ± 0.03		IL 2-1-1	0.78 ± 0.09
	IL 1-2	0.67 ± 0.09		IL 3-2	1.46 ± 0.03		IL 2-5	0.77 ± 0.01
	IL 2-1	1.70 ± 0.12		IL 3-3	0.72 ± 0.03		IL-3-1	0.68 ± 0.05
	IL 2-1-1	0.62 ± 0.09		IL 4-1	0.79 ± 0.06		IL 3-2	0.83 ± 0.04
	IL 2-4	0.70 ± 0.02		IL 4-1-1	1.38 ± 0.05		IL 3-5	0.84 ± 0.05
	IL 2-5	0.73 ± 0.05		IL 4-2	1.45 ± 0.00		IL 4-1-1	0.79 ± 0.00
	IL 2-6-5	0.67 ± 0.11		IL 5-5	0.78 ± 0.06		IL 4-4	0.86 ± 0.03
	IL-3-1	0.62 ± 0.07		IL 6-2	1.22 ± 0.07		IL 5-2	0.76 ± 0.03
	IL 3-3	0.67 ± 0.08		IL 6-2-2	0.67 ± 0.03		IL 5-5	1.17 ± 0.04
	IL 4-1-1	0.61 ± 0.01		IL 7-4	1.25 ± 0.10		IL 6-1	1.15 ± 0.03
	IL 5-2	0.41 ± 0.02		IL 7-5-5	0.78 ± 0.06		IL 6-2-2	1.55 ± 0.10
	IL 5-4	0.71 ± 0.05		IL 8-2	0.67 ± 0.01		IL 7-5	1.19 ± 0.02
	IL 5-5	1.27 ± 0.06		IL 9-1	0.81 ± 0.00		IL 8-3	1.39 ± 0.12
	IL 6-2	0.70 ± 0.06		IL 9-3-1	1.29 ± 0.05		IL 9-1	0.77 ± 0.07
	IL 7-4-1	1.63 ± 0.21		IL 10 1-1	0.62 ± 0.05		IL-9-1-2	0.79 ± 0.07
	IL 8-3	0.70 ± 0.03		IL 11-1	0.70 ± 0.02		IL 9-1-3	1.76 ± 0.20
	IL 9-1-3	2.03 ± 0.45		IL 11-2	0.78 ± 0.00		IL 9-2	0.83 ± 0.03
	IL 9-3-2	1.82 ± 0.13		IL-11-4-1	0.76 ± 0.08		IL 9-2-6	1.17 ± 0.05
	IL 10-1	1.80 ± 0.06		IL 12-1	1.37 ± 0.09		IL 9-3-1	1.56 ± 0.06
	IL 10 1-1	1.37 ± 0.14		IL 12-3	0.81 ± 0.04		IL 9-3-2	1.34 ± 0.16
	IL-11-4-1	0.54 ± 0.02		IL 12-3-1	0.77 ± 0.02		IL 10-1	1.25 ± 0.03
	IL 12-1	1.54 ± 0.27	Choline	IL 1-4-18	1.00 ± 0.12		IL 10-2	0.83 ± 0.00
	IL 12-1-1	0.66 ± 0.05		IL 3-3	0.76 ± 0.03		IL 11-2	0.81 ± 0.07
AMP	IL 1-4	1.18 ± 0.01		IL 4-1	0.78 ± 0.02		IL-11-4-1	0.81 ± 0.01
	IL 1-2	0.82 ± 0.02		IL 5-1	1.29 ± 0.04		IL 12-1	1.31 ± 0.15
	IL 1-3	0.67 ± 0.00		IL 8-2	0.64 ± 0.01		IL 12-1-1	0.71 ± 0.05
	IL 2-2	1.17 ± 0.04		IL 9-2	0.76 ± 0.00		IL 12-4-1	1.18 ± 0.07
	IL 2-5	0.76 ± 0.10	CQA	IL 1-1-3	1.70 ± 0.35	Rutin	IL 1-2	1.85 ± 0.02
	IL-3-1	0.78 ± 0.06		IL 1-3	0.66 ± 0.07		IL 3-2	2.18 ± 0.04
	IL 3-4	0.76 ± 0.05		IL 3-2	1.70 ± 0.01		IL 4-1-1	1.41 ± 0.02
	IL 5-2	0.72 ± 0.11		IL 4-2	1.83 ± 0.37		IL 4-2	1.83 ± 0.20
	IL 5-4	0.78 ± 0.08		IL 4-3-2	1.41 ± 0.07		IL 4-3	1.52 ± 0.13
	IL 5-5	0.80 ± 0.04		IL 5-4	0.69 ± 0.03		IL 5-1	1.63 ± 0.08
	IL 6-1	1.24 ± 0.05		IL 5-5	0.64 ± 0.05		IL 5-5	0.68 ± 0.00
	IL 6-2-2	0.70 ± 0.03		IL 7-5-5	0.48 ± 0.05		IL 7-4-1	0.68 ± 0.02
	IL 7-5	1.33 ± 0.09		IL 8-2	0.58 ± 0.02		IL 7-5-5	0.40 ± 0.08
	IL 8-1-1	0.67 ± 0.07		IL 10-1	2.56 ± 0.21		IL 8-1-1	0.53 ± 0.01
	IL 8-2-1	0.84 ± 0.01		IL 10 1-1	4.48 ± 1.00		IL 9-1-3	0.62 ± 0.01
	IL 8-3	1.35 ± 0.11		IL 11-1	0.57 ± 0.03		IL 9-3	0.62 ± 0.06
	IL-9-1-2	0.73 ± 0.06		IL-11-4-1	0.68 ± 0.04	Sterols	IL 1-1	1.44 ± 0.01
	IL 9-1-3	1.26 ± 0.06		IL 12-3	0.52 ± 0.00		IL 1-2	1.64 ± 0.01
	IL 9-2-6	1.42 ± 0.22		IL 12-3-1	0.58 ± 0.03		IL 2-4	0.60 ± 0.03
	IL 11-2	1.22 ± 0.06	Formic acid	IL 8-3-1	0.53 ± 0.09		IL 4-4	0.69 ± 0.09
	IL-11-4-1	0.81 ± 0.02		IL 9-2-5	1.53 ± 0.18		IL 7-3	1.11 ± 0.10
	IL 12-2	1.17 ± 0.06		IL 11-3	1.46 ± 0.00		IL 7-4-1	0.74 ± 0.01
	IL 12-3	1.18 ± 0.06	Fumaric acid	IL 1-1	0.32 ± 0.02		IL-9-1-2	1.08 ± 0.26
CGAa	IL 1-1-3	1.39 ± 0.06		IL 5-2	0.37 ± 0.00		IL 9-1-3	1.15 ± 0.15
	IL 2-6-5	1.22 ± 0.01		IL 7-4-1	1.92 ± 0.23		IL 9-3	1.11 ± 0.10
	IL 3-2	1.30 ± 0.03		IL 7-5	2.32 ± 0.20		IL 10-1	0.88 ± 0.07
	IL 4-1-1	1.33 ± 0.05		IL 8-1 D	1.84 ± 0.29		IL 12-2	1.42 ± 0.03
	IL 4-2	1.39 ± 0.07		IL 8-3	1.83 ± 0.04	Sucrose	IL 2-1	0.72 ± 0.08
	IL 7-1	1.30 ± 0.12		IL-9-1-2	0.37 ± 0.09		IL 2-6	0.83 ± 0.01
	IL 7-5-5	0.68 ± 0.10		IL 9-1-3	2.54 ± 0.05		IL 3-3	0.85 ± 0.03
	IL 8-1-1	0.75 ± 0.01		IL 9-3	2.10 ± 0.20		IL 4-3	1.22 ± 0.00
	IL 8-2	0.72 ± 0.03		IL 9-3-2	1.76 ± 0.15		IL 6-2	1.21 ± 0.05
	IL 8-3	0.73 ± 0.04		IL 10-1	1.66 ± 0.05		IL 8-1 D	0.78 ± 0.04
	IL 9-1-3	0.68 ± 0.04	GABA	IL 2-4	0.56 ± 0.07		IL 8-3	1.20 ± 0.01
	IL 10 1-1	1.35 ± 0.12		IL 3-4	1.42 ± 0.06		IL 9-1	0.79 ± 0.08
	IL 11-1	0.78 ± 0.03		IL 6-1	0.56 ± 0.07		IL 9-3	0.77 ± 0.06
	IL 11-2	0.82 ± 0.02		IL 8-3	0.51 ± 0.07		IL 11-1	0.78 ± 0.04
CGAb	IL 1-1	0.61 ± 0.11	Glucose	IL 1-1	1.32 ± 0.10		IL 11-4	1.23 ± 0.07
	IL 1-1-3	1.48 ± 0.08		IL 1-2	1.46 ± 0.02		IL 12-1-1	1.37 ± 0.01
	IL 1-4-18	1.28 ± 0.09		IL 2-1	0.61 ± 0.02	Threonine	IL 2-1	1.18 ± 0.01
	IL 2-2	1.28 ± 0.09		IL 2-4	0.52 ± 0.17		IL 3-3	0.85 ± 0.05
	IL 2-3	0.62 ± 0.19		IL 2-5	1.41 ± 0.07		IL 4-4	0.86 ± 0.05
	IL 2-6-5	1.24 ± 0.04		IL-3-1	1.25 ± 0.04		IL 5-2	0.89 ± 0.00
	IL 3-2	1.39 ± 0.06		IL 3-3	0.48 ± 0.03		IL 7-1	1.10 ± 0.01
	IL 4-1-1	1.34 ± 0.01		IL 4-3	1.64 ± 0.28		IL 9-3-1	1.11 ± 0.02

Metabolite	Locus	x-Fold	Metabolite	Locus	x-Fold	Metabolite	Locus	x-Fold
	IL 4-2	1.44 ± 0.12		IL 5-4	1.45 ± 0.19		IL 9-3-2	1.10 ± 0.01
	IL 7-4	1.25 ± 0.10		IL 5-5	0.69 ± 0.04		IL 10 1-1	1.10 ± 0.01
	IL 7-5-5	0.73 ± 0.12		IL 6-1	0.61 ± 0.06		IL-11-4-1	0.83 ± 0.03
	IL 8-1-1	0.78 ± 0.02		IL 6-2-2	0.66 ± 0.07	Trigonelline	IL 2-1	1.17 ± 0.00
	IL 8-2	0.73 ± 0.01		IL 8-1 D	0.55 ± 0.02		IL 3-2	1.39 ± 0.00
	IL 8-3	0.76 ± 0.08		IL 8-1-3	0.62 ± 0.06		IL 3-3	0.20 ± 0.00
	IL 9-1-3	0.72 ± 0.03		IL 8-2	0.65 ± 0.06		IL 3-4	0.30 ± 0.11
	IL 11-1	0.77 ± 0.00		IL 8-3	0.73 ± 0.07		IL 4-1	1.20 ± 0.06
	IL-11-4-1	0.66 ± 0.04		IL 9-2-5	0.59 ± 0.01		IL 6-2-2	1.68 ± 0.09
				IL 9-3	0.36 ± 0.04		IL 6-3	1.18 ± 0.03
				IL 10 1-1	1.33 ± 0.05		IL 8-1-1	0.67 ± 0.01
				IL 11-1	0.63 ± 0.01		IL 9-2-5	1.25 ± 0.06
				IL 11-3	0.64 ± 0.11		IL 9-3-1	1.69 ± 0.02
				IL 12-1-1	1.76 ± 0.14		IL 10-1	0.75 ± 0.04
				IL 12-2	0.67 ± 0.03		IL 12-2	0.79 ± 0.01
							IL 12-3	0.83 ± 0.01
						UDPG	IL 1-1	0.93 ± 0.32
							IL 2-3	0.58 ± 0.10
							IL 3-2	1.54 ± 0.06
							IL 6-2-2	2.87 ± 0.05
							IL 8-1-3	0.62 ± 0.09
							IL 8-3-1	0.61 ± 0.08
							IL 11-3	1.36 ± 0.00
							IL-11-4-1	0.63 ± 0.07
						UFA	IL 1-2	1.15 ± 0.06
							IL 7-4-1	0.81 ± 0.07
							IL-9-1-2	1.15 ± 0.06
							IL 9-1-3	0.84 ± 0.02

UFA: unsaturated fatty acids, GABA: γ-aminobutyric acid, CQA: caffeoylquinic acid (chlorogenic acid), CGA: caffeoylglucaric acid, PAL: phenyalanine, UDPG: uridine diphosphoglucose, AMP: adenosine monophosphate.

in the introgression population were obtained (Table 2). Confirmed QTL involved in the foliar accumulation of phenolics were mapped as presented in Figure 6. The overall concentration ratios varied between 0.2 and 2 times relative to M82, with a few exceptional cases such as CQA, which reached an increase of 2.6 and up to 4.4 times in ILs 10-1 and 10-1-1, respectively. These two lines define therefore a quantitative locus for increased content of CQA of about 12 centimorgans located between markers TG230 and TG303 on chromosome 10 (Fig. 6). CQA is a ubiquitous secondary metabolite in the plant kingdom with a broad spectrum of alleged and proven biological activities. Its bioavailability (Scalbert et al., 2002; Williamson et al., 2000) and antioxidant properties (Rice-Evans et al., 1997) have drawn a lot of attention to CQA not only for its potential in nutraceutics (Wildman, 2007) but also as a plant defense compound (Leiss et al., 2009b; Summers and Felton, 1994). The identified QTL for increased levels of CQA represents a very important finding as it may provide one of the missing links to solve the controversial puzzle of CQA biosynthesis and accumulation in plants (Comino et al., 2009). Expression of this genetic segment may help identify the key enzyme(s) necessary for a plant to accumulate CQA. Three main routes for the synthesis of CQA in plants have been proposed, involving caffeoyl-CoA quinate caffeoyl transferase, HQT (Ulbrich and Zenk, 1979), caffeoyl D-glucose:quinate caffeoyl transferase, HCGQT (Villegas and Kojima, 1986) and *p*-coumaroyl-CoA quinate *p*-coumaroyl transferase, HCT (Hoffmann et al., 2003) as the respective rate-limiting enzymes. Although Niggeweg et al. (2004) demonstrated that HQT catalyzes the main route of CQA production in Solanaceous spp. genes encoding for these hydroxycinnamoyl-CoA shikimate/quinate tranferases have been detected only on chromosome 7 of tomato (Sol Genomics Network, http://solgenomics.net), which did not bear any QTL for high levels of CQA (Fig 6). No other phenolic compounds, rutin or CGA, colocalized on IL10-1-1, suggesting that this locus may control aspects of quinic acid metabolism, either synthesis, accumulation or, most likely, condensation to the hydroxycinnamic group.

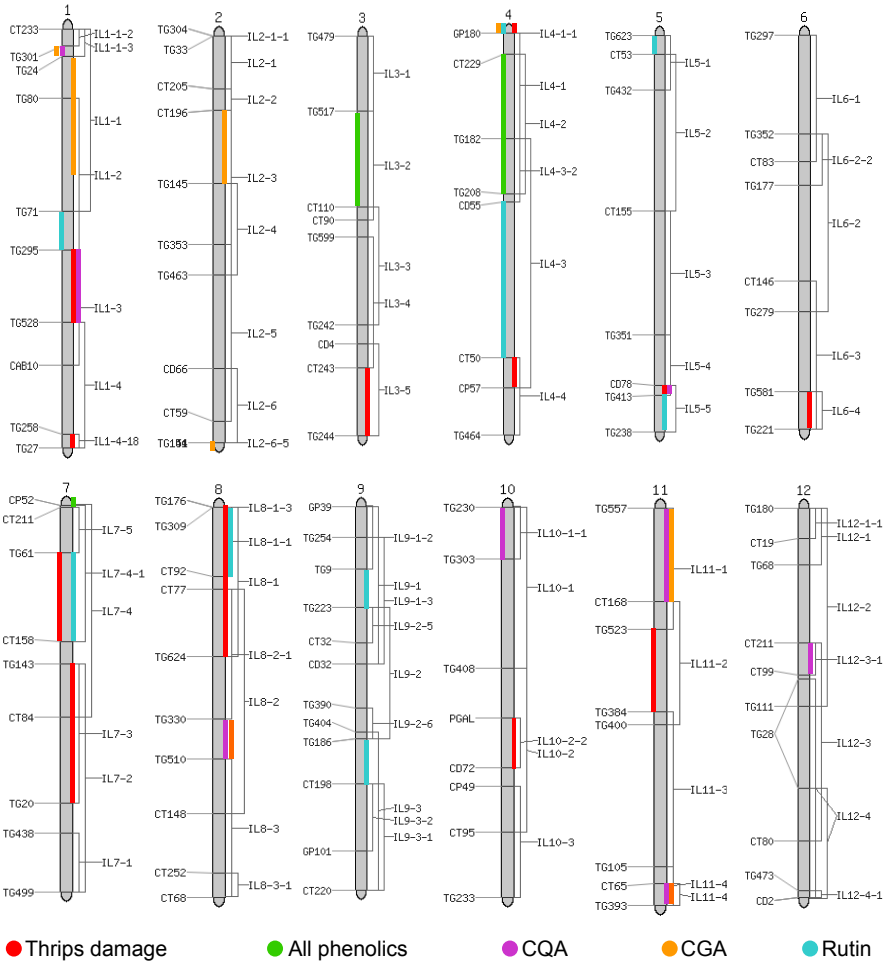

Figure 6. Genetic map of QTL for catechol-like phenolics in the *Solanum pennellii* x *lycopersicum* introgression population. Thrips damage has been included to facilitate co-localization analysis. Bars on the left side of each chromosome match QTL for increased levels of phenolics while those on the right side correspond to QTL for decreased levels relative to the parent *S. lycopersicum* M82.

QTL for phenolics in tomato fruits have also been identified before in this same *pennellii* x *lycopersicum* introgression population (Rousseaux et al., 2005). Authors identified a total of 20 QTL associated with antioxidants, five for antioxidant capacity, six for ascorbic acid and nine for total phenolics, of which only IL10-1 for antioxidant capacity overlapped with our QTL map for catechol-like phenolics, specifically for higher content of CQA. In some cases QTL are in contradiction. For instance, IL7-4 for increased total phenolics coincides with one of our QTL for lower rutin levels. Such discrepancies may indicate that different unlinked sets of genes control these same traits on leaves and fruits, or may partly result from the fact that Rouseaux et al. (2005) did not profile any specific phenolics. Antioxidant activity and total phenolic content were determined through general non-chromatographic methods, which on the one hand include more compounds than those quantified in our study and on

the other hand are more susceptible to interference from other unrelated metabolites. High variation between year trials in the work of Rouseaux et al. (2005) may account as well for the discrepancies.

QTL analysis confirmed that thrips resistance was not correlated with any of the phenolic compounds. Silver damage did not map consistently with any metabolic trait. In some cases opposing associations were observed.

In order to validate our findings their stability against external variables such as radiation, humidity, air-borne elicitors and pollutants, among others, must be verified. Ideally several harvests from different years should be considered in this kind of studies. Using the same introgression population it has been shown in similar metabolic genomics studies conducted on tomato fruit that environmental conditions have a greater impact on the metabolome compared to genotype (Phuc et al., 2010).

Introgression populations represent an excellent tool to study the genetics of not only pest resistance but also many other relevant traits, including the accumulation of phytochemicals of high added value. The application of NMR metabolomics to a stable marker-assisted introgression population enabled us to discover QTL for differential expression of important secondary metabolites, such as chlorogenic acid and rutin, that could easily be incorporated into new domesticated tomatoes for crop improvement purposes. Therefore, this kind of approaches represents an effective alternative to genetic manipulation in plant metabolic engineering.

Chapter 5

Salting-out gradients in centrifugal partition chromatography for the isolation of chlorogenic acids from green coffee beans

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Abstract

In addition to sample solubility constraints, the use of polarity gradients in normal-phase centrifugal partition chromatography (CPC) for the purification of complex mixtures is also limited by the instability of biphasic systems as a consequence of dramatic changes in the settling times along the gradient, leading in many cases to column bleeding when working under maximum efficiency conditions. In this paper an electrostriction approach is proposed as a strategy in reversed-phase CPC to fractionate intermediate polarity extracts in a single run by bringing its components into the "sweet spot" in a controlled fashion through a stepwise reduction of salt concentration in the aqueous mobile phase. The salting-out gradient method was successfully tested with the separation of the major chlorogenic acids (hydroxycinnamoylquinic acids, HCQAs) present in green coffee beans (5-caffeoylquinic acid, 5-CQA, 5-feruloylquinic acid, 5-FQA, and 3,5-dicaffeoylquinic acid, 3,5-diCQA) using ethyl acetate-hexane as the stationary phase and an ionic gradient of LiCl (5.0, 2.5 and 0.1 M) as the mobile phase in one case and $(NH_4)_2SO_4$-KNO_3 (3.0 and 1.5 M-1.5 M) in another. Regioisomers of each chlorogenic acid obtained by base-catalysed isomerisation were also separated by CPC using isocratic elution. The best resolution for both FQAs and diCQAs was achieved with a chloroform-*n*-butanol/0.01 M pH 2.5 phosphate buffer (84:16:100, v/v) system, while CQAs were best isolated using chloroform-*n*-butanol/0.01 M pH 2.5 phosphate buffer-5.0 M LiCl (82:18:100, v/v).

