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### Insight on the inside

*Phloem-based whitefly resistance in tomato*

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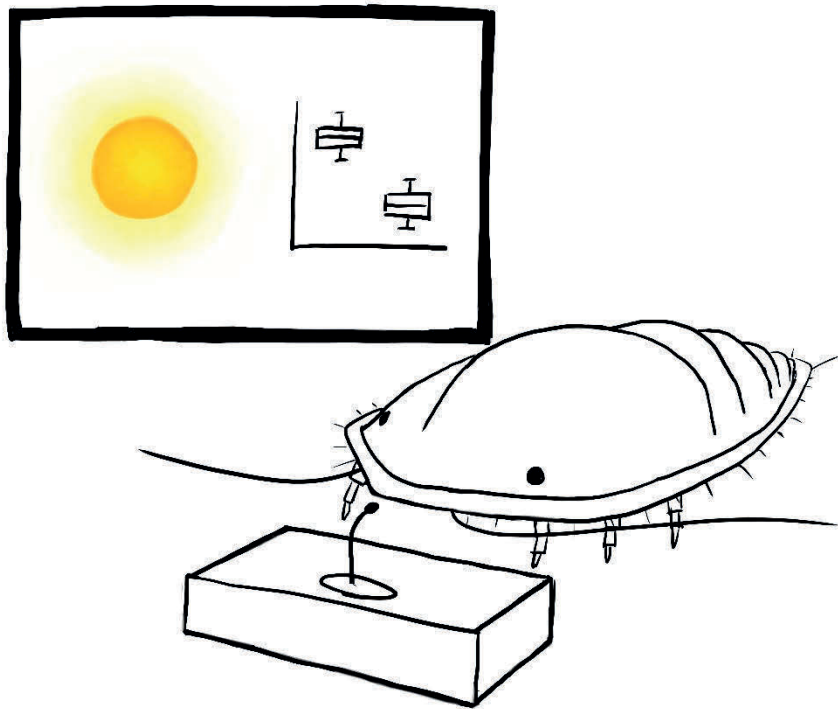
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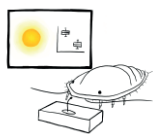
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# Chapter 6

## General discussion



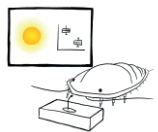
The phloem is one of the most difficult to reach tissues in a plant. Not only for insects, some of which have developed specialised mouthparts to reach the phloem, but also for researchers, who obviously lack such tools. Yet, the phloem is also one of the most fascinating tissues in a plant. It plays a key role in the transport of photosynthesis products from their source tissue to a sink, like flowers, fruit or meristems, as well as signalling molecules involved in long distance signalling, such as RNAs and peptides (Tegeeder & Hammes, 2018; De Marco *et al.*, 2018; Jensen, 2018; Lezzhov *et al.*, 2019; Schulze *et al.*, 2019). Most excitingly, the phloem also forms a battleground, as it is the site of interaction between plants and phloem-feeding insects. These phloem-based defence mechanisms include proteins and callose plugs that block sieve plates (Jiang *et al.*, 2019; Twayana *et al.*, 2022), but also specialised metabolites, as discussed in this thesis. Before reaching the phloem however, insects must overcome the defences on the outside of a plant. These outside defence layers can include physical barriers like thorns, spikes and cuticles (Lucas *et al.*, 2000; Zhao *et al.*, 2020), and chemical defences in the form of specialised metabolites produced and stored in dedicated structures, such as glandular trichomes, which can in some cases lead to especially strong resistances against phloem-feeding insects (Bleeker *et al.*, 2011; Alquézar *et al.*, 2017; Kortbeek *et al.*, 2021). It is thus not surprising that phloem-based defence mechanisms have not been considered ‘low-hanging fruit’ and have not been given the same scientific attention as the easier to reach trichomes, especially when it comes to specialised metabolites in those tissues. While there are 142 hits on Web of Science when searching for specialised metabolites in tomato trichomes, the same input with ‘phloem’ instead of ‘trichome’ results in only 11 hits, none of which are about the phloem itself, but about phloem-feeding



insects<sup>1</sup>. Nonetheless, we were able to find several examples in literature of other plant genera with phloem-based specialised metabolites that affect plant-insect interactions, as reviewed in **chapter 2**. Furthermore, previous findings, like a certain whitefly resistance in the wild tomato species *Solanum galapagense* that is not related to trichomes (Santegoets *et al.*, 2021), suggest that phloem-based defence mechanisms could play a role. The aim of this thesis was therefore to gain insight in the existence of phloem-based defence mechanisms against whiteflies (*Bemisia tabaci*) in wild tomato species and the involvement of specialised metabolites in such defence mechanisms. In this chapter, I will discuss why our findings indicate that such phloem-based defences can indeed be found in wild tomato independently of trichome-based defence and formulate a hypothesis for the underlying processes of a phloem-based resistance mechanism.