Introduction

The liquid nature of the stationary phase in counter-current separations (CCS) confers a greater versatility to this technique compared to HPLC. This unique feature has led to a fascinating new spectrum of instrumentation setups, column designs, separation methods and polarities that have been successfully applied and there is still potential for further developments. From the preparative point of view this characteristic makes CCS even more powerful as it enables the usage of the stationary phase to increase the solubility of analytes. Allowing in this way the loading of highly concentrated complex samples, *e.g.* plant extracts, for fractionation or isolation purposes. However, in contrast to adsorption chromatography, polarity elution gradients used for sample fractionation are not very common in CCS. The significant changes in the physical properties of the mobile phase associated with the change in solvent composition can lead to instability of the biphasic system in CCS and cause inconvenient column bleeding. Several ingenious strategies have been developed to solve this practical downside that limits the competitiveness of CCS. The most common is perhaps the elution-extrusion mode (Berthod, 2007; 2003; Lu et al., 2008), in which the stationary phase containing retained analytes is entirely pumped out of the system preserving its separation pattern. The alternation of the mobile/stationary roles between the upper and lower phases of the solvent system is another widespread elution method, first introduced as the dual-mode in high-speed counter-current chromatography (HSCCC) (Agnely and Thiebaut, 1997) and later on as a multiple dual-mode in centrifugal partition chromatography (CPC) (Delannay et al., 2006). Nevertheless both polarity range and chromatographic resolution of CCS could be increased with the implementation of elution gradients. As an alternative to a polarity gradient an electrostriction or salting-out gradient is used in this case to control the distribution constant (*D*) of analytes and hence their relative elution times or resolution.

Salting-out is a very common but not simple physical phenomenon extensively exploited by biopolymer science and ion-exchange chromatography. It has also been applied in CCS, specifically by Ito (Ito, 2002), using a gradient elution CPC method for the selective precipitation of proteins called centrifugal precipitation chromatography, and at single salt concentrations to either stabilize the solvent system or to adjust distribution constants. In the method described in this paper the electrostriction effect is achieved by a stepwise reduction of the ionic strength of the mobile phase to separate the different types of chlorogenic acids present in green coffee beans in a reversed-phase mode.

Although the term chlorogenic acid originally referred exclusively to 5-*O*-caffeoylquinic acid (5-CQA), nowadays it is often used to refer to the whole family of *trans* hydroxycinnamoylquinic acids (HCQAs), one of the most important groups of phenolics in the plant kingdom particularly abundant in many foods and medicinal herbs. These catechol-like compounds have a broad range of proven and purported biological activities such as anti-bacterial (Li and Steffens, 2002), anti-fungal (Shadle et al., 2003), anti-insect (Beninger et al., 2004), anxiolytic (Bouayed et al., 2007), hepatoprotective (Xiang et al., 2001), anti-thrombotic (Satake et al., 2007) and anti-viral (Chiang et al., 2002), including HIV inhibition (Robinson et al., 1996). Their extensively documented antioxidant properties render them beneficial against several oxidative stress-related conditions such as atherosclerosis (Cheng et al., 2007), cancer (Lee and Lee, 2006) and Alzheimer's disease (Silva et al., 2005). Ubiquitous compounds such as these always have very crucial functions in Nature. Surprisingly, however, in spite of the vast amount of research conducted on them some of their roles are not yet fully understood. The dietary importance of catechol-like polyphenolics (flavonoids and HCQAs) for instance is still quite controversial. Some recent studies claim that their health benefits in humans do not result from their antioxidant properties but instead from their toxicity (Galati and O'Brien, 2004) which triggers the physiological production of the endogenous actual antioxidant uric acid (Lotito and Frei, 2006).

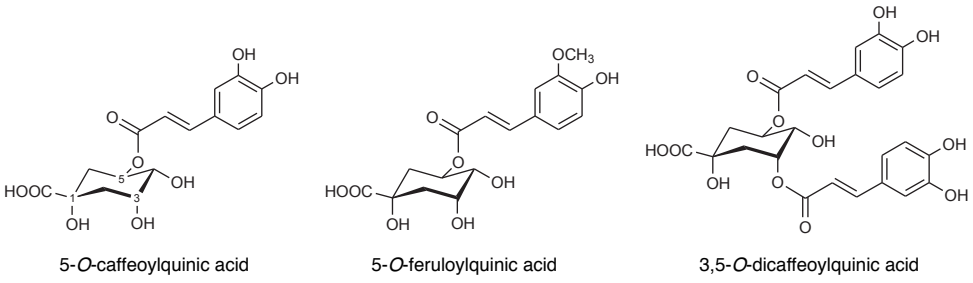

Figure 1. Molecular structure of major representative chlorogenic acids.

Chlorogenic acid, 5-CQA, has been previously isolated from *Flos lonicerae* by CCS, using multiple step isocratic HSCCC (Lu et al., 2004) and from *Lonicera japonica* with a simple pH-gradient CCC (countercurrent chromatography) method (Wang et al., 2008). However, no attempt has been made to either isolate the compounds belonging to the different CGA subfamilies, which are concomitantly present in most sources, or separate their regioisomers. Apart from 5-CQA and cynarine (1,3-dicaffeoylquinic acid) these conjugates and their isomers are not commercially available as reference standards and whenever required, for structure-activity relationship studies for instance, they have to be either isolated by tedious conventional column chromatographic procedures (Nakatani et al., 2000; Parejo et

al., 2004) or obtained in limited amounts through preparative HPLC. Since coffee is the richest and most readily available source of HCQAs an alternatively method based on preparative CPC was developed for their isolation from green coffee beans.

Methods

Reagents

Chlorogenic acid (97%) and all inorganic salts were purchased from Sigma (St. Louis, MO, USA). Solvents, including formic and acetic acid, were purchased from Merk Biosolve (Valkenswaard, The Netherlands). Those used for extractions and CPC were of analytical grade while methanol and acetonitrile were of chromatographic grade. Ultra-pure deionized water was used. Green coffee beans of Ethiopian *Coffea arabica* cv. Tipica were purchased locally (Leiden, The Netherlands).

Apparatuses

Centrifugal partition chromatography experiments were performed with a hydrostatic counter-current chromatograph model HPCPC LLB-M (Sanki Engineering, Kyoto, Japan) equipped with a 4-way ascending-descending-mode switching valve and a rotor of 110 ± 3 mL as total capacity operating at a maximum speed of 2000 rpm. This CPC system was connected to a Knauer 10 mL (Knauer, Berlin, Germany) pump and a Rheodyne (Cotati, CA, USA) manual injector with a 5 mL or 30 mL loop. The eluate was monitored at 330 nm with a CPC UVIS 200 (Sanki Engineering) UV-Vis detector and a Kipp&Zonen BD40 (Kipp&Zonen, Delft, The Netherlands) recorder. HPLC analyses were performed on an Agilent 1200 series system comprising an autosampler, low-pressure mixing pump and a diode array detection (DAD) system.

Green coffee bean extract

The major hydroxycinnamoylquinic acid in green coffee beans is by far 5-CQA, with a relative concentration of around 72% on dry basis. This is followed by diCQAs (dicaffeoylquinic acids) with 17% and 5% FQAs (feruloylquinic acids). Diverse CFQAs (caffeoylferuloylquinic acids) and triCQAs (tricaffeoylquinic acids) account for the remaining 6%. The extraction method described below was developed with the aim of obtaining an extract enriched in the two minor subfamilies of compounds, *i.e.* the FQAs and the diCQAs, ideal for the successive chromatographic steps. The best protocol worked out for this purpose was the following: green beans of Ethiopian *Coffea arabica* tipica var. (250 g) were soaked overnight in acidic water (300 mL, pH 4.0) and then ground and extracted with 80% EtOH (3x500 mL). The resulting extract was taken to dryness under vacuum and the residue was redissolved in 50% MeOH (200 mL) and defatted with hexane (3x200 mL). Methanol was removed by evaporation under vacuum. The remaining water extract was diluted to a volume of 250 mL with sufficient amount of 0.1 M pH 2.0 phosphate buffer and 2.0 M LiCl. This extract was partitioned against EtOAc (2x250 mL) and the organic phases were combined and taken to dryness, yielding 10 g of the crude blend of HCQAs. This residue was subsequently redissolved in hot water (200 mL), washed twice with $CHCl_3$ to remove caffeine and extracted twice with $CHCl_3$/18% BuOH. The resulting extract was

taken to dryness yielding a residue (3.4 g) composed of about 60% in HCQAs, of which, CQAs represented around 41%, FQAs 21% and diCQAs 38%.

Chlorogenic acid isomerisation

Chlorogenic acids can be easily converted into their respective regioisomers by means of a simple base-catalysed intramolecular migration of the cinnamoyl group around the vicinal hydroxyls on the quinic acid moiety (Clifford et al., 1989). Reaction conditions, *i.e.* base nature, base-substrate concentration ratio and time, were optimized using 5-CQA as a general reference for the preparation of a total of nine HCQAs from the coffee extract: 3-CQA (neochlorogenic acid), 4-CQA (cryptochlorogenic acid), 5-CQA, the three analogous feruloyl conjugates and three isochlorogenic acids 3,5-diCQA, 3,4-diCQA and 4,5-diCQA. Independent isomerization of the major HCQAs present in coffee beans, once separated by CPC in a first step, was carried out then by treating an aqueous solution of 5-CQA, 5-FQA or 3,5-diCQA (30 mL, 10 mg mL^{-1}) with sodium hydroxide (15 mL, 1 M) for five seconds while stirring. The mixture was immediately acidified by adding enough 6.0 M HCl and desalted on a polyamide column eluted with water and acidic methanol. Evaporation under reduced pressure of the organic solvent yielded in all cases a nearly equimolar isomeric mixture of the CQAs.

Distribution constants and settling times

Aliquots of 0.5 mg of either extracts or reference compounds were placed in 1.5 mL Eppendorf tubes along with 300 µL of each partitioning phase and vortexed for 60 s. After centrifugation for 30 s at 6000 rpm 200 µL of each layer were transferred to vials, taken to 500 µL with methanol and analysed by HPLC as described below. Settling times were determined by vigorously shaking 2 mL of each phase for 10 s in a 10 mL measuring cylinder and registering the time needed for the system to separate into two clear layers. This was done in triplicate.

CPC procedures, solvents and samples

Biphasic systems were prepared by thoroughly mixing convenient volumes of the different solvents in their nominal ratios and letting them settle overnight. In all CPC runs the aqueous layer was the mobile phase, whose pH was adjusted to 2.5 with either H_3PO_4-Na_2HPO_4 or H_2SO_4-$(NH_4)_2SO_4$ buffering systems. The stationary phase for the gradient procedures was saturated in both cases with a middle concentrated salt solution to minimize composition changes along the gradient. The stationary phase was always loaded first into the column at 100 rpm and then the mobile phase, in the adequate mode (ascending or descending according to density) at 600-1000 rpm at a flow of 1-2 mL min^{-1}. Samples were injected only when the dynamic equilibrium was reached. The volume composition of the biphasic system for the different experiments was: (a) green coffee bean extract LiCl salting-out gradient: ethyl acetate-hexane/0.01 M phosphate buffer-LiCl (68:32:100, v/v) with a stepwise gradient of 5.0 M (60 mL), 2.5 M (40 mL) and 0.1 M (250 mL) LiCl; (b) green coffee bean extract $(NH_4)_2SO_4$-KNO_3 salting-out gradient: ethyl acetate-hexane/$(NH_4)_2SO_4$-KNO_3 (70:30:100, v/v) with a stepwise gradient of 3.0 M (85 mL) and 1.5 M (105 mL) $(NH_4)_2SO_4$, and a final step of 1.5 M (260 mL) KNO_3; (c) scale-up of green coffee bean extract $(NH_4)_2SO_4$-KNO_3 salting-out gradient: ethyl acetate-hexane/$(NH_4)_2SO_4$-KNO_3 (70:30:100, v/v) with a stepwise gradient of 3.0 M (110 mL) and 1.5 M (170 mL) $(NH_4)_2SO_4$, and a final

step of 1.5 M (270 mL) KNO_3; (d) CQA regioisomers: chloroform-*n*-butanol/0.01 M phosphate buffer-5.0 M LiCl (82:18:100, v/v); (e) FQA regioisomers: chloroform-*n*-butanol/0.01 M phosphate buffer (84:16:100, v/v); (f) diCQA regioisomers: chloroform-*n*-butanol/0.01 M phosphate buffer (84:16:100, v/v). Injection volume was 5 mL in all cases except for the scale-up run (30 mL). In order to maximize solubility and yield in most cases samples were prepared by adding suitable volumes of the biphasic systems and vortexed or heated up to 60 °C, if necessary. The stationary-like phases of the resulting limpid or cloudy samples were injected firstly, followed by the aqueous ones. In all runs fractions were collected manually on a peak elution basis.

HPLC analyses

Fractions and standards were analysed by HPLC using a ODS-Luna 150x4.6 mm 3 µm column (Phenomenex, Torrance, CA, USA) and eluted at 0.7 mL min^{-1} flow with a gradient of water-0.5% formic acid (solvent A) and methanol-0.5% formic acid (solvent B) according to the following gradient program (v/v): 0 min 25% B, 40 min 50% B linear, 43 min 60% B linear and 45 min 25% B linear, followed by 10 min for reequilibration. With the exception of 5-CQA all HCQAs were identified by means of their relative elution pattern and DAD spectrum analysis as consistently found in the literature (Clifford et al., 2003; Farah et al., 2005; Guerrero and Suarez, 2001), while quantification was achieved using a 5-CQA calibration curve in combination with the relative molar absorptivity coefficients reported for HCQAs (Trugo and Macrae, 1984).

Results and discussion

In an octanol-water system the separation factor (α) between 5-CQA and 3,5-diCQA, respectively the most and the least polar molecules in the targeted group of coffee HCQAs, was above 30. This implied that their separation evidently required either gradient, extrusion or dual-mode elution. In an effort to preserve the same column for possible multiple injections while keeping the maximum efficiency conditions and avoid the phase redistribution that takes place in dual-modes, an electrostriction gradient was chosen instead.

The main criterion for salt choice in these gradients is the salt overall electrostrictive or salting-out capacity. Even though salting effects are basic principles ubiquitous in chemistry and biology, the mechanism of their action has not been fully explained yet, relying on several different theories for their full description (Grover and Ryall, 2004), out of which the ones based on ionic hydration shells, while unsatisfactory, are the most widely accepted.

Figure 2 shows the electrostrictive power of some effective salting-out electrolytes on the distribution constant of 5-CQA in an ethyl acetate-$salt_{(aq,\ pH\ 2.5)}$ biphasic system. The relative salting-out power observed for the different salts is very much in agreement with the Hofmeister series for cations and anions usually reported as: $F^- > PO_4^{3-} > SO_4^{2-} > AcO^- > Cl^- > NO_3^- > I^- > CNS^-$ and $Mg^{2+} > Ca^{2+} > Ba^{2+} > Li^+ > Na^+ > K^+ > NH_4^+ > Cs^+$ (Cacace et al., 1997; Napper and Netschey, 1971; Zhang and Cremer, 2006), and directly correlated with the ion hydration enthalpies. As predicted by these series ions with the greatest charge density, e.g. HPO_4^{2-}, SO_4^{2-} and Li^+, proved to be the most electrostrictive, with KNO_3 on the lower limit in the set showing a slight salting-in effect.

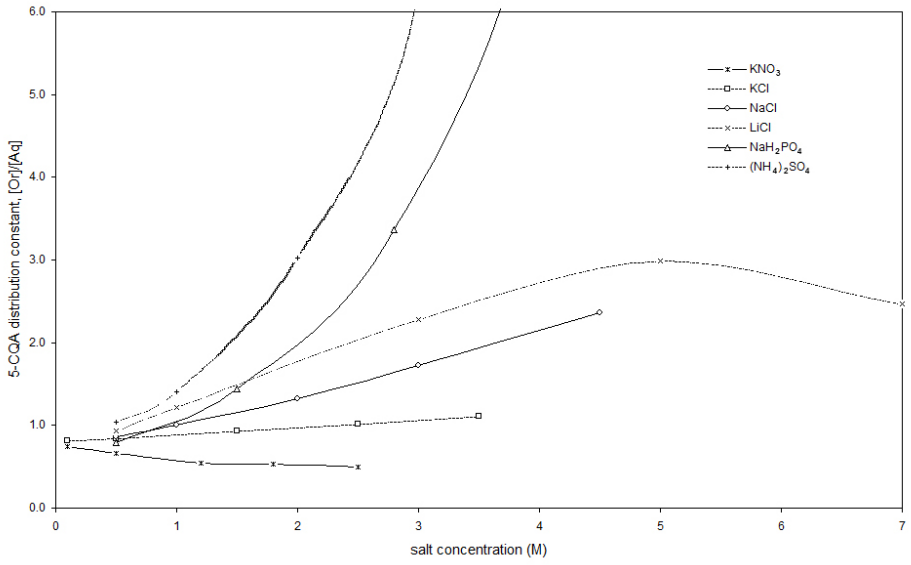

Figure 2. Salting effect of selected salts on chlorogenic acid, 5-CQA, in an ethyl acetate-aqueous salt solution (pH 2.5) biphasic system. Distribution constants are defined as the concentration ratio between the most lipophilic phase and the most hydrophilic one. Maximum salt concentrations are determined by their solubility limits.

Most likely due to metal complex-derived salting-in effects on the catechol group (Ghasemi and Shamsipur, 1995; Lapouge and Cornard, 2007) some tightly hydrated and hence supposedly good kosmotropic cations, such as Mg^{2+}, Ca^{2+} and Al^{3+}, were not effective salting-out agents for 5-CQA. Similar phenomena may explain the inverted effect observed for Li^{+} at extremely high salt concentrations (Fig. 2).

Lithium chloride -the lightest among the most electrostrictive salts- was chosen for the solvent system optimization and resolution studies. It is important to note that the selection of solvent systems for this group of compounds was particularly laborious due to their peculiar solubility behaviour, ranging from the affinity of CQAs with very polar organic solvents such as methanol to more lipophilic ones such as methyl tert-butyl ether for diCQAs, making it almost impossible to devise single stable biphasic systems suitable for all classes of HCQAs without compromising yield. The distribution constants for all isomers in the three most efficient candidate solvent systems found for HCQAs are summarized in Table 1. In the disk-type HPCPC rotor used in this case minimally satisfactory resolutions were obtained at optimal working conditions only for compounds with an experimental value of $\alpha \geq 2$, which unfortunately could not be achieved by any of the biphasic combinations for all 10 phenolics, including caffeic acid. The chloroform-*n*-butanol system for instance provided good α values between the regioisomers of all subfamilies (CQAs, FQAs and diCQAs) but not between 5-FQA and 3,5-diCQA, thus ruling out its application in the gradient experiments. A similar situation occurred in the methyl tert-butyl ether system for 5-CQA and 4-FQA. As an additional drawback in this system the polarity differences between CQAs and diCQAs are accentuated, as evidenced by an average $\alpha > 55$, exceeding then the electrostriction capacity of LiCl to afford practical distribution constants ($0.4 < D < 3$) for most isomers. Surprisingly, the ethyl acetate-hexane system, while apparently the least discriminant

(no satisfactory α were obtained for the regioisomers of either subfamily), provided convenient values for the main representatives, that is 5-CQA, 5-FQA and 3,5-diCQA. At this point a two-step purification approach was taken for the HCQAs, separating 5-CQA, 5-FQA and 3,5-diCQA-4,5-diCQA from the green coffee bean extract in a gradient run with the ethyl acetate-hexane system in a first stage and secondly using independent CPC experiments to purify the regioisomers, obtained by isomerization, with suitable chloroform-butanol mixtures.

Table 1. Salting-out effect of lithium chloride on chlorogenic acids in different solvent systems.