#### Phloem-based resistance: is it a resistance and is it based in the phloem?

Although the defence mechanism I studied did not provide the plants with a full resistance against whiteflies, I called it ‘phloem-based resistance’ and the resulting phenotype a ‘resistance phenotype’. Cambridge Dictionary defines ‘resistance’ as: “*the act of fighting against something that is attacking you, or refusing to accept something*” or “*a force that acts to stop the progress of something or make it slower*”, whereas ‘tolerance’ is defined as: “*the ability to deal with something unpleasant or annoying, or to continue existing despite bad or difficult conditions*” or “*an animal's or plant's ability not to be harmed by a drug or poison over a long period of time*”. I interpret the difference between ‘resistance’ and ‘tolerance’ as the first being a mechanism to stop or decrease the (spread of) an infestation,

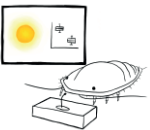


<sup>1</sup> The search results on Web of Science for the inputs “(tomato OR solanum) AND (specialised OR specialized OR secondary) AND metabolites AND trichome” and “(tomato OR solanum) AND (specialised OR specialized OR secondary) AND metabolites AND phloem” were compared on 18 November 2024.

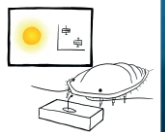
while the latter is an ability to reduce the impact of an infestation on the plants' fitness. In other words, the difference between fighting back on one hand, and damage control on the other. As discussed in **chapter 3**, a hampered whitefly nymph development will result in a substantial reduction in population size over multiple generations compared to a population with undisturbed development.

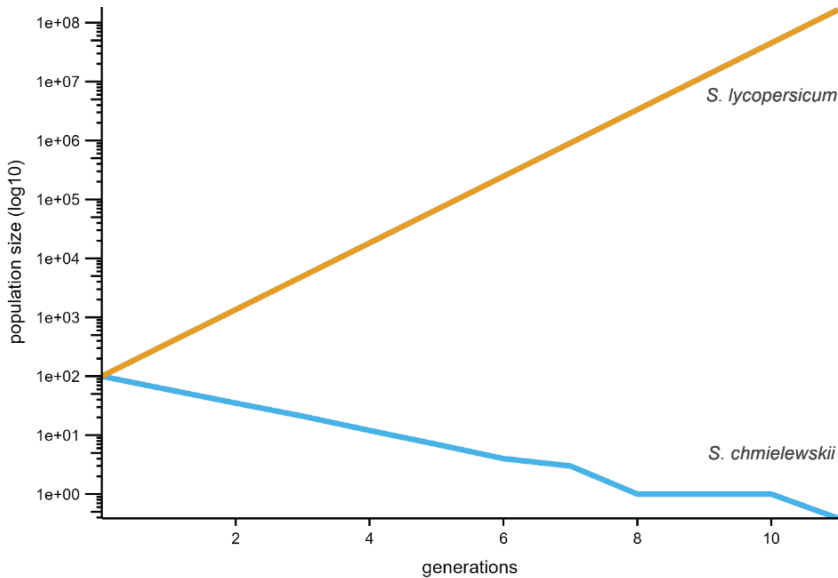
The tomato growing season, from young plants to the end of harvest, lasts between four and 11 months (Heuvelink, 2018). Based on the observations for this thesis, the average generation time of *B. tabaci* on tomato is one month, which translates to four to 11 generations per growing season. Let us imagine a hypothetical whitefly population in which half of the population is female, each female lays eggs for 10 days, and all individuals reaching fourth instar stage also develop to fertile adults. In this simplified setting there is no effect of other variables like temperature and the sex ratio. From this population, 100 healthy adults take flight and land on a set of *S. lycopersicum* cv Moneymaker plants, whilst another 100 of the whiteflies end up on *S. chmielewskii* LA1840 plants. The oviposition and percentage of eggs developing to adults on these plants would be the same as those found in **chapter 3** (Table 3.1). On the *S. lycopersicum* plants, the whiteflies would lay 19 eggs per female in 10 days, and 38.7% of those eggs would develop into adults, while the 100 whiteflies on the *S. chmielewskii* plants, which would have an oviposition of 13 eggs per female in 10 days and a development to adults of 9.09%. We can use this to calculate the number of whiteflies in the population ( $N$ ) in a generation ( $g$ ) as