		3-CQA	5-CQA	4-CQA	CA	5-FQA	4-FQA	3,4-diCQA	3,5-diCQA	4,5-diCQA
[LiCl] (M)	***St* (s)**	**Distribution constant (*D*)**								
Chloroform/*n*-butanol (82:18)										
0.0	14	0.01	0.12	0.06	2.92	1.77	0.74	0.54	1.53	5.49
0.1		0.01	0.13	0.06	2.94	1.83	0.76	0.53	1.52	5.63
0.5	15	0.02	0.14	0.07	3.60	2.25	0.94	0.71	2.08	7.57
2.0		0.03	0.30	0.15	7.19	4.57	2.01	2.35	7.03	22.83
5.0	18	0.09	0.87	0.43	27.63	12.92	5.78	18.37	>>	>>
7.0		0.12	1.03	0.49	41.60	13.78	6.00	24.82	>>	>>
Methyl tert-butyl ether										
0.0	10	0.02	0.12	0.05	12.15	0.21	0.11	2.44	6.81	11.83
0.1		0.02	0.12	0.06	13.77	0.23	0.12	2.59	7.51	13.52
0.5	8	0.03	0.18	0.09	17.41	0.34	0.18	4.18	10.76	20.24
2.0		0.04	0.27	0.13	32.51	0.58	0.29	10.19	27.79	47.46
5.0	6	0.07	0.46	0.23	>>	1.19	0.58	36.20	>>	>>
7.0		0.06	0.35	0.16	>>	1.02	0.47	37.12	>>	>>
Ethyl acetate-hexane (70:30)										
0.0	17	0.01	0.04	0.02	3.15	0.15	0.09	0.41	1.27	1.76
0.1		0.01	0.03	0.02	3.03	0.15	0.08	0.38	1.29	1.80
0.5	15	0.01	0.05	0.03	4.55	0.22	0.13	0.67	2.14	3.09
2.0		0.02	0.10	0.06	8.93	0.44	0.26	2.16	7.04	10.20
5.0	11	0.03	0.22	0.13	24.27	1.14	0.64	12.78	>>	>>
7.0		0.03	0.16	0.09	33.04	0.91	0.50	10.57	>>	>>

St, settling time; CQA, caffeoylquinic acid; CA, caffeic acid; FQA, caffeoylferulic acid; diCQA, dicaffeoylquinic acid. >>= *D* above 100

Using the salty aqueous layer as a mobile phase in descending mode, the green coffee bean extract rich in HCQAs was successfully fractionated into the major subfamilies of HCQAs by means of a LiCl salting-out gradient (Fig. 3). The stepwise variation included three salt concentrations, 5.0, 2.5, and 0.1 M, applied in decreasing electrostrictive order, emulating thus a conventional reversed-phase gradient, aiming at the respective elution of the three major subclasses, CQAs, FQAs and diCQAs. The length of each step, as indicated by the dashed horizontal lines in the figures, was arbitrary, depending only on the elution of the major part of each peak. The maximum amount

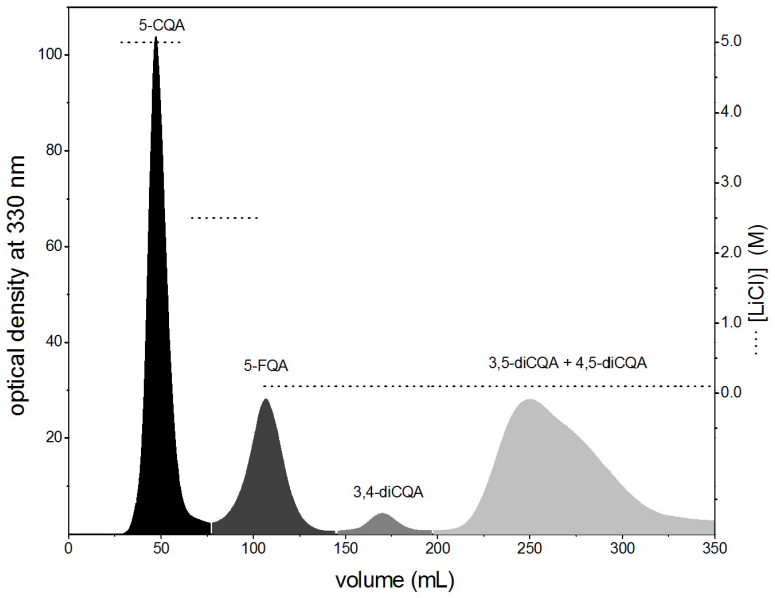

Figure 3. CPC separation of the major chlorogenic acids present in green coffee beans with a LiCl salting-out gradient. Experimental conditions: rotation speed: 800 rpm; solvent system: ethyl acetate-hexane/0.01 M pH 2.5 phosphate buffer-LiCl (68:32:100; v/v); salting-out gradient: 5.0 M (60 mL), 2.5 M (40 mL) and 0.1 M (250 mL) LiCl; mobile phase: lower aqueous phase; flow rate: 2.4 mL min^{-1}; retention of stationary phase: 76%; injection volume: 5 mL; back pressure: 50 bar (5.0 M LiCl), 40 bar (2.5 M LiCl) and 30 bar (0.1 M LiCl); sample: 50 mg CGA-enriched green coffee bean extract dissolved in 2.5 mL of mobile phase and 2.5 mL of stationary phase.

of extract that could apparently be dissolved in a 5 mL portion of a 1:1 biphasic system was 50 mg, yielding 10.3 mg of CQAs (88% recovery of the total content in 50 mg of extract), 5.1 mg of FQAs (85% recovery), 0.7 mg of 3,4-diCQA (70% recovery) and 5.8 mg of a 3,5-diCQA-4,5-diCQA mixture (53% recovery). The low recoveries registered for the least hydrophilic compounds reveal a clear solubility preference of this solvent system towards the more hydrophilic molecules and a general loading limitation common to most gradient chromatographic techniques.

A second gradient run was designed for ammonium sulphate, $(NH_4)_2SO_4$), as a more electrostrictive, less expensive and more environmentally friendly salt. This salt however is much heavier and hence prone to bring about significant changes in the settling times of the biphasic system along the gradient. The most suitable concentrations of $(NH_4)_2SO_4$ for a similar set of distribution constants compared to LiCl were 3.0, 1.5 and 0.1 M, but unfortunately the settling time net variation was around 10 s, enough to cause undesirable stationary phase bleeding. To overcome this difficulty another common application of salts in CCS was used: non electrostrictive salts such as KNO_3 can be introduced to stabilize the biphasic system as the $(NH_4)_2SO_4$ concentration is reduced. Based on the settling times determined for several KNO_3 concentrations (data not presented) the last step of this $(NH_4)_2SO_4$ gradient was replaced by a KNO_3 1.5 M solution. The result of this multistep and multisalt method (Fig. 4) was a separation with a resolution comparable to that obtained with LiCl (Fig. 3). With this system same amount of extract yielded 9.5 mg of CQAs (81% recovery), 3.6 mg of FQAs (60% recovery), 0.8 mg of 3,4-diCQA (80% recovery) and 6.7 mg of 3,5-4,5-diCQA (61% recovery). Observed differences in recoveries when compared to the LiCl experiment were again

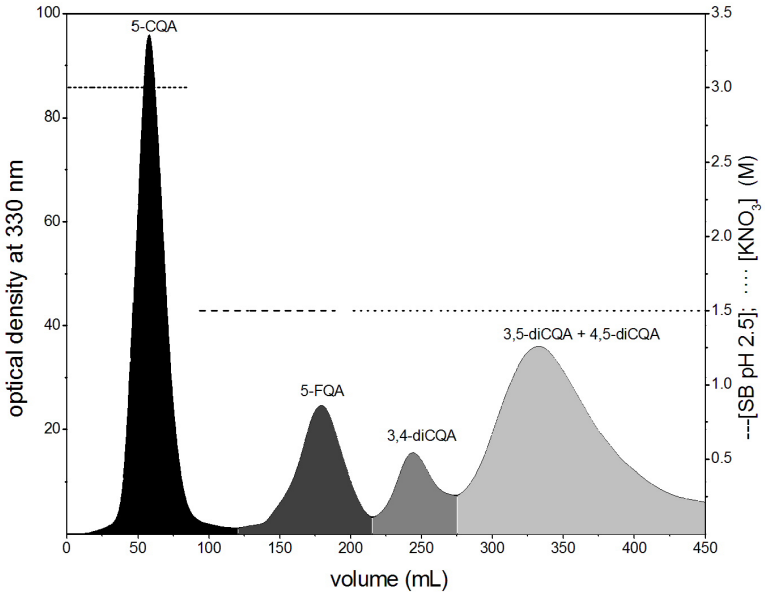

Figure 4. CPC separation of the major chlorogenic acids present in green coffee beans with a $(NH_4)_2SO_4$-KNO_3 salting-out gradient. Experimental conditions: rotation speed: 600 rpm; solvent system: ethyl acetate-hexane/pH 2.5 $(NH_4)_2SO_4$ (70:30:100; v/v); salting-out gradient: 3.0 M (85 mL) and 1.5 M (105 mL) $(NH_4)_2SO_4$, and 1.5 M (260 mL) KNO_3; mobile phase: lower aqueous phase; flow rate: 2.0 mL min^{-1}; retention of stationary phase: 80%; injection volume: 5 mL; back pressure: 22 bar (3.0 M $(NH_4)_2SO_4$), 18 bar (1.5 M $(NH_4)_2SO_4$) and 16 bar (1.5 M KNO_3); sample: 50 mg CGA-enriched green coffee bean extract dissolved in 2.5 mL of mobile phase and 2.5 mL of stationary phase.

most likely a result of preferential solubilisation.

As mentioned before, CCS have the advantage, in contrast to HPLC, of being able to count on the liquid stationary phase to deal with a major solubility drawback, partly dissolving complex samples in as much stationary phase as the retained column volume allows. An attempt in this direction was made using a second $(NH_4)_2SO_4$-KNO_3 gradient, injecting in this case 250 mg of green coffee bean extract dissolved in 12.5 mL of mobile phase and 12.5 mL of stationary phase. In spite of the five-time scale-up, the separation obtained was as satisfactory as that obtained with the previous system as can be appreciated in the CPC chromatogram in Figure 5. It is important to note that in this case the second step ($(NH_4)_2SO_4$ 1.5 M) was extended until all FQAs eluted, achieving in this way a remarkable increase in the resolution between the FQAs and 3,4-diCQA peaks. The 250 mg extract sample yielded in this case 46.7 mg of CQAs (80% recovery), 17.6 mg of FQAs (59% recovery), 3.7 mg of 3,4-diCQA (74% recovery) and 28.3 mg of 3,5-4,5-diCQA (52% recovery), for an average yield increase of 4.5 times.

The best solvent system for the separation of regioisomer of all subfamilies was chloroform-*n*-butanol based. Unfortunately the salt-free composition polar enough to give distribution constants around 1 for CQA regioisomers (chloroform-*n*-butanol, 82:12; v/v) was relatively unstable (settling time: 27 s) and hence inefficient. In consequence a chloroform-*n*-butanol/0.01 M phosphate buffer-5.0 M LiCl system was implemented in order to achieve a good purification of CQA isomers (Fig. 6). A sample of 70 mg of isomerized mixture dissolved in 1.0 mL of mobile phase was injected into the column, yielding 19.6 mg of neochlorogenic acid (3-CQA), 20.3 mg of cryptochlorogenic acid (4-CQA) and 25.0 mg of chlorogenic acid (5-CQA), all over 95% pure. Fractions containing the pure compounds were desalted by loading them on a polyamide column, which was washed with deionized water to

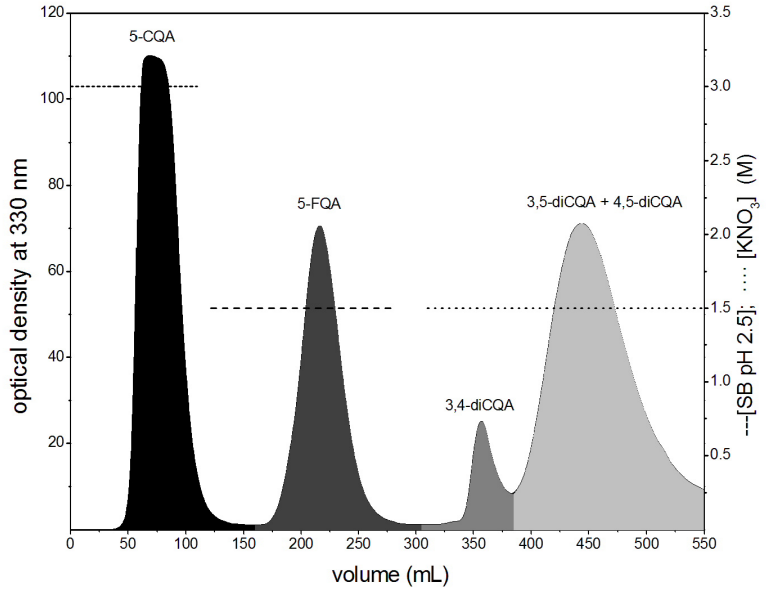

Figure 5. CPC scale-up separation of the major chlorogenic acids present in green coffee beans with a $(NH_4)_2SO_4$-KNO_3 salting-out gradient. Experimental conditions: rotation speed: 600 rpm; solvent system: ethyl acetate-hexane/pH 2.5 $(NH_4)_2SO_4$ (70:30:100; v/v); salting-out gradient: 3.0 M (110 mL) and 1.5 M (170 mL) $(NH_4)_2SO_4$, and 1.5 M (270 mL) KNO_3; mobile phase: lower aqueous phase; flow rate: 2.0 mL min^{-1}; retention of stationary phase: 82%; injection volume: 30 mL; back pressure: 22 bar (3.0 M $(NH_4)_2SO_4$), 18 bar (1.5 M $(NH_4)_2SO_4$) and 16 bar (1.5 M KNO_3); sample: 250 mg CGA-enriched green coffee bean extract dissolved in 12.5 mL of mobile phase and 12.5 mL of stationary phase.

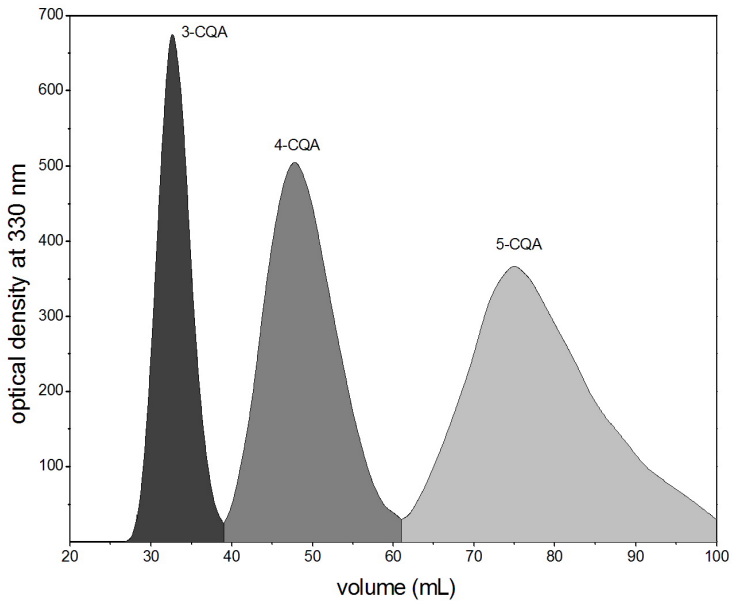

Figure 6. CPC separation of the regioisomers of chlorogenic acid (CQA). Experimental conditions: rotation speed: 1000 rpm; solvent system: chloroform-*n*-butanol/0.01 M pH 2.5 phosphate buffer-5.0 M LiCl (82:18:100; v/v); mobile phase: lower aqueous phase; flow rate: 1.0 mL min^{-1}; retention of stationary phase: 73%; injection volume: 5 mL; back pressure: 42 bar; sample: 70 mg of a mixture of chlorogenic acid regioisomers dissolved in 1.0 mL of mobile phase.

remove the salts after which pure compounds were eluted with acidic methanol.
Regioisomers of the FQA and diCQA subfamilies were submitted to CPC using a chloroform-*n*-butanol/0.01 M phosphate buffer (84:16:100; v/v) solvent system. A 33 mg FQAs sample yielded 11.4 mg of 3-FQA (83% pure, with an impurity consisting mainly of CQAs) (Fig. 7), 9.3 mg of 4-FQA (92% pure, contaminated mainly with an unidentified flavonoid also present in the 3-FQA fraction) and 8.4 mg of 5-FQA (96% pure).

Separation of isochlorogenic acids (diCQAs) was less satisfactory as can be observed in the CPC chromatogram in Figure 8. Resolution between 3,4-diCQA and 3,5-diCQA was low and HPLC analysis of the 3,4-diCQA peak (10.2 mg) showed only 63% of purity, co-eluted with CQAs (resulting from the partial hydrolysis of diCQAs during the isomerization procedure) and 3,5-diCQA.

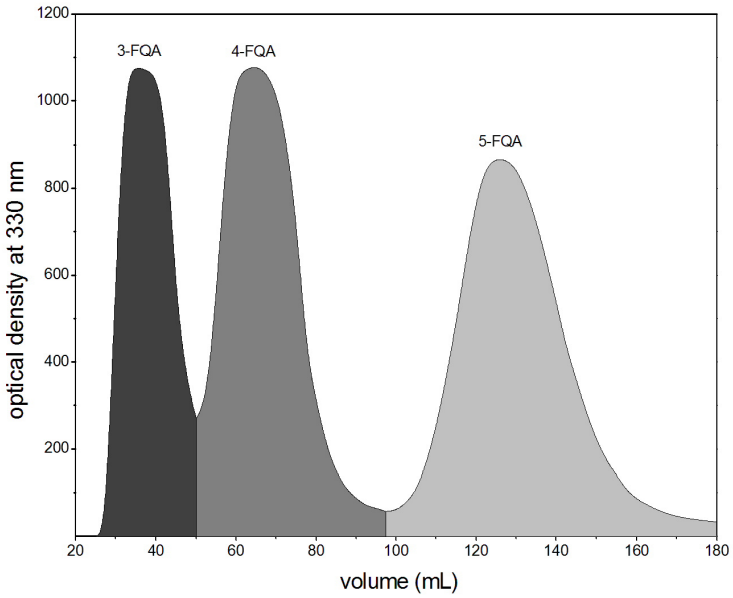

Figure 7. CPC separation of the regioisomers of feruloyquinic acid (FQA). Experimental conditions: rotation speed: 900 rpm; solvent system: chloroform-*n*-butanol/0.01 M pH 2.5 phosphate buffer (84:16:100; v/v); mobile phase: lower aqueous phase; flow rate: 1.0 mL min^{-1}; retention of stationary phase: 80%; injection volume: 5 mL; back pressure: 39 bar; sample: 33 mg of a mixture of feruloylquinic acid regioisomers dissolved in 2.0 mL of mobile phase and 2.0 mL of stationary phase.

The latter was obtained with a 76% of purity (15.4 mg) and 4,5-diCQA (4.3 mg, 43% pure) contaminated with caffeic acid (6.8 mg), the main hydrolysis product. The purity of all compounds could have been increased in detriment of yield by collecting partial chromatographic regions instead of the entire eluting peaks.

With the separation of CQAs, FQAs and diCQAs from green coffee beans it has been proven that the manipulation of electrostriction in an aqueous solution by changing the concentration of one or more salts can constitute an effective method of performing a reversed-phase-like gradient elution in CCS. A major contribution of these salting-out gradients lies on the fast single-column elution of compounds largely differing on their distribution constants without experiencing significant column bleeding. Although this achievement is also possible with a conventional normal-phase gradient in CCC, very few biphasic systems meet the conditions to actually do it. In addition, notwithstanding the fact that this proposed method may suffer from some impracticability for routine application

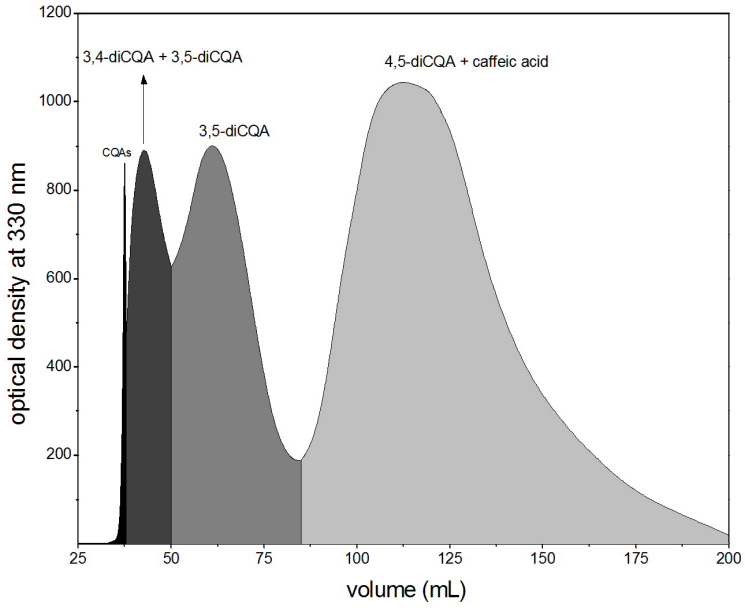

Figure 8. CPC separation of the regioisomers of isochlorogenic acid (diCQA). Experimental conditions: rotation speed: 900 rpm; solvent system: chloroform-*n*-butanol/0.01 M pH 2.5 phosphate buffer (84:16:100; v/v); mobile phase: lower aqueous phase; flow rate: 1.0 mL min^{-1}; retention of stationary phase: 80%; injection volume: 5 mL; back pressure: 42 bar; sample: 38 mg of a mixture of isochlorogenic acid regioisomers dissolved in 1.5 mL of mobile phase and 3.0 mL of stationary phase.

when compared to other powerful ones like the dual (Agnely and Thiebaut, 1997; Delannay et al., 2006), the elution-extrusion (Berthod, 2007; 2003; Lu et al., 2008) and the cocurrent (Berthod and Hassoun, 2006) modes, these ionic gradients represent a proof and a reminder of how salting effects could open new possibilities in poor resolution cases limited by solvent system stability.

Chapter 6

Effect of chlorogenic acid on thrips performance in artificial diet bioassays

Manuscript in preparation

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Abstract

With the separation of CQAs, FQAs and diCQAs from green coffee beans it has been proven that the manipulation of electrostriction in an aqueous solution by changing the concentration of one or more salts can constitute an effective method of performing a reversed-phase-like gradient elution in CCS. A major contribution of these salting-out gradients lies on the fast single-column elution of compounds largely differing on their distribution constants without experiencing significant column bleeding. Although this achievement is also possible with a conventional normal-phase gradient in CCC, very few biphasic systems meet the conditions to actually do it. In addition, notwithstanding the fact that this proposed method may suffer from some impracticability for routine application when compared to other powerful ones like the dual (Agnely and Thiebaut, 1997; Delannay et al., 2006), the elution-extrusion (Berthod, 2007; 2003; Lu et al., 2008) and the cocurrent (Berthod and Hassoun, 2006) modes, these ionic gradients represent a proof and a reminder of how salting effects could open new possibilities in poor resolution cases limited by solvent system stability.

Introduction

Phenolics are a very diverse and prominent group of secondary metabolites spread throughout the entire plant kingdom. They play various key roles in plants, behaving as antioxidants, building blocks of secondary cell walls, allelochemicals, UV-protectants, antimicrobials, signal molecules and insect defenses (Treutter, 2006). Many plant scientists have unquestionably accepted the defensive role of phenolics, to such extent that these compounds are by default a variable in almost every single study on fitness costs of plant defense and represent a pillar in the resource availability theory (Coley et al., 1985). However, still after several decades of intense research no consensus about their efficacy in providing protection against attackers, in particular against herbivores, has been reached. Part of this controversy originates in the fact that phenolics have multiple modes of action, which can have positive and negative effects on pathogens and insects. The following have been proposed as some of the main modes of action of phenolics: radical scavenging, pro-oxidation, covalent and hydrogen bonding to proteins and free amino acids, enzyme inhibition and crosslinking, chelation of enzyme metal cofactors and wound sealing (Fig. 1). Due to these chemical properties phenolics have been shown to act as feeding deterrents, phagostimulants, digestion inhibitors, digestion stimulants, toxins, toxicity reducers, signal inhibitors and signal transducers (Appel, 1993; Crozier et al., 2009). In addition, their activity is also dose-dependent, structure-sensitive and organism-specific.

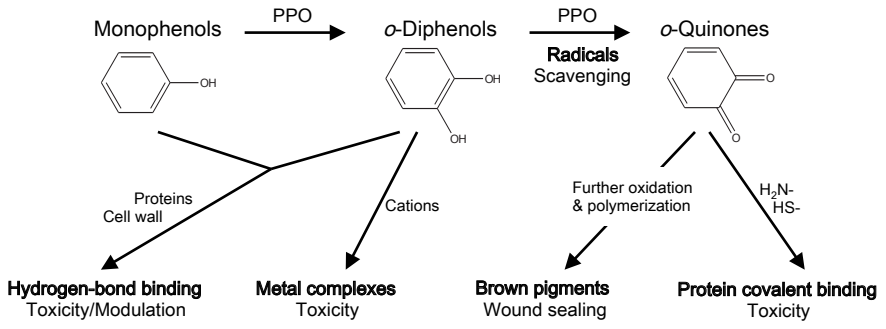

Figure 1.Main modes of action of simple phenolics. PPO: oxidase.

Many studies claiming adverse effects of phenolics on herbivores rely on correlations between insect performance and any phenolic content as the single factor responsible for the observed variation in the dependent variable (Bi et al., 1997a; Felton, 1989; Ikonen et al., 2001; Isman and Duffey, 1982a; Johnson and Felton, 2001). These studies overlook in this way covariables that could have a stronger and hence overriding influence on the performance of the insect. Moreover, correlation analysis does not prove causation, requiring additional evidence to invoke a cause-effect relationship. Results from other studies in which the number of factors has been minimized, e.g. artificial diet bioassays, were inconsistent or contradictory. Most research on the role of phenolics in host-plant resistance has been conducted on chewing insects. Along the course of more than a decade researchers collected partial evidence implicating phenolic compounds in host-plant resistance against caterpillars. A lot of these studies focused on chlorogenic acid (5-caffeoylquinic acid, CQA) as the most common and abundant representative compound among simple phenolics in plants. Isman and Duffey (1982a) observed that semi-purified extracts of phenolics from tomato leaves inhibited growth of the fruitworm, *Heliothis zea*, but could not detect a correlation between foliar phenolic content and larval growth among several tomato cultivars. Authors underlined the potential danger of comparing biological activities from artificial diet bioassays and *in situ* experiments. Rutin and chlorogenic acid inhibited early larval growth of *H. zea* but had no adverse effect on third and fifth-instar larvae at concentrations of up to 1% in artificial diets (Isman and Duffey, 1982b). Felton et al. (1989) showed that polyphenol oxidase (PPO) was required for CQA to exhibit anti-insect activity against *Spodoptera exigua*. Performance of larval *H. zea* barely correlated with PPO activity (R=0.55) but did not correlate with the content of either rutin or chlorogenic acid. Polyphenol oxidase catalyzes the conversion of *o*-diphenols into *o*-quinones (Fig. 1). These quinones are very reactive electrophiles and oxidizing agents that can covalently bond more than one amino or thio group, causing protein cross-linkage and indigestibility (Kalyanaraman et al., 1987). In a later study using proteins from different sources Felton et al. (1992) not only confirmed the enzymatic requisite but also observed that CQA had a beneficial impact on the performance of *S. exigua* in the absence of PPO. Although CQA induced oxidative stress in midgut tissue of the generalist *Helicoverpa zea* no deleterious effect was observed on the performance of larvae acutely exposed to this phenolic. Chronic exposure to CQA did cause a significant reduction of *H. zea* growth but had no effect on survival (Summers and Felton, 1994). Differences in activity were observed among different phenolic compounds but no explanations were offered. Using transgenic tobacco with significant differential expression of both CQA and PPO Bi et al. (1997a) showed that phenolics are not involved in host-plant resistance to either the generalist *Heliothis virescens* or the specialist *Manduca sexta*. In a choice setup *M. sexta* did not discriminate between tobacco leaves with a differential CQA content of more than ten times (Eichenseer et al., 1998). Likewise, neither phenolic content, including CQA, nor PPO activity correlated with resistance to the coffee leaf miner, *Leucoptera coffeella*, across 15 genotypes of coffee (Melo et al., 2006). In a study involving 13 Finnish willow species and four willow-feeding beetles only one of the coleopteran species was apparently affected by CQA (Ikonen et al., 2001). Measuring the total Trolox equivalent antioxidant capacity in hemolymph of *H. virescens* larvae fed foliage with high phenolic content Johnson and Felton (2001) showed that phenolics may actually serve as antioxidants for herbivorous insects. Although these studies constitute only a small fraction of the vast literature on phenolics the sample shows how in the course of about a decade the image of these metabolites could diametrically change from "anti-herbivore" to "insect-beneficial", proving how complex and debatable still is the manifold role of phenolic compounds in plant-insect interactions.

In contrast to chewing insects very few experiments have been performed on sucking herbivores. In artificial diet bioassays chlorogenic acid significantly deterred the apple aphid, *Aphis pomi*, only in combination with ascorbic acid (Miles and Oertli, 1993), while catechin significantly reduced feeding of the rose aphid, *Macrosiphum rosae* (Peng and Miles, 1991), and the spotted alfalfa aphid, *Theoriaphis trifolii maculata* (Miles and Oertli, 1993). However, phenolics were phagostimulant to aphids at very low concentrations (Peng and Miles, 1991). Aphids polymerize phenolic compounds with salivary polyphenol oxidase to strengthen the stylet and facilitate feeding (Miles and Peng, 1989; Peng and Miles, 1988a; Peng and Miles, 1988b). A similar dual behavior was observed for quercetin and chlorogenic acid against the redlegged earth mite, *Halotydeus destructor* (Ridsdill-Smith et al., 1995).

Using an artificial diet setup developed to monitor thrips larvae (de Jager et al., 1996) we intended hereby to assess the impact of phenolic compounds in general and CQA in particular on the performance of one of the most economically important pests nowadays, the western flower thrips, *Frankliniella occidentalis* (Pergande; Thysanoptera: Thripidae). Isomers of CQA were tested as well to explore the potential influence of structural features on the activity of CQA against larvae of this piercing-sucking generalist herbivore.

Methods

Reagents

All reagents were of analytical grade. Mushroom tyrosinase (polyphenol oxidase, PPO), casein, Vanderzant vitamin mixture, chlorogenic acid (97%), linoleic acid, sucrose, sodium citrate and hydrochloric acid were purchased from Sigma (St. Louis, MO, USA). Cholesterol, casein hydrolysate, potassium phosphate salts and pH-indicator strips were purchased from Merck KGaA (Darmstadt, Germany). Potassium carbonate salts were purchased from J.T.Baker (Deventer, Netherlands) and Wesson's salt was purchased from ICN Biochemicals (Cleveland, USA). Regioisomers of chlorogenic acid were obtained through base-catalyzed isomerization of chlorogenic acid followed by centrifugal partition chromatography as described in chapter 5. Ultra-pure deionized water was used to prepare all solutions. A Hanna PH20 pH-meter (Hanna Instruments, Ann Arbor, Michigan, USA) was used to measure the pH of all buffers.

Artificial diet

The thrips diet was based on a general dietary mixture for insects formulated by Singh (1983). We adapted the original formula to obtain a liquid diet on which piercing-sucking insects, like WFT, could feed (Table 1). All ingredients were mixed with a specific volume of either ultra-pure deionized water or buffer solution. The mixture was vortexed (1 min), sonicated (5 min, 20 °C) and centrifuged to remove remaining insoluble residues. A solution of chlorogenic acid (5-caffeoylquinic acid, 5-CQA) was added at this point to obtain a concentration of 1%. The solubility of 5-CQA in water is ca. 2% at 20 °C. However, amorphous crystals of 5-CQA do not disintegrate easily at room temperature and the solution must be heated up to 70 °C to achieve complete dissolution. The concentration of PPO used was 450 units mL^{-1}. Fresh diets were prepared right before each bioassay and their final pH values were measured with pH-indicator strips of 0.5 units of precision.

Table 1. Composition of the artificial diet for larval thrips based on the generalist insect formula by Singh (1983).

Ingredient		Concentration (%)
Casein		3.50
Cholesterol		0.05
Linoleic acid		0.25
Sucrose		3.00
Vanderzant vitamins		2.00
α-Tocopherol	2.26	
Ascorbic Acid	76.43	
Biotin	0.01	
Calcium Pantothenate	0.28	
Choline Chloride	14.15	
Folic Acid	0.07	
Inositol	5.66	
Vitamin B3 amide	0.28	
Vitamin B6 HCl	0.07	
Vitamin B2	0.14	
Vitamin B1 HCl	0.07	
Vitamin B12	0.57	
Wesson's salt		1.00

Deionized water was used as solvent. Mixture was vortexed, sonicated and centrifuged

Insects and bioassay

Thrips larvae were reared on fresh Italian string beans, *Phaseolus vulgaris*, in a climate room (12/12 hr photoperiod, 20 °C). Adults of a virus-free WFT biotype, reared for several months on chrysanthemum flowers, were let to feed and oviposit on the beans for 24 hrs. After 5-6 days the larvae emerged. For the artificial diet bioassay first-instar larvae were transferred to special growth plates made of clear plastic. These plates consisted of three detachable pieces. The first piece had a cylindrical well (1mm, 4 mm i.d.) where the liquid dietary treatment was poured. The well was covered each time with maximally stretched parafilm, through which thrips can feed (Teulon, 1992). The second block had a cylindrical orifice (3 mm, 6 mm i.d.) that constituted the chamber for the larva. A 3 mm-thick lid was used as the top layer. All pieces were tightly kept together with strong clips. A total of 30 replicates were prepared for each treatment. Larvae were let to feed for 72 hrs in the climate room. At the beginning and at the end of the bioassay pictures of each larva were taken under an Optika SZM-45B2 stereomicroscope (Optica Microscopes, Ponteranica, Italy) equipped with an Optikam 3 digital camera. Initial and final larval lengths were measured with the aid of the public-domain software ImageJ 1.44 (Rasband, 1997) and growth rates were calculated. Mortality was also recorded.

Statistical analysis

The significance of differences in growth rates between treatments was investigated with ANOVA followed by Duncan's multiple range test, whereas mortality rates were analyzed with Fisher exact tests. ANOVA was performed using SPSS v. 17.0 (SPSS Inc., Chicago, IL, USA) while the Fisher tests were conducted through the online service SISA (Uitenbroek, 1997).

Results and discussion

Chlorogenic acid had a clear negative effect on the performance of thrips larvae when tested at a concentration of 1% in the artificial diet (Fig. 2). The addition of 5-CQA to the liquid dietary solution significantly reduced larval growth (F=8.0, df=3, p=0.0001) and increased mortality compared to the plain diet and to the polyphenol oxidase (PPO) used as controls. The presence of PPO did not improve the deleterious effect of 5-CQA on larval thrips, suggesting that its mode of action does not involve the formation of reactive quinones or other reactive oxygen species. However, at the concentration of 1% 5-CQA caused a decrease of more than 2 units on the pH of the dietary solution, from 5.5 to 3.0. This raised the question of a possible influence of pH on the observed negative effect. Such pH changes may be sufficient to disrupt the digestion of thrips and significantly reduce nutrient bioavailability. Thus a buffered artificial diet was introduced.

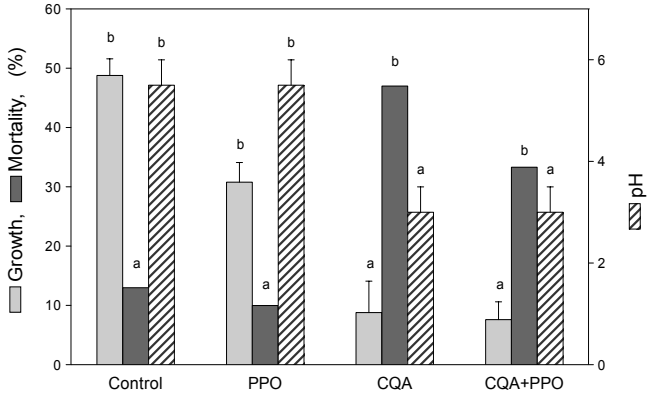

Figure 2. Effect of chlorogenic acid on the performance of first-instar larvae of *Frankliniella occidentalis* in un-buffered artificial diet bioassays. The concentration of chlorogenic acid and polyphenol oxidase (PPO) were 1% and 450 units mL^{-1} respectively. Letters refer to significant differences at the 0.05 level as analyzed with ANOVA.

The ideal buffer concentration had to be high enough to maintain the pH of the diet after the addition of 1% 5-CQA but could not exceed the electrolyte tolerance limit of thrips larvae. To determine this threshold value we tested a series of diets with increasing concentrations of potassium phosphate buffer pH 7.0 in the range of 25-900 mM. Buffer toxicity to larval thrips was detected above 200 mM (Fig. 3). A sharp decline in growth and a concomitant increase in mortality were observed between 200 and 500 mM, beyond which insect survival was zero. Therefore, the buffer concentration for the following bioassays was cautiously set at 150 mM.

When chlorogenic acid was retested in a pH-controlled diet, with all other variables remaining unchanged, its previously observed anti-insect effect was abolished (Fig. 4). No significant differences were detected in neither growth nor mortality of thrips compared to control groups. In this experiment the final pH values of the CQA-containing diet was 6.0, only one unit more acidic than the controls. These results indicate that the observed underperformance of thrips larvae was connected to the strong acidification of the diet and not to the reactive *o*-diphenol moiety on CQA. Most digestive enzymes have been selected to work optimally in a relatively narrow pH range. Out of this range enzyme denaturation or inactivation takes place, precluding the insect from extracting nutrients out

of the food source. Increased vacuolar accumulation of organic acids, including CQA, in otherwise susceptible foliar tissue may therefore represent a contributing factor to host-plant resistance against herbivores with neutral or alkaline digestive tracts.

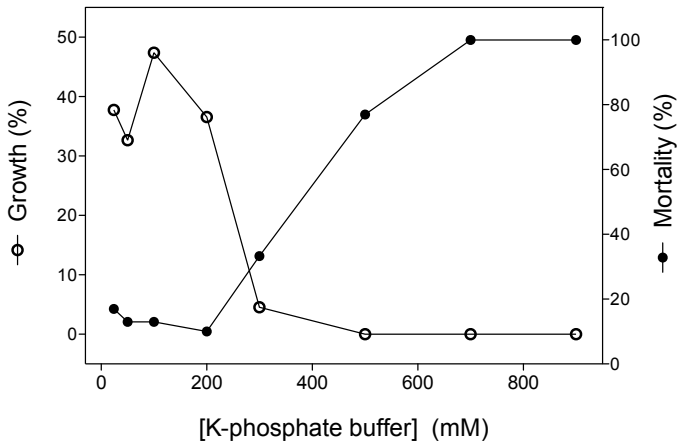

Figure 3. Influence of the concentration of phosphate buffer pH 7.0 on the performance of first-instar larvae of *Frankliniella occidentalis*.

Polyphenol oxidase has its activity maximum at a pH of 7.0 (Felton, 1989). Yet, the addition of this enzyme to the neutral dietary treatment did not trigger any anti-insect effect in CQA. The browning of the liquid diet upon addition of PPO evidenced the oxidation of CQA. It has been shown that activation of phenolics by PPO is necessary for these metabolites to exhibit anti-insect activity in artificial diet setups (Felton, 1989; 1992). However, both PPO activity and phenolic levels have failed to correlate with host-plant resistance in different plant-insect systems (Bi et al., 1997a; Isman and Duffey, 1982a; Melo et al., 2006). Considering our results we wonder whether insufficient control of diet pH could account in some cases for such discrepancy between *in situ* and *in vitro* herbivory tests.

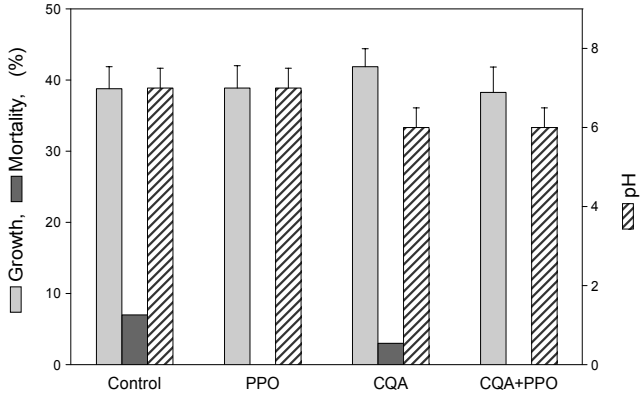

Figure 4. Effect of chlorogenic acid on the performance of first-instar larvae of *Frankliniella occidentalis* in pH-controlled artificial diet bioassays. The concentration of chlorogenic acid and polyphenol oxidase (PPO) were 1% and 450 units mL^{-1} respectively.

To further examine the influence of hydrogen ion concentration on thrips performance we tested dietary solutions prepared with phosphate buffers of eight different pH values, ranging from 2 to 12, at a total phosphate concentration of 150 mM. As evidence by the growth and mortality profiles thrips larvae performed best at pHs between 5 and 7 (Fig. 5), suggesting that the digestive tract of larval thrips is in the acidic-neutral range. To the best of our knowledge this represents the first indirect determination of the physiological midgut pH of *F. occidentalis*. Using a series of indicators fed to larvae and adults Day and Irzykiewicz (1954) determined a narrow range of possible midgut pH values for two other thrips species, the onion thrips, *Thrips tabaci*, and the apple blossom thrips, *T. imaginis*. Larval and adult thrips of both species had a digestive pH between 5.0 and 5.6. Acidic to neutral digestive tracts are also characteristic of species in the orders Orthoptera, Diptera, Hymenoptera, and in some cases Coleoptera. Whereas alkaline digestive conditions are typical in Lepidopteran larvae (Johnson and Felton, 1996).

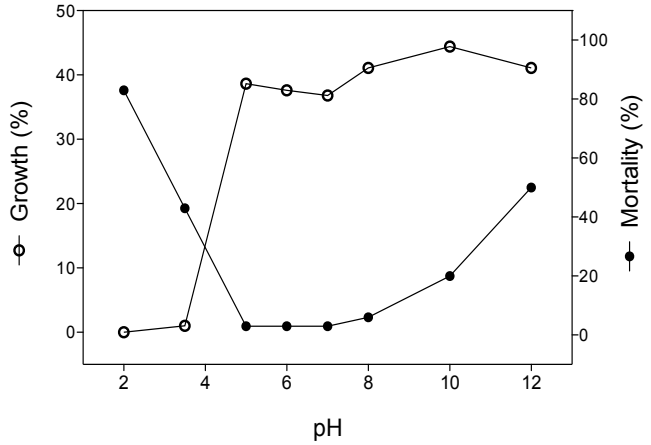

Figure 5. Influence of diet pH on the performance of first-instar larvae of *Frankliniella occidentalis*. All buffer systems are based on potassium phosphate salts with a total phosphate concentration of 150 mM.

Other diet parameters, such as high nutrient levels, may partially counteract potential adverse physiological effects of CQA. For instance, the oxidation of CQA into reactive *o*-quinone would be inhibited by other metabolites with higher reducing potentials, such as the ascorbic acid present in the vitamin mixture (Table 1). To investigate this hypothesis the relative concentrations of protein and vitamins in the liquid diet were reduced. As expected, thrips larvae grew significantly less (F=8.3, df=8, p=0.0001) when the contents of protein and vitamins were cut down to a fifth and an eighth of the full-diet values respectively (Fig. 6). Growth did not decrease much more when the concentrations of protein and vitamins were reduced further to a tenth and a fortieth of the full diet content, respectively. Larval performance was not affected by CQA in any of the low-nutrient diets, confirming thus that CQA does not have acute negative effects on larval thrips. The potential antioxidant benefits of phenolics, claimed in some cited literature, did not reflect on thrips performance either.

Concentrations of CQA higher than 1% could have been tested with the aid of suitable ratios of organic solvents. However, such values are usually considered physiologically unrealistic and lack in evident ecological meaning. The highest foliar concentrations of CQA reported to date are 2% of dry weight for tomato, *Solanum lycopersicum* (Jansen and Stamp, 1997) and 3.3% of dry weight for

tobacco, *Nicotiana tabacum* (Camacho-Cristobal et al., 2004). Metabolic engineering of the phenylpropanoid biosynthetic pathway in tomato increased the foliar accumulation of CQA from 0.9% to 1.85% of dry weight (Niggeweg et al., 2004). The engineered plants showed improved resistance to the bacterial pathogen *Pseudomonas syringae* but were not challenged with herbivores.

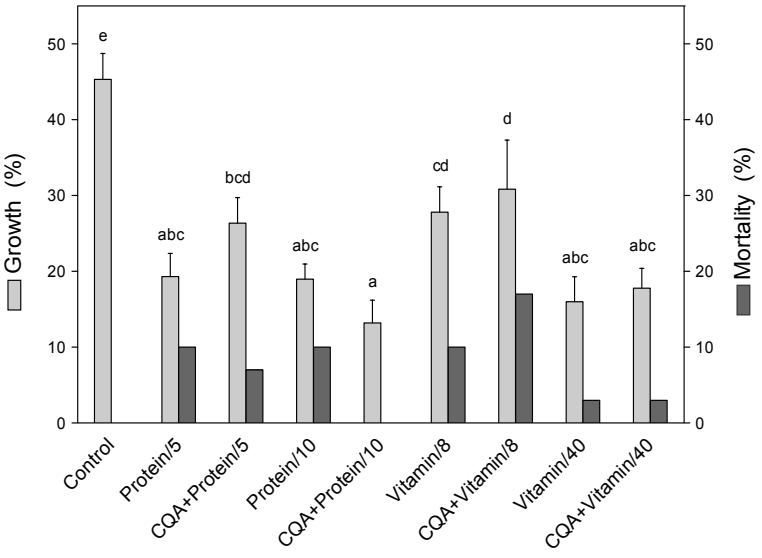

Figure 6. Effect of the nutrient/chlorogenic acid ratio on the performance of first-instar larvae of *Frankliniella occidentalis* in pH-controlled artificial diet bioassays. Concentrations of protein and vitamin in the control diet were 3.5 and 2 % respectively. For the treatments protein content was reduced to 1/5 and 1/10 whereas the vitamin content was reduced to 1/8 and 1/40. The concentration of chlorogenic acid was 1%.