$$N_g = N_{g-1} * 0.5 * oviposition * development \quad (6.1)$$



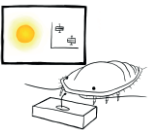
On *S. lycopersicum* this would result in the growth of the population of initially 100 whiteflies to 18,270 whiteflies after four generations and 165,874,747 whiteflies in the 11<sup>th</sup> generation at the end of the growing season (Fig. 6.1). If we now look at the 100 whiteflies on the *S. chmielewskii* plants, we would find only 12 adult whiteflies after four generations and there would be no whiteflies left at the end of the season. When comparing the explosive population growth on *S. lycopersicum* to the dwindling population on *S. chmielewskii*, the effectiveness of this type of resistance becomes clear. This example is of course a simplified estimation of population growth, and the omitted variables would likely affect the growth of a real population. For example, the sex ratio is assumed to be balanced in all generations despite the haplodiploid sex determination in whiteflies that would likely lead to a skewed sex ratio, especially in smaller populations. Haplodiploid sex determination entails a system in which unfertilised (haploid) eggs become males, and fertilised (diploid) eggs become females. Thus, a female whitefly can asexually reproduce male offspring. Still, the general dynamic in population size would remain the same and a whitefly population would not be able to grow on plants on which the nymphal development is as severely impeded as on *S. chmielewskii*, and whiteflies could therefore not become an uncontrollable pest on these plants.





**Figure 6.1** Projected population development of *Bemisia tabaci* on *Solanum lycopersicum* and *S. chmielewskii* plants over 11 generations, scaled logarithmically. Population size ( $N$ ) at generation  $g$  is calculated as  $N_g = N_{g-1} * 0.5 * oviposition * development$ .

Furthermore, why do I use the word ‘phloem’ when describing the origin of the resistance mechanism instead of ‘vasculature’ or ‘xylem’? Indeed, the xylem might also have a role in the resistance mechanism that I studied, since the phloem and xylem regularly interact, as also discussed in **chapter 3**. This makes it impossible to completely rule out effects of the xylem when studying phloem-based mechanisms, but also to rule out the phloem when studying the xylem. Although I am aware that (part of) the resistance mechanism might at some point also be present in the xylem, the mechanism must be phloem-based for it to reach the whitefly nymphs during their feeding. Adult whiteflies are known to occasionally feed from the xylem (Pollard, 1955; Milenovic *et al.*, 2019), but this has not been observed for nymphs (Jiang & Walker, 2003). An untargeted metabolomics study on the xylem, like that on phloem in **chapter 5**, could give more insight into the role of putative xylem-based specialised metabolites in resistance

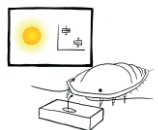


mechanisms against phloem-feeding insects as well as xylem infecting pathogens. The xylem of poplar, for example, was shown to contain defence related specialised metabolites including, but not limited to, coumarins and salicinoids (Kasper *et al.*, 2022).

In this thesis I studied a mechanism that reduces the growth of a whitefly population, fitting the definition of resistance. It is possible that the resistant wild tomato plants are also tolerant to whitefly infestations, but additional experiments would be required to draw any conclusions about the effect of infestations on the fitness of the plants. As a resistance mechanism that interacts with phloem-feeding insects during the feeding must be based in the phloem, I think it appropriate to use the term phloem-based when describing such resistance. Taken together, the defence mechanism studied in this thesis could be called a phloem-based resistance mechanism.