When thrips larvae were fed diets containing either one of CQA regioisomers, 3-CQA (neochlorogenic acid) and 4-CQA (cryptochlorogenic acid), no significant changes in their performance were observed (Fig. 7). This result indicates that the differences in physical properties between these molecules are irrelevant as well to the potential anti-insect activity of CQA.

Researchers often draw the inference that phenolic compounds are involved in host-plant resistance against herbivores on the ground of their induction or increase upon herbivory or wounding. This connection is in many cases a misinterpretation of incomplete evidence, especially when polyphenol oxidases are concomitantly induced (Bi et al., 1997b). One of the most essential roles of phenolics and oxidases in plants is to seal off damaged tissue and reduce thereby the plant vulnerability to pathogen attack. The induced accumulation of phenolics after injury by pathogens or pests has been extensively reviewed (Freucht and Treutter, 1999). Our results suggest that in the course of plant-insect coevolution thrips may have indeed evolved adaptations similar to those in mammals to circumvent the predicted adverse effects of simple phenolics like chlorogenic acid. In addition, our study joins the mounting body of evidence compelling us to abandon the assumption that phenolics are in general effective defenses against herbivores. Interactions between insects and phenolic compounds should be evaluated on a case-to-case basis as long as the specific molecular modes of action of these secondary metabolites are not elucidated and fully understood.

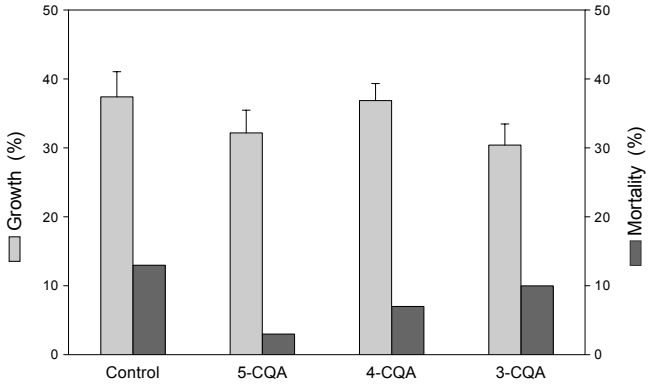

Figure 7. Effect of the chlorogenic acid regioisomers on the performance of first-instar larvae of *Frankliniella occidentalis* pH-controlled artificial diet bioassays. The concentration of chlorogenic acids was 1%. Letters refer to significant differences at the 0.05 level as analyzed with ANOVA.

References

Agnely, M. and Thiebaut, D. (1997) Dual-mode high-speed counter-current chromatography: retention, resolution and examples. Journal of Chromatography A 790(1-2), 17-30.

Allen, W.R. and Broadbent, A.B. (1986) Transmission of tomato spotted wilt virus in ontario greenhouses by *Frankliniella-occidentalis*. Canadian Journal of Plant Pathology 8(1), 33-38.

Allwood, J.W., Ellis, D.I. and Goodacre, R. (2008) Metabolomic technologies and their application to the study of plants and plant-host interactions. Physiologia Plantarum 132(2), 117-135.

Allwood, J.W., Ellis, D.I., Heald, J.K., Goodacre, R. and Mur, L.A.J. (2006) Metabolomic approaches reveal that phosphatidic and phosphatidyl glycerol phospholipids are major discriminatory non polar metabolites in responses by Brachypodium distachyon to challenge by Magnaporthe grisea. The Plant Journal 46(3), 351-368.

Annadana, S., Peters, J., Gruden, K., Schipper, A., Outchkourov, N. and Beekwilder, M. (2002) Effects of cysteine protease inhibitors on oviposition rate of the western flower thrips, *Frankliniella occidentalis*. Journal of Insect Physiology 48(7), 701-706.

Antonious, G.F., Dahlman, D.L. and Hawkins, L.M. (2003) Insecticidal and acaricidal performance of methyl ketones in wild tomato leaves. Bulletin of Environmental Contamination and Toxicology 71(2), 400-407.

Antonious, G.F., Kochhar, T.S. and Simmons, A.M. (2005) Natural products: Seasonal variation in trichome counts and contents in Lycopersicum hirsutum f. glabratum. Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes 40(4), 619-631.

Appel, H.M. (1993) Phenolics in ecological interactions - The importance of oxidation. Journal of Chemical Ecology 19(7), 1521-1552.

Arany, A., de Jong, T., Kim, H., van Dam, N., Choi, Y., Verpoorte, R. and van der Meijden, E. (2008) Glucosinolates and other metabolites in the leaves of *Arabidopsis thaliana* from natural populations and their effects on a generalist and a specialist herbivore. Chemoecology 18(2), 65-71.

Bai, Y., Huang, C.-C., van der Hulst, R., Meijer-Dekens, F., Bonnema, G. and Lindhout, P. (2003) QTLs for tomato powdery mildew resistance (*Oidium lycopersici*) in *Lycopersicon parviflorum* G1.1601 co-localize with two qualitative powdery mildew resistance genes. Molecular Plant-Microbe Interactions 16(2), 169-176.

Bai, Y.L. and Lindhout, P. (2007) Domestication and breeding of tomatoes: What have we gained and what can we gain in the future? Annals of Botany 100, 1085-1094.

Baldin, E.L.L., Vendramim, J.D. and Lourencao, A.L. (2005) Resistance of tomato genotypes to the whitefly *Bemisia tabaci* (Gennadius) biotype B (Hemiptera : Aleyrodidae). Neotropical Entomology 34(3), 435-441.

Ben-Israel, I., Yu, G., Austin, M., Bhuiyan, N., Auldridge, M., Nguyen, T., Schauvinhold, I., Noel, J., Pichersky, E. and Fridman, E. (2009) Multiple biochemical and morphological factors underlie the production of methylketones in tomato trichomes. Plant Physiology 151(4), 1952.

Beninger, C.W., Abou-Zaid, M.M., Kistner, A.L.E., Hallett, R.H., Iqbal, M.J., Grodzinski, B. and Hall, J.C. (2004) A flavanone and two phenolic acids from *Chrysanthemum morifolium* with phytotoxic and insect growth regulating activity. Journal of Chemical Ecology 30(3), 589-606.

Berthod, A. (2007) Countercurrent chromatography and the journal of liquid chromatography: A love story. Journal of Liquid Chromatography & Related Technologies 30(9-12), 1447-1463.

Berthod, A. and Hassoun, M. (2006) Using the liquid nature of the stationary phase in countercurrent chromatography - IV. The cocurrent CCC method. Journal of Chromatography A 1116(1-2), 143-148.

Berthod, A., Ruiz-Angel, M.J. and Carda-Broch, S. (2003) Elution-extrusion countercurrent chromatography. Use of the liquid nature of the stationary phase to extend the hydrophobicity window. Analytical Chemistry 75(21), 5886-5894.

Bi, J.L., Felton, G.W., Murphy, J.B., Howles, P.A., Dixon, R.A. and Lamb, C.J. (1997a) Do plant phenolics confer resistance to specialist and generalist insect herbivores? Journal of Agricultural and Food Chemistry 45(11), 4500-4504.

Bi, J.L., Murphy, J.B. and Felton, G.W. (1997b) Antinutritive and oxidative components as mechanisms of induced resistance in cotton to Helicoverpa zea. Journal of Chemical Ecology 23(1), 97-117.

Bleeker, P.M., Diergaarde, P.J., Ament, K., Guerra, J., Weidner, M., Schutz, S., de Both, M.T.J., Haring, M.A. and Schuurink, R.C. (2009) The role of specific tomato volatiles in tomato-whitefly interaction. Plant Physiology 151(2), 925-935.

Bleeker, P.M., Diergaarde, P.J., Ament, K., Sch¸tz, S., Johne, B., Dijkink, J., Hiemstra, H., de Gelder, R., de Both, M.T.J., Sabelis, M.W., Haring, M.A. and Schuurink, R.C. (2011) Tomato-produced 7-epizingiberene and R-curcumene act as repellents to whiteflies. Phytochemistry 72(1), 68-73.

Bouayed, J., Rammal, H., Dicko, A., Younos, C. and Soulimani, R. (2007) Chlorogenic acid, a polyphenol from *Prunus domestica* (Mirabelle), with coupled anxiolytic and antioxidant effects. Journal of the Neurological Sciences 262(1-2), 77-84.

Breeden, D.C. and Coates, R.M. (1994) 7-Epizingiberene, a novel bisabolane sesquiterpene from wild tomato leaves. Tetrahedron 50(38), 11123-11132.

Brodbeck, B.V., Stavisky, J., Funderburk, J.E., Andersen, P.C. and Olson, S.M. (2001) Flower nitrogen status and populations of *Frankliniella occidentalis* feeding on *Lycopersicon esculentum*. Entomologia Experimentalis et Applicata 99(2), 165-172.

Brodsgaard, H. (1994) Insecticide resistance in european and african strains of western flower thrips (Thysanoptera, Thripidae) tested in a new residue-on-glass test. Journal of Economic Entomology 87(5), 1141-1146.

Burke, A., B., Goldsby, G., Mudd and B., J. (1987) Polar epicuticular lipids of *Lycopersicon pennellii*. Phytochemistry 26(9).

Cacace, G., M., Landau, M., E., Ramsden and J., J. (1997) The Hofmeister series: salt and solvent effects on interfacial phenomena. Quarterly Reviews of Biophysics 30(03), 241-277.

Camacho-Cristobal, J., Lunar, L., Lafont, F., Baumert, A. and Gonzalez-Fontes, A. (2004) Boron deficiency causes accumulation of chlorogenic acid and caffeoyl polyamine conjugates in tobacco leaves. Journal of Plant Physiology 161(7), 879-881.

Campbell, J., Canene-Adams, K., Lindshield, B., Boileau, T., Clinton, S. and Erdman, J. (2004) Tomato phytochemicals and prostate cancer risk. The Journal of Nutrition 134(12), 3486S-3492S.

Canady, M.A., Ji, Y. and Chetelat, R.T. (2006) Homeologous recombination in *Solanum lycopersicoides* introgression lines of cultivated tomato. Genetics 174(4), 1775-1788.

Carter, C., Sacalis, J. and Gianfagna, T. (1989) Zingiberene and resistance to Colorado potato beetle in *Lycopersicon hirsutum* f. *hirsutum*. Journal of Agricultural and Food Chemistry 37(1), 206-210.

Chatzivasileiadis, E., Boon, J. and Sabelis, M. (1999) Accumulation and turnover of 2-tridecanone in Tetranychus urticae and its consequences for resistance of wild and cultivated tomatoes. Experimental and Applied Acarology 23(12), 1011-1021.

Cheng, J.C., Dai, F., Zhou, B., Yang, L. and Liu, Z.L. (2007) Antioxidant activity of hydroxycinnamic acid derivatives in human low density lipoprotein: Mechanism and structure-activity relationship. Food Chemistry 104(1), 132-139.

Chiang, L.C., Chiang, W., Chang, M.Y., Ng, L.T. and Lin, C.C. (2002) Antiviral activity of *Plantago major* extracts and related compounds *in vitro*. Antiviral Research 55(1), 53-62.

Childers, C.C. (1997) Feeding and oviposition injuries to plants. In: T. Lewis (Ed), Thrips as crop pests, pp. 505-537. CAB International, New York, USA.

Choi, Y.H., Tapias, E.C., Kim, H.K., Lefeber, A.W.M., Erkelens, C., Verhoeven, J.T.J., Brzin, J., Zel, J. and Verpoorte, R. (2004) Metabolic discrimination of Catharanthus roseus leaves infected by phytoplasma using 1H-NMR spectroscopy and multivariate data analysis. Plant Physiology 135(4), 2398.

Clifford, M.N., Johnston, K.L., Knight, S. and Kuhnert, N. (2003) Hierarchical scheme for LC-MSn identification of chlorogenic acids. Journal of Agricultural and Food Chemistry 51(10), 2900-2911.

Clifford, M.N., Kellard, B. and Birch, G.G. (1989) Characterisation of chlorogenic acids by simultaneous isomerisation and transesterification with tetramethylammonium hydroxide. Food Chemistry 33(2), 115-123.

Coates, R.M., Denissen, J.F., Juvik, J.A. and Babka, B.A. (1988) Identification of alpha-santalenoic and endo-beta-bergamotenoic acids as moth oviposition stimulants from wild tomato leaves. Journal of Organic Chemistry 53(10), 2186-2192.

Coley, P.D., Bryant, J.P. and Chapin, F.S. (1985) Resource availability and plant antiherbivore defense. Science 230(4728), 895-899.

Comino, C., Hehn, A., Moglia, A., Menin, B., Bourgaud, F., Lanteri, S. and Portis, E. (2009) The isolation and mapping of a novel hydroxycinnamoyltransferase in the globe artichoke chlorogenic acid pathway. BMC Plant Biology 9(1), 30-43.

Crozier, A., Jaganath, I.B. and Clifford, M.N. (2009) Dietary phenolics: chemistry, bioavailability and effects on health. Natural Product Reports 26(8), 1001-1043.

Daughtrey, M.L., Jones, R.K., Moyer, J.W., Daub, M.E. and Baker, J.R. (1997) Tospoviruses strike the greenhouse industry - INSV has become a major pathogen on flower crops. Plant Disease 81(11), 1220-1230.

Davis, J., Yu, D.Z., Evans, W., Gokirmak, T., Chetelat, R.T. and Stotz, H.U. (2009) Mapping of loci from *Solanum lycopersicoides* conferring resistance or susceptibility to *Botrytis cinerea* in tomato. Theoretical and Applied Genetics 119(2), 305-314.

Day, M.F. and Irzykiewicz, H. (1954) Physiological studies on Thrips in relation to transmission of tomato spotted wilt virus. Australian Journal of Biological Sciences 7(3), 274-281.

de Jager, C., Butôt, R., Klinkhamer, P., de Jong, T., Wolff, K. and van der Meijden, E. (1995) Genetic variation in chrysanthemum for resistance to *Frankliniella occidentalis*. Entomologia Experimentalis et Applicata 77(3), 277-287.

de Jager, C.M., Butot, R.P.T., van der Meijden, E. and Verpoorte, R. (1996) The role of primary and secondary metabolites in chrysanthemum resistance to *Frankliniella occidentalis*. Journal of Chemical Ecology 22(11), 1987-1999.

de Resende, J.T.V., Maluf, W.R., Faria, M.V., Pfann, A.Z. and do Nascimento, E.R. (2006) Acylsugars in tomato leaflets confer resistance to the South American tomato pinworm, *Tuta absoluta* Meyr. Scientia Agricola 63(1), 20-25.

de Resendel, J.T.V., Maluf, W.R., Cardoso, M.D., Faria, M.V., Goncalves, L.D. and do Nascimento, I.R. (2008) Resistance of tomato genotypes with high level of acylsugars to *Tetranychus evansi* Baker & Pritchard. Scientia Agricola 65(1), 31-35.

Deborde, C., Maucourt, M., Baldet, P., Bernillon, S., Biais, B., Talon, G., Ferrand, C., Jacob, D., Ferry-Dumazet, H., de Daruvar, A., Rolin, D. and Moing, A. (2009) Proton NMR quantitative profiling for quality assessment of greenhouse-grown tomato fruit. Metabolomics 5(2), 183-198.

Delannay, E., Toribio, A., Boudesocque, L., Nuzillard, J.M., Zeches-Hanrot, M., Dardennes, E., Le Dour, G., Sapi, J. and Renault, J.H. (2006) Multiple dual-mode centrifugal partition chromatography, a semi-continuous development mode for routine laboratory-scale purifications. Journal of Chromatography A 1127(1-2), 45-51.

Dimock, M.B., Kennedy, G.G. and Williams, W.G. (1982) Toxicity studies of analogs of 2-tridecanone, a naturally-occurring toxicant from a wild tomato. Journal of Chemical Ecology 8(5), 837-842.

Duffey, S. (1986) Plant glandular trichomes: their potential role in defense against insects. In: B. Juniper and R. Southwood (Eds), Insects and plant surface, pp. 151-172. Arnold, London, UK.

Eichenseer, H., Bi, J.L. and Felton, G.W. (1998) Indiscrimination of *Manduca sexta* larvae to overexpressed and underexpressed levels of phenylalanine ammonia-lyase in tobacco leaves. Entomologia Experimentalis et Applicata 87(1), 73-78.

Eigenbrode, S., Trumble, J., Millar, J. and White, K. (1994) Topical toxicity of tomato sesquiterpenes to the beet armyworm and the role of these compounds in resistance derived from an accession of *Lycopersicon hirsutum f. typicum*. Journal of Agricultural and Food Chemistry 42(3), 807-810.

Eshed, Y. and Zamir, D. (1995) An introgression line population of *Lycopersicon pennellii* in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. Genetics 141(3), 1147-1162.

FAO. (2008) FAO Statistical Database. http://faostat.fao.org/.

Farah, A., de Paulis, T., Trugo, L.C. and Martin, P.R. (2005) Effect of roasting on the formation of chlorogenic acid lactones in coffee. Journal of Agricultural and Food Chemistry 53(5), 1505-1513.

Felton, G.W. (1989) Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. Journal of Chemical Ecology 15(12), 2667.

Felton, G.W., Donato, K.K., Broadway, R.M. and Duffey, S.S. (1992) Impact of oxidized plant phenolics on the nutritional quality of dietary-protein to a noctuid herbivore, *Spodoptera exigua*. Journal of Insect Physiology 38(4), 277-285.

Ferreres, F., Sousa, C., Valentao, P., Pereira, J.A., Seabra, R.M. and Andrade, P.B. (2007) Tronchuda cabbage flavonoids uptake by Pieris brassicae. Phytochemistry 68(3), 361-367.

Fobes, J.F., Mudd, J.B. and Marsden, M.P.F. (1985) Epicuticular lipid-accumulation on the leaves of *Lycopersicon-Pennellii* (Corr) Darcy and *Lycopersicon-Esculentum* Mill. Plant Physiology 77(3), 567-570.

Forkner, R.E. and Hare, J.D. (2000) Genetic and environmental variation in acyl glucose ester production and glandular and nonglandular trichome densities in *Datura wrightii*. Journal of Chemical Ecology 26(12), 2801-2823.

Frei, A., Blair, M.W., Cardona, C., Beebe, S.E., Gu, H. and Dorn, S. (2005) QTL mapping of resistance to thrips palmi Karny in common bean. Crop Science 45(1), 379-387.

Frelichowski Jr, J. and Juvik, J. (2001) Sesquiterpene carboxylic acids from a wild tomato species affect larval feeding behavior and survival of *Helicoverpa zea* and *Spodoptera exigua* (Lepidoptera: Noctuidae). Journal of Economic Entomology 94(5), 1249-1259.

Freucht, W. and Treutter, D. (1999) The role of flavan-3-ols and proanthocyanidins in plant defence In: S. Inderjit, K.M.M. Dakshini and C.L. Foy (Eds), Principles and practices in plant ecology, pp. 307-338. CRC Press, Boca Raton, USA.

Galati, G. and O'Brien, P.J. (2004) Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free Radical Biology and Medicine 37(3), 287-303.

Galili, G. and Hofgen, R. (2002) Metabolic engineering of amino acids and storage proteins in plants. Metabolic Engineering 4(1), 3-11.

Galvez, H.F., Fernandez, E.C. and Hautea, D.M. (2005) Molecular mapping of resistance to thrips in potato. Philippine Agricultural Scientist 88(3), 268-280.

Gentile, A.G. and Stoner, A.K. (1968) Resistance in *Lycopersicon* and *Solanum* species to potato aphid. Journal of Economic Entomology 61(5), 1152-&.

German, T.L., Ullman, D.E. and Moyer, J.W. (1992) Tospoviruses-diagnosis, molecular-biology, phylogeny, and vector relationships. Annual Review of Phytopathology 30, 315-348.

Ghasemi, J. and Shamsipur, M. (1995) Spectrophotometric study of the thermodynamics of interaction of some metal ions with murexide in binary acetonitrile-dimethylsulfoxide mixtures. Journal of Coordination Chemistry 36(3), 183-194.

Giovannucci, E., Ascherio, A., Rimm, E.B., Stampfer, M.J., Colditz, G.A. and Willett, W.C. (1995) Intake of carotenoids and retinol in relation to risk of prostate-cancer. Journal of the National Cancer Institute 87(23), 1767-1776.

Goffreda, J. and Mutschler, M. (1987) Potato aphid feeding response to *lycopersicon-pennellii* type-IV trichome exudate and trichome exudate components. HortScience 22(5), 1073-1073.

Goffreda, J.C., Steffens, J.C. and Mutschler, M.A. (1990) Association of epicuticular sugars with aphid resistance in hybrids with wild tomato. Journal of The American Society for Horticultural Science 115(1), 161-165.

Goldbach, R. and Peters, D. (1994) Possible causes of the emergence of tospovirus diseases. Seminars in Virology 5(2), 113-120.

Grover, P.K. and Ryall, R.L. (2004) Critical appraisal of salting-out and its implications for chemical and biological sciences. Chemical Reviews 105(1), 1-10.

Guerrero, G. and Suarez, M. (2001) Chlorogenic acids as a potential criterion in coffee genotype selections. Journal of Agricultural and Food Chemistry 49(5), 2454-2458.

Gur, A., Semel, Y., Cahaner, A. and Zamir, D. (2004) Real time QTL of complex phenotypes in tomato interspecific introgression lines. Trends in Plant Science 9(3), 107-109.

Harrewijn, P., Tjallingii, W.F. and Mollema, C. (1996) Electrical recording of plant penetration by western power thrips. Entomologia Experimentalis et Applicata 79(3), 345-353.

Hawthorne, D.J., Shapiro, J.A., Tingey, W.M. and Mutschler, M.A. (1992) Trichome-borne and artificially applied acylsugars of wild tomato deter feeding and oviposition of the leafminer *Liriomyza trifolii*. Entomologia Experimentalis et Applicata 65(1), 65-73.

Heinz, K.M. and Zalom, F.G. (1995) Variation in trichome-based resistance to bemisia-argentifolii (Homoptera, Aleyrodidae) oviposition on tomato. Journal of Economic Entomology 88(5), 1494-1502.

Hoffmann, L., Maury, S., Martz, F., Geoffroy, P. and Legrand, M. (2003) Purification, cloning, and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. Journal of Biological Chemistry 278(1), 95 - 103.

Hunter, W.B. and Ullman, D.E. (1989) Analysis of mouthpart movements during feeding of *Frankliniella occidentalis* (pergande) and *F. schultzei trybom* (Thysanoptera : Thripidae). International Journal of Insect Morphology and Embryology 18(2-3), 161-171.

Hutton, S.F., Scott, J.W., Yang, W.C., Sim, S.C., Francis, D.M. and Jones, J.B. (2010) Identification of QTL associated with resistance to bacterial spot race T4 in tomato. Theoretical and Applied Genetics 121(7), 1275-1287.

Ikonen, A., Tahvanainen, J. and Roininen, H. (2001) Chlorogenic acid as an antiherbivore defence of willows against leaf beetles. Entomologia Experimentalis et Applicata 99(1), 47-54.

Immaraju, J., Paine, T., Bethke, J., Robb, K. and Newman, J. (1992) Western flower thrips (Thysanoptera, Thripidae) resistance to insecticides in coastal california greenhouses. Journal of Economic Entomology 85(1), 9-14.

Isman, M.B. and Duffey, S.S. (1982a) Phenolic compounds in foliage of commercial tomato cultivars as growth-inhibitors to the fruitworm, *Heliothis zea*. Journal of The American Society for Horticultural Science 107(1), 167-170.

Isman, M.B. and Duffey, S.S. (1982b) Toxicity of tomato phenolic-compounds to the fruitworm, *Heliothis zea*. Entomologia Experimentalis et Applicata 31(4), 370-376.

Ito, Y. (2002) Centrifugal precipitation chromatography: Novel fractionation method for biopolymers, based on their solubility. Journal of Liquid Chromatography & Related Technologies 25(13-15), 2039-2064.

Janmaat, A.F., de Kogel, W.J. and Woltering, E.J. (2002) Enhanced fumigant toxicity of p-cymene against Frankliniella occidentalis by simultaneous application of elevated levels of carbon dioxide. Pest Management Science 58(2), 167-173.

Jansen, J.J., Allwood, J.W., Marsden-Edwards, E., van der Putten, W.H., Goodacre, R. and van Dam, N.M. (2009) Metabolomic analysis of the interaction between plants and herbivores. Metabolomics 5(1), 150-161.

Jansen, M.P.T. and Stamp, N.E. (1997) Effects of light availability on host plant chemistry and the consequences for behavior and growth of an insect herbivore. Entomologia Experimentalis et Applicata 82(3), 319-333.

Jensen, S.E. (2000) Insecticide resistance in the western flower thrips, *Frankliniella occidentalis*. Integrated Pest Management Reviews 5(2), 131-146.

Johnson, K.S. and Felton, G.W. (1996) Potential influence of midgut pH and redox potential on protein utilization in insect herbivores. Archives of Insect Biochemistry and Physiology 32(1), 85-105.

Johnson, K.S. and Felton, G.W. (2001) Plant phenolics as dietary antioxidants for herbivorous insects: A test with genetically modified tobacco. Journal of Chemical Ecology 27(12), 2579-2597.

Kalyanaraman, B., Premovic, P.I. and Sealy, R.C. (1987) Semiquinone anion radicals from addition of amino-acids, peptides, and proteins to quinones derived from oxidation of catechols and catecholamines - an electron-spin-resonance spin stabilization study. Journal of Biological Chemistry 262(23), 11080-11087.

Kamenetzky, L., Asis, R., Bassi, S., de Godoy, F., Bermudez, L., Fernie, A.R., Van Sluys, M.-A., Vrebalov, J., Giovannoni, J.J., Rossi, M. and Carrari, F. (2010) Genomic analysis of wild tomato introgressions determining metabolism- and yield-associated traits. Plant Physiol. 152(4), 1772-1786.

Kang, J.H., Liu, G.H., Shi, F., Jones, A.D., Beaudry, R.M. and Howe, G.A. (2010) The tomato odorless-2 mutant is defective in trichome-based production of diverse specialized metabolites and broad-spectrum resistance to insect herbivores. Plant Physiology 154(1), 262-272.

Kennedy, G.G. and Barbour, J.D. (1992) Resistance variation in natural and managed systems. In: R.S. Fritz and E.L. Slimms (Eds), Plant resistance to herbivores and pathogens: ecology, evolution, and genetics, pp. 29. The University of Chicago Press, Chicago, USA.

Kennedy, G.G. and Henderson, W.R. (1978) Laboratory assay for resistance to tobacco hornworm in *Lycopersicon* and *Solanum*-Spp. Journal of The American Society for Horticultural Science 103(3), 334-336.

Kennedy, G.G. and Sorenson, C.F. (1985) Role of glandular trichomes in the resistance of *Lycopersicon hirsutum* f *glabratum* to colorado potato beetle (Coleoptera, Chrysomelidae). Journal of Economic Entomology 78(3), 547-551.

King, R.R., Calhoun, L.A., Singh, R.P. and Boucher, A. (1990) Sucrose esters associated with glandular trichomes of wild *Lycopersicon* species. Phytochemistry 29(7), 2115-2118.

Kirk, W.D.J. and Terry, L.I. (2003) The spread of the western flower thrips *Frankliniella occidentalis* (Pergande). Agricultural and Forest Entomology 5(4), 301-310.

Kirsh, V.A., Mayne, S.T., Peters, U., Chatterjee, N., Leitzmann, M.F., Dixon, L.B., Urban, D.A., Crawford, E.D. and Hayes, R.B. (2006) A prospective study of lycopene and tomato product intake and risk of prostate cancer. Cancer Epidemiology Biomarkers & Prevention 15(1), 92-98.

Kohler, G.R. and St Clair, D.A. (2005) Variation for resistance to aphids (Homoptera : Aphididae) among tomato inbred backcross lines derived from wild *Lycopersicon* species. Journal of Economic Entomology 98(3), 988-995.

Krishnan, P., Kruger, N.J. and Ratcliffe, R.G. (2005) Metabolite fingerprinting and profiling in plants using NMR. Journal of Experimental Botany 56(410), 255-265.

Kumar, N.K.K., Ullman, D.E. and Cho, J.J. (1995) Resistance among lycopersicon species to *frankliniella-occidentalis* (thysanoptera, thripidae). Journal of Economic Entomology 88(4), 1057-1065.

Lang, S.P. and Tibbitts, T.W. (1983) Factors controlling intumescence development on tomato plants. Journal of the American Society for Horticultural Science 108(1), 93-98.

Lapouge, C. and Cornard, J.P. (2007) Reaction pathways involved in the mechanism of Al-III chelation with caffeic acid: Catechol and carboxylic functions competition. ChemPhysChem 8(3), 473-479.

Lawson, D., Lunde, C. and Mutschler, M. (1997) Marker-assisted transfer of acylsugar-mediated pest resistance from the wild tomato, Lycopersicon pennellii, to the cultivated tomato, Lycopersicon esculentum. Molecular breeding 3(4), 307-317.

Lawton, J.H., Brown, K.C., Crawley, M.J., Way, M.J., Holdgate, M.W., May, R.M., Southwood, R. and O'Connor, R.J. (1986) The population and community ecology of invading insects. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 314(1167), 607-617.

Le Gall, G., Colquhoun, I.J., Davis, A.L., Collins, G.J. and Verhoeyen, M.E. (2003) Metabolite profiling of tomato (*Lycopersicon* esculentum) using H NMR spectroscopy as a tool to detect potential unintended effects following a genetic modification. Journal of Agricultural and Food Chemistry 51(9), 2447-2456.

Lee, K.W. and Lee, H.J. (2006) The roles of polyphenols in cancer chemoprevention. BioFactors 26(2), 105-121.

Leiss, K., Choi, Y., Verpoorte, R. and Klinkhamer, P. (2010) An overview of NMR-based metabolomics to identify secondary plant compounds involved in host plant resistance. Phytochemistry Reviews. In press.

Leiss, K.A., Choi, Y.H., Abdel-Farid, I.B., Verpoorte, R. and Klinkhamer, P.G.L. (2009a) NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in senecio hybrids. Journal of Chemical Ecology 35(2), 219-229.

Leiss, K.A., Maltese, F., Choi, Y.H., Verpoorte, R. and Klinkhamer, P.G.L. (2009b) Identification of chlorogenic acid as a resistance factor for thrips in chrysanthemum. Plant Physiology 150(3), 1567-1575.

Lewis, T., ed. (1997) Thrips as crop pests. CAB international, Wallingford, UK.

Li, L. and Steffens, J.C. (2002) Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. Planta 215(2), 239-247.

Li, X.C., Schuler, M.A. and Berenbaum, M.R. (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annual Review of Entomology 52, 231-253.

Liedl, B.E., Lawson, D.M., White, K.K., Shapiro, J.A., Cohen, D.E., Carson, W.G., Trumble, J.T. and Mutschler, M.A. (1995) Acylsugars of wild tomato *Lycopersicon pennellii* alters settling and reduces oviposition of *Bemisia argentifolii* (Homoptera : Aleyrodidae). Journal of Economic Entomology 88(3), 742-748.

Lindshield, B.L., Canene-Adams, K. and Erdman, J.W. (2007) Lycopenoids: Are lycopene metabolites bioactive? Archives of Biochemistry and Biophysics 458(2), 136-140.

Lotito, S.B. and Frei, B. (2006) Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: Cause, consequence, or epiphenomenon? Free Radical Biology And Medicine 41(12), 1727-1746.

Lu, H.T., Jiang, Y. and Chen, F. (2004) Application of preparative high-speed counter-current chromatography for separation of chlorogenic acid from *Flos lonicerae*. Journal of Chromatography A 1026(1-2), 185-190.

Lu, Y., Liu, R., Berthod, A. and Pan, Y. (2008) Rapid screening of bioactive components from *Zingiber cassumunar* using elution-extrusion counter-current chromatography. Journal of Chromatography A 1181(1-2), 33-44.

Luckwill, L.C. (1943) The genus *Lycopersicon*: An historical, biological and taxonomic survey of the wild and domesticated tomatoes. Aberdeen University Press, Aberdeen, UK.

Macdonald, O.C. (1993) Susceptibility of western flower thrips, Frankliniella-occidentalis (Pergande) to fumigation with methyl-bromide. Annals of Applied Biology 123(3), 531-537.

Macel, M., Bruinsma, M., Dijkstra, S.M., Ooijendijk, T., Niemeyer, H.M. and Klinkhamer, P.G.L. (2005) Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. Journal of Chemical Ecology 31(7), 1493-1508.

Mantel, W.P. and van de Vrie, M. (1988) De Californische trips, *Frankliniella occidentalis*, een nieuwe schadelijke tripssoort in de tuinbouw onder glas in Nederland. Entomologische Berichten 48(9), 140-144.

Melo, G.A., Shimizu, M.M. and Mazzafera, P. (2006) Polyphenoloxidase activity in coffee leaves and its role in resistance against the coffee leaf miner and coffee leaf rust. Phytochemistry 67(3), 277-285.

Miles, P.W. and Oertli, J.J. (1993) The significance of antioxidants in the aphid-plant interaction - The redox hypothesis. Entomologia Experimentalis et Applicata 67(3), 275-283.

Miles, P.W. and Peng, Z. (1989) Studies on the salivary physiology of plant bugs - Detoxification of phytochemicals by the salivary peroxidase of aphids. Journal of Insect Physiology 35(11), 865-872.

Minorsky, P.V. (2002) The hot and the classic. Plant Physiology 130(3), 1077-1078.

Mirnezhad, M., Romero-Gonzalez, R.R., Leiss, K.A., Choi, Y.H., Verpoorte, R. and Klinkhamer, P.G.L. (2010) Metabolomic analysis of host plant resistance to thrips in wild and cultivated tomatoes. Phytochemical Analysis 21(1), 110-117.

Moco, S., Bino, R.J., Vorst, O., Verhoeven, H.A., de Groot, J., van Beek, T.A., Vervoort, J. and de Vos, C.H.R. (2006) A liquid chromatography-mass spectrometry-based metabolome database for tomato. Plant Physiology 141(4), 1205-1218.

Mollema, C. and Cole, R.A. (1996) Low aromatic amino acid concentrations in leaf proteins determine resistance to *Frankliniella occidentalis* in four vegetable crops. Entomologia Experimentalis et Applicata 78(3), 325-333.

Momotaz, A., Scott, J.W. and Schuster, D.J. (2010) Identification of quantitative trait loci conferring resistance to *Bemisia tabaci* in an F-2 population of *Solanum lycopersicum* x *Solanum habrochaites* accession LA1777. Journal of the American Society for Horticultural Science 135(2), 134-142.

Monforte, A.J. and Tanksley, S.D. (2000) Development of a set of near isogenic and backcross recombinant inbred lines containing most of the Lycopersicon hirsutum genome in a L-esculentum genetic background: A tool for gene mapping and gene discovery. Genome 43(5), 803-813.

Mound, L.A. (2005) Thysanoptera: Diversity and interactions. Annual Review of Entomology 50, 247-269.

Muchero, W., Ehlers, J.D. and Roberts, P.A. (2010) QTL analysis for resistance to foliar damage caused by Thrips tabaci and Frankliniella schultzei (Thysanoptera: Thripidae) feeding in cowpea [Vigna unguiculata (L.) Walp.]. Molecular Breeding 25(1), 47-56.

Muigai, S.G., Bassett, M.J., Schuster, D.J. and Scott, J.W. (2003) Greenhouse and field screening of wild Lycopersicon germplasm for resistance to the whitefly Bemisia argentifolii. Phytoparasitica 31(1), 27-38.

Muigai, S.G., Schuster, D.J., Snyder, J.C., Scott, J.W., Bassett, M.J. and McAuslane, H.J. (2002) Mechanisms of resistance in Lycopersicon germplasm to the whitefly Bemisia argentifolii. Phytoparasitica 30(4), 347-360.

Mutschler, M.A., Doerge, R.W., Liu, S.C., Kuai, J.P., Liedl, B.E. and Shapiro, J.A. (1996) QTL analysis of pest resistance in the wild tomato Lycopersicon pennellii: QTLs controlling acylsugar level and composition. Theoretical and Applied Genetics 92(6), 709-718.

Nakatani, N., Kayano, S., Kikuzaki, H., Sumino, K., Katagiri, K. and Mitani, T. (2000) Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (*Prunus domestica* L.). Journal of Agricultural and Food Chemistry 48(11), 5512-5516.

Napper, D.H. and Netschey, A. (1971) Studies of the steric stabilization of colloidal particles. Journal of Colloid and Interface Science 37(3), 528-535.

Nicholson, J.K., Lindon, J.C. and Holmes, E. (1999) Metabonomics: understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 29(11), 1181-1189.

Niggeweg, R., Michael, A.J. and Martin, C. (2004) Engineering plants with increased levels of the antioxidant chlorogenic acid. Nature Biotechnology 22(6), 746-754.

Nonomura, T., Xu, L., Wada, M., Kawamura, S., Miyajima, T., Nishitomi, A., Kakutani, K., Takikawa, Y., Matsuda, Y. and Toyoda, H. (2009) Trichome exudates of Lycopersicon pennellii form a chemical barrier to suppress leaf-surface germination of Oidium neolycopersici conidia. Plant Science 176(1), 31-37.

Oksman-Caldentey, K.-M. and Inzé, D. (2004) Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. Trends In Plant Science 9(9), 433-440.

Omo-Ikerodah, E.E., Fawole, I. and Fatokun, C.A. (2008) Genetic mapping of quantitative trait loci (QTLs) with effects on resistance to flower bud thrips (Megalurothrips sjostedti) identified in recombinant inbred lines of cowpea (Vigna unguiculata (L.) Walp). African Journal of Biotechnology 7(3), 263-270.

Outchkourov, N.S., De Kogel, W.J., Schuurman-de Bruin, A., Abrahamson, M. and Jongsma, M.A. (2004) Specific cysteine protease inhibitors act as deterrents of western flower thrips, *Frankliniella occidentalis* (Pergande), in transgenic potato. Plant Biotechnology Journal 2(5), 439-448.

Overy, S.A., Walker, H.J., Malone, S., Howard, T.P., Baxter, C.J., Sweetlove, L.J., Hill, S.A. and Quick, W.P. (2005) Application of metabolite profiling to the identification of traits in a population of tomato introgression lines. Journal of Experimental Botany 56(410), 287-296.

Paini, D.R., Funderburk, J.E., Jackson, C.T. and Reitz, S.R. (2007) Reproduction of four thrips species (Thysanoptera : Thripidae) on uncultivated hosts. Journal of Entomological Science 42(4), 610-615.

Parejo, I., Jauregui, O., Sanchez-Rabaneda, F., Viladomat, F., Bastida, J. and Codina, C. (2004) Separation and characterization of phenolic compounds in fennel (*Foeniculum vulgare*) using liquid chromatography-negative electrospray ionization tandem mass spectrometry. Journal of Agricultural and Food Chemistry 52(12), 3679-3687.

Parrella, G., Gognalons, P., Gebre-Selassie, K., Vovlas, C. and Marchoux, G. (2003) An update of the host range of tomato spotted wilt virus. Journal of Plant Pathology 85(4), 227-264.

Peng, Z. and Miles, P. (1988a) Acceptability of catechin and its oxidative condensation products to the rose aphid, macrosiphum-rosae. Entomologia Experimentalis et Applicata 47(3), 255-265.

Peng, Z. and Miles, P. (1988b) Studies on the salivary physiology of plant bugs - function of the catechol oxidase of the rose aphid. Journal of Insect Physiology 34(11), 1027-1033.

Peng, Z. and Miles, P.W. (1991) Oxidases in the gut of an aphid, *Macrosiphum rosae* (L) and their relation to dietary phenolics. Journal of Insect Physiology 37(10), 779-787.

Pereira, G.V.N., Maluf, W.R., Goncalves, L.D., do Nascimento, D.R., Gomes, L.A.A. and Licursi, V. (2008) Selection towards high acylsugar levels in tomato genotypes and its relationship with resistance to spider mite (*Tetranychus evansi*) and to the South American pinworm (*Tuta absoluta*). Ciencia e Agrotecnologia 32(3), 996-1004.

Phuc, T.D., Prudent, M., Sulpice, R., Causse, M. and Fernie, A.R. (2010) The Influence of fruit load on the tomato pericarp metabolome in a *Solanum chmielewskii* introgression line population. Plant Physiology 154(3), 1128-1142.

Rasband, W.S. (1997) ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/ USA.

Reitz, S. (2009) Biology and ecology of the western flower thrips (Thysanoptera: Thripidae): the making of a pest. The Florida Entomologist 92(1), 7-13.

Reitz, S.R. (2002) Seasonal and within plant distribution of Frankliniella thrips (Thysanoptera : Thripidae) in north Florida tomatoes. Florida Entomologist 85(3), 431-439.

Reitz, S.R. (2005) Biology and ecology of flower thrips in relation to tomato spotted wilt virus. Acta Horiculturae (ISHS) 695, 75-84.

Rice-Evans, C., Miller, N. and Paganga, G. (1997) Antioxidant properties of phenolic compounds. Trends in Plant Science 2(4), 152-159.

Ridsdill-Smith, T., Jiang, Y. and Ghisalberti, E. (1995) A method to test compounds for feeding deterrence towards redlegged earth mite (Acarina: Penthaleidae). Annals of Applied Biology 127(3), 593-600.

Riley, D.G. and Pappu, H.R. (2004) Tactics for management of thrips (Thysanoptera : Thripidae) and tomato spotted wilt virus in tomato. Journal of Economic Entomology 97(5), 1648-1658.

Robinson, W.E., Cordeiro, M., AbdelMalek, S., Jia, Q., Chow, S.A., Reinecke, M.G. and Mitchell, W.M. (1996) Dicaffeoylquinic acid inhibitors of human immunodeficiency virus integrase: Inhibition of the core catalytic domain of human immunodeficiency virus integrase. Molecular Pharmacology 50(4), 846-855.

Rossi, M., Goggin, F.L., Milligan, S.B., Kaloshian, I., Ullman, D.E. and Williamson, V.M. (1998) The nematode resistance gene Mi of tomato confers resistance against the potato aphid. Proceedings of the National Academy of Sciences of the United States of America 95(17), 9750-9754.

Rousseaux, M.C., Jones, C.M., Adams, D., Chetelat, R., Bennett, A. and Powell, A. (2005) QTL analysis of fruit antioxidants in tomato using Lycopersicon pennellii introgression lines. Theoretical and Applied Genetics 111(7), 1396-1408.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V. and Quackenbush, J. (2003) TM4: A free, open-source system for microarray data management and analysis. Biotechniques 34(2), 374-378.

Saeidi, Z., Mallik, B. and Kulkarni, R. (2007) Inheritance of glandular trichomes and two-spotted spider mite resistance in cross *Lycopersicon esculentum* "Nandi" and *L. pennellii* "LA2963". Euphytica 154(1), 231-238.

Satake, T., Kamiya, K., An, Y., Oishi, T. and Yamamoto, J. (2007) The anti-thrombotic active constituents from Centella asiatica. Biological & Pharmaceutical Bulletin 30(5), 935-940.

Scalbert, A., Morand, C., Manach, C. and Remesy, C. (2002) Absorption and metabolism of polyphenols in the gut and impact on health. Biomedecine & Pharmacotherapy 56(6), 276-282.