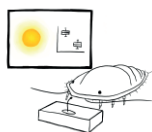
#### What makes you stronger might also kill you

In **chapter 3** we identified a phloem-based resistance mechanism in *S. chmielewskii* LA1840 that could be linked to the high riboflavin concentration in these plants. This is a surprising link, because riboflavin, also known as vitamin B2, is an essential nutrient for whiteflies. In fact, whiteflies require riboflavin producing endosymbionts for normal fertility (Wang *et al.*, 2020). For this paradoxical resistance to whiteflies deriving from a compound that is an essential nutrient for those same whiteflies, we might find an explanation in resistance mechanisms to microbial pathogens. Riboflavin was previously found to induce a disease resistance mechanism against a wide range of pathogens (Dong & Beer, 2000; Zhang *et al.*, 2009; Taheri & Tarighi, 2010; Liu *et al.*, 2010; Azami-Sardooei *et al.*, 2010; Boubakri *et al.*, 2013; Nie & Xu, 2016). Next to riboflavin, two more plant-produced vitamins have been found to induce resistance in plants. Both

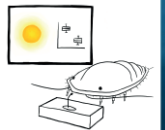


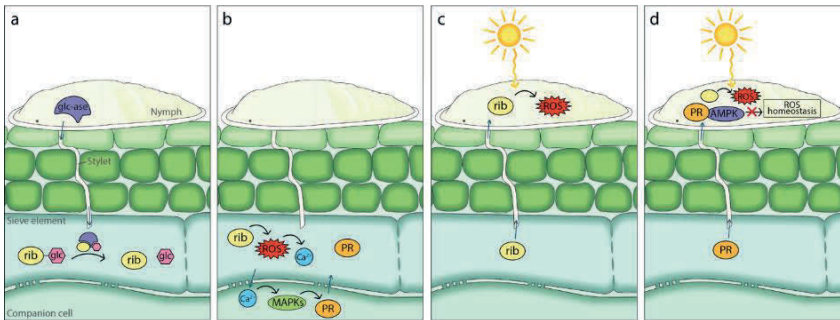
thiamine (vitamin B1) and folic acid (vitamin B9) induce a salicylic acid (SA) dependent disease resistance (Ahn *et al.*, 2005; Wittek *et al.*, 2015). Furthermore, thiamine induces resistance against aphids possibly through the same SA dependent resistance mechanism (Hamada & Jonsson, 2013). Interestingly, the riboflavin-induced resistance mechanism appears to be independent of SA, despite the involvement of pathogenesis related (PR) proteins in this mechanism (Dong & Beer, 2000). Although the resistance mechanism also seems to be independent of the other classical defence related hormones jasmonic acid (JA), ethylene, and abscisic acid in *Arabidopsis thaliana* (Zhang *et al.*, 2009), riboflavin-induced resistance against *Botrytis cinerea* in bean (Azami-Sardooei *et al.*, 2010), *Rhizoctonia solani* in rice (Taheri & Tarighi, 2010), and *Plasmopara viticola* in grapevine (Boubakri *et al.*, 2013) is mediated by JA.

In **chapter 3** we argued that the resistance mechanism we studied might be the same as the riboflavin-induced disease resistance first introduced by Dong and Beer (Dong & Beer, 2000) and a defence mechanism against whitefly linked to PR proteins (Puthoff *et al.*, 2010). Both the riboflavin treatment of plants and a whitefly infestation caused an upregulation of *PR* gene expression, seemingly independent of SA or JA (Dong & Beer, 2000; Puthoff *et al.*, 2010). I therefore propose a model consisting of four parts for a riboflavin-induced whitefly resistance in wild tomato (Fig. 6.2). As a first step, whiteflies might trigger a local increase in the riboflavin concentration in their host plant, activating the riboflavin-induced defence mechanism. This could for example happen through increased biosynthesis or a local deglycosylation of riboflavin glycosides at the site of interaction by glycosidases (Fig. 6.2a). The deglycosylation of riboflavin glycosides could resemble the so called two-component defence mechanisms like cyanogenic glucosides and glucosinolates (Morant *et al.*, 2008). The glycoside and



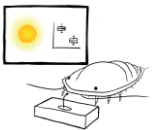
enzyme are stored in separate compartments and can interact only upon cell damage. Whereas cell damage is inevitable during feeding by chewing herbivores, whiteflies cause little to no damage while manoeuvring their stylets around cells on their way to the phloem vessel (Pollard, 1955). Yet, whiteflies were found to trigger the hydrolysis of cyanogenic glucosides and glucosinolates, before deactivating the toxins through glucosylation and phosphorylation (Malka *et al.*, 2020; Easson *et al.*, 2021). Perhaps whiteflies cause sufficient tissue damage to elicit the plant's defence mechanism in the same manner as chewing insects, but some component of the insects' saliva might also set off the response in the plant. Next to enzymes for the glucosylation and phosphorylation of toxic compounds, whitefly saliva might also contain multiple types of glycosidases that are released into the phloem prior to or during feeding that might function as the second component in the two-component mechanism (Huang *et al.*, 2021). These salivary enzymes play vital roles for phloem-feeding insects. The  $\alpha$ -glucosidase in the saliva of green peach aphids (*Myzus persicae*) for example, is crucial for the extra-intestinal digestion of phloem (Yang *et al.*, 2022). Although we still do not know whether there are riboflavin glycosides present in the phloem of tomato, we do know that many compounds in the phloem are glycosylated, and the glycosylation of riboflavin would likely improve its solubility in the aqueous phloem sap. As exemplified by glucosinolates and cyanogenic glucosides, glycosylation of plant specialised metabolites plays an important role in i.a. the stability and bioactivity of compounds (Louveau & Osbourn, 2019). Similarly, glycosylation would improve the stability of riboflavin and thereby reduce its reactivity. The degradation of riboflavin causes the production of reactive oxygen species (ROS) and cell damage, so riboflavin might be better suited for phloem transport and distribution through the plant.





**Figure 6.2** Visualisation of proposed riboflavin-induced whitefly resistance. Reactions in panels might function as alternative mechanisms or sequentially as steps of a single mechanism. **a:** A whitefly nymph injects glycosidases into the phloem during feeding, leading to deglycosylation of riboflavin. **b:** Riboflavin in the phloem triggers the activation of a MAPK signalling cascade and thereby upregulation of *PR* gene expression. **c:** The whitefly nymph ingests phloem sap with riboflavin, after which the translucent nymphal body allows for photodegradation of riboflavin and subsequent ROS production. **d:** The nymph ingests *PR* proteins along with the phloem sap, which bind the AMPK subunits in the nymph and in that way block the proper functioning of AMPK in ROS homeostasis in the nymph.

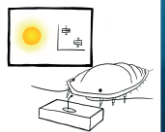
Following an increase in the riboflavin concentration, a ROS burst and mitogen-activated protein kinase (MAPK) cascades are presumably part of the pathway that results in the upregulation of *PR* expression (Zhang *et al.*, 2009; Taheri & Tarighi, 2010; Azami-Sardoei *et al.*, 2010; Boubakri *et al.*, 2013; Nie & Xu, 2016). If this pathway is involved in the here hypothesised role of riboflavin in whitefly resistance, at least part of this pathway would likely take place in the phloem companion cells rather than the sieve elements (Fig. 6.2b). Fully developed sieve elements lack a nucleus and depend on the companion cells for gene expression. A response in *PR* expression upon whitefly infection was previously found in *S. lycopersicum* (Puthoff *et al.*, 2010), so if *PR* expression is indeed induced by riboflavin, the increased riboflavin concentration in *S. chmielewskii* could be hypothesised to induce a stronger, or faster, defence response. To test the involvement of *PR* proteins in the resistance mechanism, one could repeat the riboflavin feeding essays in **chapter 3** with *PR* knock-out or knock-down mutants. At this moment, there are CRISPR/Cas9 knockout lines of *S.*



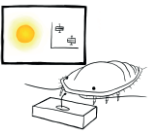
*lycopersicum* for *PR1* (Zhang *et al.*, 2025) and *PR5* (Šimkovicová *et al.*, 2025) that could be used for this experiment. In the same experiment, NahG (a transgenic plant incapable of SA accumulation; Brading *et al.*, 2000) and *jail* (a JA insensitive mutant; Li *et al.*, 2004) plants could be included to gain insight in dependence on SA and JA signalling, respectively.