Schauer, N. and Fernie, A.R. (2006) Plant metabolomics: towards biological function and mechanism. Trends in Plant Science 11(10), 508-516.

Schauer, N., Semel, Y., Roessner, U., Gur, A., Balbo, I., Carrari, F., Pleban, T., Perez-Melis, A., Bruedigam, C., Kopka, J., Willmitzer, L., Zamir, D. and Fernie, A.R. (2006) Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. Nature Biotechnology 24(4), 447-454.

Schauer, N., Zamir, D. and Fernie, A.R. (2005) Metabolic profiling of leaves and fruit of wild species tomato: a survey of the Solanum lycopersicum complex. Journal of Experimental Botany 56(410), 297-307.

Schilmiller, A., Shi, F., Kim, J., Charbonneau, A.L., Holmes, D., Jones, A.D. and Last, R.L. (2010) Mass spectrometry screening reveals widespread diversity in trichome specialized metabolites of tomato chromosomal substitution lines. Plant Journal 62(3), 391-403.

Shadle, G.L., Wesley, S.V., Korth, K.L., Chen, F., Lamb, C. and Dixon, R.A. (2003) Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of L-phenylalanine ammonia-lyase. Phytochemistry 64(1), 153-161.

Shapiro, J.A., Steffens, J.C. and Mutschler, M.A. (1994) Acylsugars of the wild tomato *Lycopersicon Pennellii* in relation to geographic distribution of the species. Biochemical Systematics And Ecology 22(6), 545-561.

Shipp, J.L., Wang, K. and Binns, M.R. (2000) Economic injury levels for western flower thrips (Thysanoptera : Thripidae) on greenhouse cucumber. Journal of Economic Entomology 93(6), 1732-1740.

Silva, B.A., Ferreres, F., Malva, J.O. and Dias, A.C.P. (2005) Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. Food Chemistry 90(1-2), 157-167.

Silva, V.D., Cardoso, M.D., de Moraes, J.C., Pimentel, F.A., Goncalves, L.D. and Neri, D.K.P. (2008) Characterization and evaluation of synthetic acylsugar on the behavior of the whitefly *Bemisia tabaci* (Gennadius, 1886) b biotype (Hemiptera: Aleyrodidae) in tomato plant. Ciencia e Agrotecnologia 32(5), 1408-1412.

Simmons, A.M. (1994) Oviposition on vegetables by *Bemisia tabaci* (Homoptera, Aleyrodidae) - Temporal and leaf surface factors. Environmental Entomology 23(2), 381-389.

Simmons, A.T. and Gurr, G.M. (2005) Trichomes of *Lycopersicon* species and their hybrids: effects on pests and natural enemies. Agricultural and Forest Entomology 7(4), 265-276.

Simmons, A.T., Gurr, G.M., McGrath, D., Martin, P.M. and Nicol, H.I. (2004) Entrapment of *Helicoverpa armigera* (Hubner) (Lepidoptera : Noctuidae) on glandular trichomes of *Lycopersicon* species. Australian Journal of Entomology 43(2), 196-200.

Simmons, A.T., Gurr, G.M., McGrath, D., Nicol, H.I. and Martin, P.M. (2003) Trichomes of *Lycopersicon* spp. and their effect on *Myzus persicae* (Sulzer) (Hemiptera : Aphididae). Australian Journal of Entomology 42(4), 373-378.

Singh, P. (1983) A general-purpose laboratory diet mixture for rearing insects. Insect Science and its Application 4(4), 357-362.

Snyder, J.C. and Carter, C.D. (1985) Trichomes on Leaves of Lycopersicon-Hirsutum, Lycopersicon-Esculentum and Their Hybrids. Euphytica 34(1), 53-64.

Snyder, J.C., Simmons, A.M. and Thacker, R.R. (1998) Attractancy and ovipositional response of adult Bemisia argentifolii (Homoptera : Aleyrodidae) to type IV trichome density on leaves of Lycopersicon hirsutum grown in three day-length regimes. Journal of Entomological Science 33(3), 270-281.

Summers, C.B. and Felton, G.W. (1994) Prooxidant effects of phenolic acids on the generalist herbivore Helicoverpa zea (Lepidoptera: Noctuidae): Potential mode of action for phenolic compounds in plant anti-herbivore chemistry. Insect Biochemistry and Molecular Biology 24(9), 943-953.

Tanksley, S.D. and McCouch, S.R. (1997) Seed banks and molecular maps: Unlocking genetic potential from the wild. Science 277(5329), 1063-1066.

Teulon, D.A.J. (1992) Laboratory technique for rearing western flower thrips (Thysanoptera, Thripidae). Journal of Economic Entomology 85(3), 895-899.

Thoquet, P., Olivier, J., Sperisen, C., Rogowsky, P., Prior, P., Anais, G., Mangin, B., Bazin, B., Nazer, R. and Grimsley, N. (1996) Polygenic resistance of tomato plants to bacterial wilt in the French West Indies. Molecular Plant-Microbe Interactions 9(9), 837-842.

Tikunov, Y., Lommen, A., de Vos, C.H.R., Verhoeven, H.A., Bino, R.J., Hall, R.D. and Bovy, A.G. (2005) A novel approach for nontargeted data analysis for metabolomics. Large-scale profiling of tomato fruit volatiles. Plant Physiology 139(3), 1125-1137.

Tommasini, M.G. and Maini, S. (1995) *Frankliniella occidentalis* and other thrips harmful to vegetables and ornamental crops in Europe. Wageningen Agricultural University Papers 95(1), 1-42.

Treutter, D. (2006) Significance of flavonoids in plant resistance: a review. Environmental Chemistry Letters 4(3), 147.

Trugo, L.C. and Macrae, R. (1984) Chlorogenic acid composition of instant coffees. Analyst 109(3), 263-266.

Tsao, R., Marvin, C.H., Broadbent, A.B., Friesen, M., Allen, W.R. and McGarvey, B.D. (2005) Evidence for an isobutylamide associated with host-plant resistance to western flower thrips, *Frankliniella occidentalis*, in chrysanthemum. Journal of Chemical Ecology 31(1), 103-110.

Uitenbroek, D.G. (1997) Binomial. SISA. http://www.quantitativeskills.com/sisa/ditributions/binomial.htm.

Ulbrich, B. and Zenk, M. (1979) Partial purification and properties of p-hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase from higher plants. Phytochemistry 18(8), 929 - 933.

van der Hoeven, R., Monforte, A., Breeden, D., Tanksley, S. and Steffens, J. (2000) Genetic control and evolution of sesquiterpene biosynthesis in Lycopersicon esculentum and L. hirsutum. The Plant Cell Online 12(11), 2283.

Verpoorte, R., Choi, Y. and Kim, H. (2007) NMR-based metabolomics at work in phytochemistry. Phytochemistry Reviews 6(1), 3-14.

Verpoorte, R., Choi, Y. and Kim, H. (2010) Metabolomics: Will it Stay? Phytochemical Analysis 21(1), 2-3.

Verpoorte, R., Choi, Y., Mustafa, N. and Kim, H. (2008) Metabolomics: back to basics. Phytochemistry Reviews 7(3), 525-537.

Villegas, R. and Kojima, M. (1986) Purification and characterization of Hydroxycinnamoyl D-Glucose: quinate hydroxycinnamoyl transferase in the root of sweet potato, Ipomoea batatas lam. The Journal of Biological Chemistry 261, 8729 - 8733.

Walters, D.S. and Steffens, J.C. (1990) Branched-chain amino-acid-metabolism in the biosynthesis of *lycopersicon-pennellii* glucose esters. Plant Physiology 93(4), 1544-1551.

Wang, T., Jiang, X., Yang, L. and Wu, S. (2008) pH-gradient counter-current chromatography isolation of natural antioxidant chlorogenic acid from *Lonicera japonica* Thumb. using an upright coil planet centrifuge with three multi-layer coils connected in series. Journal of Chromatography A 1180(1-2), 53-58.

Welter, S.C., Rosenheim, J.A., Johnson, M.W., Mau, R.F.L. and Gusukumaminuto, L.R. (1990) Effects of thrips-palmi and western flower thrips (Thysanoptera, Thripidae) on the yield, growth, and carbon allocation pattern in cucumbers. Journal of Economic Entomology 83(5), 2092-2101.

Weston, P., Johnson, D., Burton, H. and Snyder, J. (1989) Trichome secretion composition, trichome densities, and spider mite resistance of ten accessions of *Lycopersicon hirsutum*. Journal of the American Society for Horticultural Science 114(3), 492-498.

Widarto, H.T., Van Der Meijden, E., Lefeber, A.W.M., Erkelens, C., Kim, H.K., Choi, Y.H. and Verpoorte, R. (2006) Metabolomic differentiation of Brassica rapa following herbivory by different insect instars using two-dimensional nuclear magnetic resonance spectroscopy. Journal of Chemical Ecology 32(11), 2417-2428.

Wijkamp, I., VandeWetering, F., Goldbach, R. and Peters, D. (1996) Transmission of tomato spotted wilt virus by Frankliniella occidentalis; Median acquisition and inoculation access period. Annals of Applied Biology 129(2), 303-313.

Wildman, R.E.C., ed. (2007) Handbook of nutraceuticals and functional foods. 2nd ed. CRC Press, Boca Raton, FL.

Williamson, G., Day, A.J., Plumb, G.W. and Couteau, D. (2000) Human metabolic pathways of dietary flavonoids

and cinnamates. Biochemical Society Transactions 28(Part 2), 16-22.

Xiang, T., Xiong, Q.B., Ketut, A.I., Tezuka, Y., Nagaoka, T., Wu, L.J. and Kadota, S. (2001) Studies on the hepatocyte protective activity and the structure-activity relationships of quinic acid and caffeic acid derivatives from the flower buds of *Lonicera bournei*. Planta Medica 67(4), 322-325.

Yano, K. (1987) Minor components from growing buds of *Artemisia capillaris* that act as insect antifeedants. Journal of Agricultural and Food Chemistry 35(6), 889-891.

Zamir, D. (2001) Improving plant breeding with exotic genetic libraries. Nature Reviews Genetics 2(12), 983-989.

Zhang, Y. and Cremer, P.S. (2006) Interactions between macromolecules and ions: the Hofmeister series. Current Opinion in Chemical Biology 10(6), 658-663.

Summary and general discussion

Western flower thrips (WFT), *Frankliniella occidentalis*, is one of the most serious crop pests worldwide, whose control represents a major challenge for both horticulture and floriculture. The development of insecticide resistance and the unsatisfactory performance of existing control methods puts a lot of pressure on pest management for the introduction of new and complementary crop protection strategies against WFT, among which host plant resistance remains as the ideal alternative. In this context the goal of the project was to scrutinize the genus *Solanum* searching for the bases of thrips resistance in tomato.

The workings of different potential resistance mechanisms against WFT in tomato were identified, contrasted and evaluated in this thesis. In general the bases of thrips resistance in tomato were unveiled and described. The objective of this project was therefore successfully and satisfactorily met.

The first chapter contains the general introduction whereby the background and justification of the project were presented. In chapter 2 a thrips-resistance and chemical survey of *Solanum* was conducted. Foliar parts of prominent wild and domesticated tomatoes were challenged with thrips herbivory and analyzed by untargeted ^{1}H NMR metabolomics. Results showed that thrips resistance was related to acylsugars. These glycolipids are viscous metabolites exudated by the glandular trichomes, specifically by type-4 glands, in *Solanum* spp. No chemical compounds in the lamella of tomato leaves could be identified in this trial as resistance factors against WFT. In a second experiment (Chapter 3) the survey was extended to other accessions in order to explore in depth the allelochemical potential against WFT of all known trichomal defenses in *Solanum*. In this part different resistance mechanisms in which mechanical and chemical defenses work coordinately to fend thrips off were observed. In all cases thrips come in contact with exudates from glandular trichomes, mainly of the types 4 and 6. These exudates involve different defense strategies. For instance, acylsugars and sesquiterpenic carboxylic acids, as oily substances, entangle the insects in between the trichomes, preventing thus thrips from reaching the leaf surface to feed on. This resistance mechanism proved itself effective in deterring WFT provided that hairiness and exudate yield were very high. Long-chain methylketones in the exudate, as broad-spectrum insecticides, showed contact toxicity to WFT. However, efficient detoxification mechanisms in WFT limit the effectiveness of these toxins. Thrips recover quickly from the knocking out effect of these ketones once exposure to them ceases. Surprisingly, the most effective and efficient defense mechanism was that observed among the domesticated tomatoes (*Solanum lycopersicum*). Polyphenol oxidase-catalyzed polymerization of rutin upon rupture of type-6 glandular trichomes on *S. lycopersicum* literally welds thrips to the leaf surface. When the density of these glands is sufficiently high, like in the yet unidentified cultivar LXX, the insect unavoidably tears a lot of type-6 glands accumulating in consequence an amount of polymers high enough to immobilize it. This defense mechanism, described before in relation to other insect species, worked very well on WFT. In contrast to other defenses, it prevented thrips from feeding on more plants, thereby avoiding

any further virus spread. Cultivar LXX, therefore, represents a promising source of resistance to this pernicious herbivore, which may alleviate the extraordinary pressure currently existing on plant sciences to come up with alternatives for crop protection against major pests. In addition, considering that the greatest danger posed by thrips on tomato is the transmission of devastating tospoviruses, such as tomato spotted wilt virus and impatiens necrotic spot virus, this resistance strategy certainly is the ideal defense mechanism for tomato and possibly other crops. The fact that such trait is already printed in the genome of *S. lycopersicum* makes it a unique and exceptional opportunity to realize thrips resistance in all susceptible cultivars.

The genetics of chemically-based thrips resistance in tomato was also investigated in a further step (Chapter 4). A chromosomal substitution set consisting of 76 introgression lines between *S. pennellii* LA716 and *S. lycopersicum* var. Moneymaker, was chemotyped by NMR metabolic profiling and screened for thrips resistance. Nine quantitative trait loci for thrips resistance and 268 for foliar metabolites were independently identified. Unfortunately, none of the 26 detected foliar metabolites was associated with thrips resistance.

Another important candidate group of allelochemicals explored in this project was the phenolics, specifically the esters of hydroxycinnamic acids, of which chlorogenic acid (5-caffeoylquinic acid) is one of the most widely distributed in the plant kingdom. To evaluate their potential as chemical defenses against WFT and discover possible structure effects on their anti-insect activity a series of nine hydroxycinnamoylquinic acids (HCQA) were isolated from the richest source known in nature, green coffee beans (Chapter 5). The great versatility and preparative capacity of countercurrent separations, in particular centrifugal partition chromatography, were exploited in this project to isolate the major HCQAs present in green coffee beans, namely caffeoylquinic acid, feruloylquinic acid and dicaffeoylquinic acid. The respective regioisomers of these HCQAs were purified as well. Because of the significant solubility differences between these metabolites a novel gradient method based on salting-out effects was developed to control the elution of these analytes. The separation of 5-caffeoylquinic acid, 5-feruloylquinic acid, and 3,5-dicaffeoylquinic acid was successfully achieved by means of a salting-out gradient using ethyl acetate-hexane as the stationary phase and an ionic gradient of LiCl as the mobile phase in one case and $(NH_4)_2SO_4$-KNO_3 in another. Despite the greater analytical range and the scaling-up superiority of countercurrent separations compared to other chromatographic methods these techniques still have not gained wide popularity. Poor automation and laborious method development are characteristic features of countercurrent separations that discourage potential new users. Such drawbacks prevent countercurrent separations from competing with predominant liquid chromatography to find their place in every single science laboratory.

In the next and final part of the project the activity of 5-caffeoylquinic acid and its regioisomers, 3-CQA and 4-CQA, against thrips larvae was investigated (Chapter 6). These phenolic metabolites were assayed in artificial diet bioassays at a concentration of 1% with and without polyphenol oxidase. Results suggest that the anti-insect properties of CQA extensively reported in the scientific literature may not be connected to its reactive catechol moiety but to its high acidity, in contradiction with the main predicted modes of action. This supports the hypothesis that insects, as opposed to pathogens, have different ways to neutralize the adverse physiological effects known to phenolic compounds. The strong influence of pH observed on larval performance also suggests that increased vacuolar accumulation of organic acids, such as chlorogenic acid, in otherwise susceptible foliar tissue may contribute to host-plant resistance against herbivores with neutral or alkaline digestive tracts.

Many generalizations are often made about the activity of phenolic compounds. However, the results of these bioassays warn plant scientists about the dangers of such assumptions. As a very diverse class of secondary metabolites phenolics and their role in plant-insect interactions should be evaluated on a case-to-case basis as long as their specific modes of action are not fully elucidated.

Due to the complexity of the chemistry that controls the interaction between organisms as well as their response to abiotic factors, metabolomics, as the most comprehensive analytical method, should be the default tool in life sciences to investigate changes on the metabolic level. The need for holistic approaches in systems biology has made metabolic profiling a standard practice nowadays. However, metabolomics is still far from meeting its original goal since no analytical platform or combination of them can produce a full analysis of the metabolome. Nuclear magnetic resonance for example, albeit offering great throughput, analytical range, elucidation and reproducibility advantages unfortunately lacks in sensitivity compared to MS-based technologies. In this project many important primary and secondary foliar metabolites were identified in tomato but those responsible for the observed variation in thrips resistance were in some instances elusive to the ^{1}H NMR analysis. Because of this relative insensitivity drawback of NMR, an important class of allelochemicals could not be profiled to complete the whole picture of potential defenses against thrips in *Solanum*, the glycoalkaloids. Glycoalkaloids, such as tomatine, are very well known effective plant defenses especially abundant in *Solanum*. Glycoalkaloids have received little attention in the past decades as a promising anti-insect alternative due mainly to their inactivation upon binding with membrane phytosterols. However, this complex formation that certainly favors chewing insects may not take place in piercing-sucking herbivores like WFT, where alkaloid uptake may occur before sterol binding. The genetic and hence chemical diversity harbored by the numerous wild *Solanum* species offers a perfect system to conduct structure-activity relationship studies on the effect of this relevant class of allelochemicals on thrips. Glycoalkaloids with a strong negative effect on WFT could join the set of effective resistance traits hereby described to constitute a multi-target defense system whereby a sustainable and stable thrips resistance in the domesticated tomatoes could be realized.

Samenvatting

Californische thrips (WFT), *Frankliniella occidentalis*, is wereldwijd een belangrijke plaag, die onder controle te krijgen is een grote uitdaging voor de tuinbouw en de bloementeelt. De ontwikkeling van resistentie tegen insecticiden door thripsen legt een grote druk op het ontwikkelen van nieuwe en complementaire strategieën voor gewasbescherming tegen WFT. Van deze nieuwe benaderingen lijkt de waardplantresistentie de ideale oplossing. Daarom was het doel van het project binnen het geslacht *Solanum* te zoeken naar de basis van thrips resistentie in tomatenplanten.

De werking van verschillende potentiële resistentiemechanismen tegen WFT in tomaat werden geïdentificeerd, vergeleken en geëvalueerd in dit proefschrift. De basis van de weerstand tegen thrips in tomaten werd geïdentificeerd en is in dit proefschrift beschreven. Het doel van dit project is daarmee bereikt.

Het eerste hoofdstuk bevat de algemene introductie, waarbij de achtergrond en rechtvaardiging van het project worden gepresenteerd, verder wordt kort een overzicht van het proefschrift geschetst. In hoofdstuk 2 wordt een overzicht gegeven van de belangrijkste wilde en gedomesticeerde tomaten variëteiten. Delen van bladeren van alle variëteiten werden onderworpen aan thrips herbivorie en geanalyseerd met ^{1}H NMR-gebaseerde metabolomics. De resultaten toonden aan dat thrips resistentie gerelateerd is met acyl-suikers. Deze glycolipiden zijn visceuze metabolieten die uitgescheiden worden door glandulaire trichomen, met name door de type-4 klieren, die het blad van veel *Solanum* spp. bedekken. In het blad zelf van de tomatenplant konden geen stoffen konden worden geïdentificeerd die gerelateerd waren met de resistentie tegen de WFT.

In een tweede experiment (hoofdstuk 3) werd het onderzoek uitgebreid tot andere variëteiten om in meer detail de allelochemische potentie van de trichomale afweer in *Solanum* tegen WFT te onderzoeken. In dit onderzoek bleken verschillende resistentiemechanismen gebaseerd op mechanische en chemische afweer samen te werken in de afweer tegen thrips. Op het blad komen thrips in contact met het exudaat van de glandulaire trichomen, met name van het type 4 en 6. Deze exudaten vertegenwoordigen verschillende verdedigingsstrategieën. Bijvoorbeeld de olieachtige acylsuikers en sesquiterpeencarbonzuren verstrikken de insecten tussen de trichomen, waardoor thrips het bladoppervlak niet meer kunnen bereiken. Dit mechanisme bleek effectief in de afweer tegen WFT bij een dichte beharing en een hoge exudaat opbrengst. Lange-keten methylketonen uit het exudaat, bekend als breed-spectrum insecticiden, hebben contact-toxiciteit op WFT. Echter, efficiënte ontgiftingsmechanismen in WFT beperken de effectiviteit van deze gifstoffen. Thrips herstellen snel van het effect van deze ketonen nadat blootstelling aan de insecticiden ophoudt. Verrassend is dat het meest effectieve en efficiënte verdedigingsmechanisme werd ontdekt bij gedomesticeerde tomaten, *Solanum lycopersicum*. Polyfenoloxidase-gekatalyseerde polymerisatie van rutine die vrijkomt bij de breuk van het type-6 glandulaire trichomen lassen letterlijk thrips vast aan het bladoppervlak. Wanneer de dichtheid van deze klieren hoog genoeg is, zoals in de nog niet-geïdentificeerde cultivar LXX, beschadigt

het insect onvermijdelijk type-6 klieren waarbij voldoende polymeer gevormd wordt om het insect te immobiliseren. Dit afweermechanisme werkt zeer goed op WFT en voorkomt het voeden van de insecten op het blad, in tegenstelling tot andere verdedigingssystemen, voorkomt het ook de verdere verspreiding van het insect naar andere planten en daarmee ook van virussen. Cultivar LXX vormt derhalve een veelbelovende bron van de resistentie tegen deze verderfelijke herbivoor, en verlicht daarmee de druk op de plantwetenschappen om te komen met alternatieven voor gewasbescherming tegen belangrijke plagen. Deze vorm van resistentie is het ideale afweermechanisme voor tomaat en eventueel andere gewassen omdat het grootste gevaar van thrips de overdracht is van verwoestende tospoviruses, zoals tomaat wilt virus en impatiens necrotische vlek virus naar de plant. Het feit dat deze eigenschap al is vastgelegd in het genoom van *S. lycopersicum* maakt het tot een unieke kans om thrips resistentie te realiseren in alle gevoelige cultivars.

De genetica van chemie-gebaseerde thrips resistentie in tomaat werd ook onderzocht (hoofdstuk 4). Een chromosomale substitutie set bestaande uit 76 introgressie lijnen tussen *S. pennellii* LA716 en *S. lycopersicum* var. Moneymaker werd gechemotypeerd met behulp van NMR gebaseerde metaboliet profilering en gescreend voor thrips resistentie. Negen kwantitatieve trait loci (QTL) voor thrips resistentie en 268 voor metabolieten in het blad werden onafhankelijk van elkaar geïdentificeerd. Helaas is geen van de 26 geïdentificeerde metabolieten in het blad geassocieerd met thrips resistentie. Hogere gevoeligheid is vereist om mogelijk de verbindingen te identificeren die verantwoordelijk zijn voor de verschillen in herbivorie in de introgressie populatie.

Een andere belangrijke groep van kandidaat-allelochemicals die werd onderzocht in dit project was die van de fenolen, in het bijzonder de esters van hydroxykaneelzuren. Chlorogeenzuur (5-caffeoylquinic zuur) is een van de meest wijd verspreide vertegenwoordigers van deze klasse van stoffen in het plantenrijk. Om hun potentieel als chemische afweerstoffen tegen WFT en structuur-activiteits effecten te onderzoeken werd een serie van negen hydroxycinnamoylquinic zuren (HCQA) geïsoleerd uit de rijkste bekende natuurlijke bron: groene koffiebonen (hoofdstuk 5). De grote veelzijdigheid en de preparatieve mogelijkheden van counter-current vloeistofchromatografie, en in het bijzonder van centrifugale verdelingschromatografie, werden uitgebuit in dit project om de HCQAs te isoleren uit groene koffiebonen, en met name caffeoylquinic zuur, feruloylquinic zuur en dicaffeoylquinic zuur. De respectievelijke regio-isomeren van deze HCQAs werden ook gezuiverd. Vanwege de aanzienlijke verschillen in de oplosbaarheid van deze metabolieten werd een nieuwe, op uit-zouten gebaseerde, gradient methode ontwikkeld voor de scheiding van deze stoffen. De scheiding van de 5-caffeoylquinic zuur, 5-feruloylquinic zuur, en 3,5-dicaffeoylquinic zuur werd bereikt met ethyl acetaat-hexaan als de stationaire fase en een ionische gradiënt van LiCl of $(NH_4)_2SO_4$-KNO_3 in water als de mobiele fase. Ondanks het grotere analytische bereik en de superieure opschalingsmogelijkheden van counter-current chromatografie in vergelijking met andere chromatografische methoden genieten deze technieken nog geen wijde populariteit. Slechte automatisering en bewerkelijke methodeontwikkeling zijn kenmerken van counter-current chromatografie die potentiële nieuwe gebruikers ontmoedigen. Deze nadelen voorkomen dat counter-current chromatografie een concurrent is van de nu overheersende vloeistofchromatografie voor een plaats in elk laboratorium.

In het laatste deel van het project werd de activiteit van 5-caffeoylquinic zuur en de regio-isomeren, 3-CQA en 4-CQA, tegen thrips larven onderzocht (hoofdstuk 6). Deze fenolische metabolieten werden getest in een kunstmatige voeding bioassay bij een concentratie van 1% met en zonder polyfenoloxidase. De resultaten suggereren dat de uitvoerig in de wetenschappelijke literatuur gerapporteerde

anti-insecten eigenschappen van CQA niet gerelateerd zijn aan de reactieve catechol groep, maar aan de sterk zure eigenschappen, dus in tegenspraak met het nu belangrijkste gedachte werkingsmechanisme. Dit ondersteunt de hypothese dat insecten, in tegenstelling tot pathogenen, op verschillende manieren de negatieve fysiologische effecten van fenolische verbindingen kunnen neutraliseren. De sterke invloed van de pH die werd waargenomen op de prestaties van larven, suggereert ook dat een toename van de vacuolaire accumulatie van organische zuren, zoals chlorogeenzuur in niet-resistent blad weefsel kan leiden tot resistentie van de waardplant tegen planteneters met een neutraal of alkalisch spijsverteringsstelsel. Veel generalisaties zijn er gemaakt over de biologische activiteit van fenolische verbindingen. Echter, de hier beschreven resultaten van bioassays zijn een waarschuwing voor plantenwetenschappers over de gevaren van dergelijke aannames. Zolang het specifieke werkingsmechanisme niet volledig is opgehelderd, moet voor een zeer diverse klasse van secundaire metabolieten als die van de fenolen hun rol in de plant-insect interacties van geval tot geval worden beoordeeld.

Vanwege de complexe chemie van de interactie tussen organismen en hun reactie op abiotische factoren, moet metabolomics, als de meest holistische analytische methode, het standaard gereedschap zijn in life sciences om veranderingen op in het metaboloom te onderzoeken. De noodzaak van een holistische aanpak in de systeembiologie heeft metabolomics gemaakt tot een standaard methode. Echter, metabolomics is nog ver van het ultieme doel, omdat geen enkel analytisch platform of een combinatie daarvan een volledige analyse van het metaboloom kan produceren. Nucleaire magnetische resonantie spectroscopie bijvoorbeeld, ondanks de voordelen van high-throughput, een groot analytisch bereik, structuuropheldering en reproduceerbaarheid, ontbeert gevoeligheid ten opzichte van MS-gebaseerde technologieën. In dit project zijn veel belangrijke primaire en secundaire metabolieten uit bladmateriaal geïdentificeerd in de tomatenplant, maar secondaire metabolieten die mogelijk verantwoordelijk zijn voor de waargenomen variatie in resistentie tegen thrips zijn niet geidentificeerd met de ^{1}H NMR-analyses. Vanwege deze relatieve ongevoeligheid van NMR kon een belangrijke klasse van *Solanum* allelochemicals, de glycoalkaloïden, niet worden geprofileerd. En deze ontbreken dus nog in het verhaal van de mogelijke afweermechanismen tegen thrips. Glycoalkaloïden, zoals tomatine, zijn welbekende effectieve afweerstoffen van planten, en komen veel voor in *Solanum* soorten. In de afgelopen decennia hebben glycoalkaloïden weinig aandacht gekregen als veelbelovend alternatieve anti-insecten stoffen. Dit is voornamelijk te wijten aan hun inactivering door plantensterolen. Deze beperking is zeker een voordeel voor blad -etende insecten, maar niet voor WFT die alleen de vloeibare celinhoud opzuigen. De genetische en dus chemische diversiteit aanwezig in de talrijke wilde *Solanum* soorten biedt dus een perfect systeem voor onderzoek naar de structuur-activiteits relaties van deze relevante klasse van allelochemicals op thrips en dit te exploiteren als een aanvullend afweersysteem tegen WFT in de tomatenplant. Glycoalkaloïden met een sterk effect tegen WFT zouden een aanvulling kunnen zijn op de set van de hierboven beschreven effectieve afweersystemen en deel zijn van een multi-target afweersysteem voor een duurzame en stabiele thrips resistentie in de gedomesticeerde tomatenplanten.

Resumen

El trips occidental de las flores (TOF), *Frankliniella occidentalis*, es hoy dia una de las plagas mas perniciosas a nivel mundial, cuyo control representa un verdadero desafío tanto para la horticultura como para la floricultura. El desarrollo de resistencia a insecticidas y el pobre desempeño de los metodos de control existentes hacen apremiante la introducción de nuevas y complementarias estrategias de protección de cultivos contra el TOF; de entre las cuales la resistencia natural de la planta huésped sigue siendo la alternativa ideal. Dentro de éste contexto el presente proyecto tuvo como objetivo el escrutinio del género *Solanum* en busca de las bases químicas y/o morfológicas de la resistencia al trips.

Diferentes mecanismos de resistencia al trips fueron identificados, comparados y evaluados en esta tesis. Puede afirmarse que en general las bases de la resistencia al trips en tomate fueron develadas y descritas; y en consecuencia el propósito de este proyecto fue satisfactoria alcanzado.

El primer capítulo de esta tesis contiene una introducción general, donde se presentaron parte de los antecedentes y la justificación del proyecto. En el segundo capítulo se evaluó una selección diversa de especies del género *Solanum* en términos de la resistencia al trips y de la composición química foliar. La hojas de selectos tomates salvajes y domésticos fueron expuestas al trips y analizadas por metabolómica de resonancia magnética nuclear (RMN). Los resultados mostraron que la resistencia al trips en tomate está relacionada con la presencia de acilazúcares. Estos glucolípidos son compuestos viscosos exudados por los tricomas glandulares del tomate, específicamente por los de tipo 4. Por otro lado, ninguno de los compuestos detectados en la lamela de las hojas de tomate fue identificado en esta experiencia como factor de resistencia al trips. Con el propósito de evaluar a profundidad el potencial aleloquímico de todas las defensas tricómicas conocidas en el género *Solanum* el número de especies y cultivares fue ampliado en un segundo experimento (capítulo 3). En esta oportunidad se observaron diferentes mecanismos de resistencia, en donde defensas químicas y mecánicas operan de forma claramente sinérgica para resguardar las hojas de la herbivoría. En cualquiera de los mecanismos los insectos entran en contacto con el exudado de los tricomas glandulares, principalmente aquellos de tipo 4 y 6. Dichos exudados implican diferentes estrategias de defensa. Los acilazúcares y los acidos carboxílicos sesquiterpénicos, por ejemplo, en su condición de sustancias aceitosas atrapan al insecto entre los tricomas, evitando asi que el mismo alcance la superficie foliar para alimentarse. Éste mecanismo de resistencia es efectivo únicamente si la densidad de tricomas y la cantidad de exudado son relativamente altas. Las metilcetonas por otro lado, conocidos insecticidas naturales de amplio espectro, mostraron toxicidad por contacto sobre el trips. Sin embargo, los mecanismos de destoxificación en el TOF limitan la efectividad de dichas toxinas. Los insectos se recuperan rápidamente de los efectos anestésicos de las metilcetonas una vez que cesa la exposición a las mismas. Inesperadamente, el mecanismo de defensa más efectivo observado contra el trips fue aquel presente en los tomates domésticos (*S. lycopersicum*). La polimerización de la rutina, catalizada por la polifenoloxidasa, que tiene lugar al romperse los tricomas tipo 6, literalmente solda las patas de

los trips a la superficie foliar. Cuando la densidad de dichos tricomas es alta, como en el caso del cultivar aun no identificado LXX, el insecto rompe numerosas glándulas acumulando sobre sus patas una cantidad de polímero suficientemente alta para inmobilizarlo. Dicho mecanismo de defensa, anteriormente descrito para otras especies de insectos, resultó ser muy efectivo también contra el TOF. A diferencia de los otros tipos de defensas, esta inmobilización evita que el trips prosiga alimentándose de más plantas, previniéndose asi la propagación de virus. El cultivar LXX representa por consiguiente una fuente promisoria de resistencia a éste pernicioso herbívoro, pudiéndo aliviarse asi la extraordinaria presión que existe hoy dia sobre la biología vegetal para que desarrolle formas alternativas de protección de cultivos. Además, si se tiene en cuenta que el TOF es vector transmisor de nefastos virus, como el virus de la mancha bronceada del tomate y el virus de la mancha necrótica del *impatiens*, esta última estrategia de defensa es sin duda el mecanismo ideal de protección contra el trips en tomate y posiblemente también en otros cultivos. El hecho de que dicha característica se encuentra ya impresa en el código genético de *S. lycopersicum* constituye una oportunidad única para el logro de la resistencia al trips en el resto de los tomates demésticos.

Las bases genéticas de la resistencia al trips en tomate fueron parcialmente investigadas en una siguiente fase (capítulo 4). Para tal efecto se tipificó químicamente a través de un análisis de perfil metabólico por RMN y evaluó en términos de resistencia al trips un sistema de sustitución cromosómica integrado por 76 lineas de introgresión entre *S. pennellii* LA716 y *S. lycopersicum* var. Moneymaker. Se identificaron de esta forma nueve (9) loci de carateres cuantitativos (QTL) para la resistencia al trips y 268 para metabolitos foliares. Desafortunadamente ninguno de los 26 metabolitos detectados mostró correlación con la resistencia al TOF.

Otro importante grupo de posibles aleloquímicos evaluados en éste proyecto fue el de los fenólicos, específicamente los ésteres de ácidos hridroxicinámicos; de entre los cuales el ácido clorogénico (ácido 5-cafeoilquínico) es el más ampliamente distribuido en el reino vegetal. Para examinar su potencial como defensas químicas contra el TOF y develar posibles efectos estructurales sobre la actividad anti-insecto de los mismo se aisló una serie de nueve (9) ácidos hidroxicinamoilquínicos (AHCQs) de la fuente natural más rica en éste grupo de fenólicos, las semillas verdes de café (capítulo 5). Los AHCQs mayoritarios de las semillas de café, es decir, ácido cafeoilquínico, ácido feruloilquínico y ácido dicafeoilquínico, fueron aislados gracias a la gran versatilidad y capacidad preparativa de la cromatografía de contracorriente, en particular la cromatografía de partición. Los correspondientes regioisómeros de dichos AHCQs también fueron aislados de la misma manera. Debido a la gran diferencia de solubilidad y/o polaridad entre éstos grupos de AHCQs se desarrolló un novedoso sistema de elución basado en un gradiente de efecto salido externo para poder controlar la elución de dichos analitos. La separación de ácido 5-cafeoilquínico, ácido 5-feruloilquínico y ácido 3,5-dicafeoilquínico se realizó satisfactoriamente gracias a un sistema cromatográfico conformado por una mezcla de acetato de etilo y hexano como fase estacionaria y un gradiente iónico de LiCl o $(NH_4)_2SO_4$-KNO_3 como fase móvil. A pesar del mayor rango analítico y de la superior capacidad de escalamiento de la cromatografía de contracorriente respecto a otros métodos de separación, dicha técnica aun no goza de amplia popularidad. El escazo nivel de automatización y la laboriosa optimización de método inherente a éste tipo de cromatografía son las características principales que disuaden a potenciales nuevos usuarios. Tales desventajas impiden que la cromatografía de contracorriente pueda competir con la predominante cromatografía líquida y encontrar asi un lugar en cada laboratorio.

En la siguiente y última parte del proyecto se investigó la actividad anti-insecto del ácido 5-cafeoilquínico y sus regioisómeros, ácido 3-cafeoilquínico y ácido 4-cafeoilquínico, contra larvas

del TOF (capítulo 6). Dichos metabolitos fueron evaluados en bioensayos de dieta artificial a una concentración de 1%, con y sin polifenoloxidasa. Los resultados de estos experimentos sugieren que la extensamente reportada actividad anti-insecto del ácido clorogénico puede no estar relacionada con el grupo reactivo catecólico sino con su alta acidez, contradiciéndo de esta manera los principales modos de acción predichos para tales compuestos. Estos resultados apoyan la hipótesis según la cual los insectos, a diferencia de los agentes patógenos, poseen diversos mecanismos con los cuales pueden neutralizar los efectos fisiológicos adversos que se le concocen a los fenólicos. El marcado efecto inhibitorio del pH observado sobre el desarrollo de las larvas sugiere al mismo tiempo que la acumulación vacular de ácidos orgánicos, tales como el ácido clorogénico, puede incrementar la resistencia a herviboros con tractos digestivos neutros o alcalinos. Con frecuencia se hacen muchas generalizaciones acerca de la bioactividad de los compuestos fenólicos. Sin embargo, los resultados de éstos bioensayos alertan a los investigadores en éste campo sobre los riesgos de tales suposiciones. Debido a la gran diversidad estructural existente en este grupo de compuestos los fenólicos y sus múltiples roles en las interaciones insecto-planta deben ser estudiados caso por caso hasta tanto sus modos de acción específicos no hayan sido dilucidados completamente.

Debido a la complejidad de la química que controla tanto las interacciones entre organismos como la respuesta de los mismos a factores abióticos, la metabolómica o análisis químico integral debería ser la herramienta analítica por omisión en las ciencias naturales para el estudio de sistemas biológicos a nivel metabólico. El enfoque holístico requerido por la biológia de sistemas ha hecho del análisis de perfil metabólico una práctica común. Sin embargo, la metabolómica dista mucho aun de su objetivo original debido a que ninguna técnica analítica, ni combinación de ellas, puede siquiera acercarse a producir un análisis del conjunto completo de metabolitos en un organismo. La RMN, por ejemplo, a pesar de ofrecer una gran capacidad de procesamiento de muestras, un amplio rango anlítico, una alta reproducibilidad y un inigualable poder de análisis estructural, desafortunadamente posee una sensibilidad considerablemente inferior a la de otras técnicas como la espectrometría de masas. En este proyecto numerosos metabolitos, primarios y secundarios, fueron identificados en las hojas de tomate. Sin embargo, aquellos responsables de la variacion en los niveles de resistencia al trips observada entre las diferentes species y cultivares en su mayoría no fueron detectados por ^{1}H RMN. Debido a esta baja sensibilidad relativa de la RMN no fue posible obtener el perfil químico de los glucoalcalides, una clase importante de aleloquímicos que pudieran constitiur una pieza importante en el conjunto de defensas químicas contra el trips en tomate. Es bien sabido que ciertos glucoalcalides, como la tomatina, son efectivos insecticidas particularmente abundantes en el género *Solanum*. En las últimas décadas estos alcaloides han recibido poca atención como posibles agentes anti-insecto debido fundamentalmente a la inactivación que sufren al acomplejarse con los fitoesteroles de las membranas celulares. No obstante, si bien la formación de estos complejos favorece a insectos masticadores, la misma pudiera no tener lugar en el tracto digestivo de insectos succionadores, como el trips, donde la absorción de los lalcaloides puede ocurrir en una extension significativa antes de unirse a los esteroles. La gran diversidad genética y por ende química albergada por las numerosas especies que integran el género *Solanum* representa un sistema idóneo para realizar estudios de estructura-actividad acerca del efecto de esta importante clase de aleloquímicos sobre el TOF. Glucoalcaloides con claros efectos negativos sobre el trips puedieran añadirse a la lista de factores de resistenca aqui descritos para asi formar un bloque de defensas multiples con el cual pudiera finalmente lograrse una resistencia estable al trips en tomate.

Curriculum Vitae

Roman R. Romero González was born on September 19, 1974 in Maracaibo, Venezuela. He attended Arzobispo Silva High School in Merida, Venezuela, between 1987 and 1992. While still in high school he obtained from the national government a prestigious scholarship to support his university education. He decided to pursue a career in chemistry and enrolled in 1993 the Chemistry Undergraduate School of Universidad de Los Andes in Merida, Venezuela, from which he graduated *Cum Laude* in 1999. For his undergraduate research project he worked on the glycation and aging of proteins. In 2000 he became a full-time faculty member of the teaching and research tenure staff of the same university. In this position he taught general and organic chemistry and conducted research in chemical ecology, more specifically on insecticides from natural sources. In 2006 he started his Ph.D. studies in the Department of Plant Ecology and Phytochemistry of Leiden University, The Netherlands, under the supervision of R. Verpoorte and P.G.L. Klinkhamer, working on the project that produced this thesis. He plans to apply in the future his knowledge of analytical chemistry and metabolomics to other challenges in systems biology.

First author

- Romero-González, R., Elwakiel M., Leiss, K., and Verpoorte, R. Chlorogenic acids and the effect of pH on thrips performance in artificial diet bioassays. *In preparation*.

- Romero-González, R., Mohammad, M., Leiss, K., and Verpoorte, R. Tomato´s best thrips shield: acylsugars, sesquiterpenes, methylketones or phenolics? *Submitted*.

- Mohammad, M., Romero-González, R., Leiss, K., Choi, Y.H., Verpoorte, R. and Klinkhamer, P.G.L. (2010) Metabolomic Analysis of Host Plant Resistance to Thrips in Wild and Cultivated Tomatoes. *Phytochem. Anal*. 21:110-117.

- Romero-González, R and Verpoorte, R. (2009) Salting-out gradients in centrifugal partition chromatography for the isolation of chlorogenic acids from green coffee beans. *J. Chrom. A*. 1216: 4245-4251.

- Romero-Gonzalez, R., Alonso-Amelot, M.E., Ávila, J.L. and Aubert, L. (2006) Labdane diterpenes from *Leonurus japonicus*. *Phytochemistry*. 67: 965-970.

Co-author

- Sumthong, P, Romero-González, R and Verpoorte, R. (2008) Identification of Anti-Wood Rot Compounds in Teak (*Tectona grandis* L.f.) Sawdust Extract. *J. Wood Chem. & Technol*. 28: 247-260.

- Gil, H., Salcedo, D. and Romero, R. (2005) Effect of phosphate buffer in the kinetics of glycation of proteins. *J. Phys. Org. Chem*. 18: 183-186.

- Alonso-Amelot, M.E., Arellano, E., Ávila, J.L., Aubert, L., Romero, R. et. al. (2004) A survey of Venezuelan plants toxic to livestock and their potential as anti-insect materials. In: Acamovic, T., Stewart, C.S. and Pennycott, T.W. (Eds), Poisonous plants and related toxins, pp. 471-476. London: CAB International.