Next to the function of riboflavin as a signalling molecule, I suggest a mechanism in which riboflavin can act as a direct defence mechanism that might be sequential to the previously discussed pathway or an alternative, separate defence mechanism. In this proposed mechanism, riboflavin would directly target whiteflies through the formation of ROS upon the photodegradation of riboflavin in the gut of whitefly nymphs (Fig. 6.2c). These two roles of riboflavin do not have to be mutually exclusive but might together cause the resistance phenotype we found in **chapter 3**. If an upregulation of *PR* expression leads to an increase in PR proteins in the phloem, these proteins would subsequently be ingested by a feeding whitefly. In *Phytophthora infestans* infected potato, PR proteins were found to be transported into the pathogen, where they bind the AMP-activated protein kinase (AMPK) subunits and thereby, among other things, inhibit the ROS homeostasis in the pathogen (Luo *et al.*, 2023). The AMPK subunit proteins are highly conserved among organisms and PR proteins might therefore also bind and inhibit the whitefly AMPK subunits. Inhibition of AMPK and ROS homeostasis in a whitefly nymph would make the nymph more vulnerable to the photodegradation of riboflavin (Fig. 6.2d).

Like plants and other animals, *B. tabaci* has a diverse microbiome (Wang *et al.*, 2019a; Goretty *et al.*, 2019; El Hamss *et al.*, 2022), which plays a role in important processes in whiteflies and can influence their fitness. The microbiome consists of free-living microbes like the gut microbiome, and

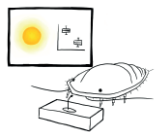


endosymbionts, microbes living inside cells of the host. For example, the endosymbiont “*Candidatus* Porteria” of *B. tabaci* is located in bacteriocytes, cells of the whitefly specialised for this endosymbiosis (Thao & Baumann, 2004). Whiteflies depend on their endosymbionts for the synthesis of essential nutrients, including riboflavin (Wang *et al.*, 2020). Their microbiome, including the endosymbionts, is in turn affected by the dietary composition of the whitefly (i.e. the host plant), but also by the application of pesticides (Selvaraj *et al.*, 2021; Bravo-Pérez *et al.*, 2024). Moreover, although the same endosymbionts are found in whiteflies of all life stages, the composition of the complete microbiome differs per life stage, suggesting that the microbiome and the nymphal development might influence each other (El Hamss *et al.*, 2024). Considering the riboflavin-induced resistance against microbial pathogens discussed above, the resistance mechanisms linked to riboflavin could also (partially) function through affecting the whitefly microbiome. The other way around, if certain endosymbionts would be better at metabolising the metabolites responsible for a resistance mechanism, the microbiome composition could also influence the efficiency of a resistance mechanism. For example, the high concentration of the alkaloid caffeine in coffee seeds is toxic to almost all insects. The only exception is the coffee berry borer (*Hypothenemus hampei*), whose caffeine degrading gut microbiome allows it to complete its entire lifecycle on coffee beans (Ceja-Navarro *et al.*, 2015). Taken together, riboflavin could have several roles in the resistance mechanism against whitefly in *S. chmielewskii* that might individually be part of alternative hypotheses but may also function in tandem as parts of a single resistance mechanism.



### Harnessing phloem-based resistance may reduce pesticide use

Although many aspects of the riboflavin-induced resistance mechanism remain to be elucidated, like whether there is vascular transport of riboflavin involved in the resistance mechanism or only an increased biosynthesis, the use of riboflavin-induced resistance as pest management strategy has the potential to become a more sustainable and safer alternative to synthetic pesticides. Riboflavin appears to be involved in a broad resistance, protecting not only against whiteflies, but also microbial pathogens (Dong & Beer, 2000; Zhang *et al.*, 2009; Taheri & Tarighi, 2010; Azami-Sardooei *et al.*, 2010; Boubakri *et al.*, 2013). During a mildew outbreak in the greenhouses, we indeed noticed that the disease symptoms on *S. chmielewskii* LA1840 plants and even grafts with an LA1840 rootstock were noticeably milder than on *S. lycopersicum* plants or grafts with an *S. lycopersicum* rootstock. Furthermore, the effect of a possible increased riboflavin concentration in tomato fruits on consumer health could be expected to be positive rather than negative. Riboflavin deficiency in humans has a high prevalence worldwide (Sivaprasad *et al.*, 2019; Aljaadi *et al.*, 2021; Jarrett *et al.*, 2022), whilst no effects of an overdose have been found to date (Turck *et al.*, 2017). Meanwhile, the environmental impact of riboflavin is likely to be limited, due to its rapid degradation. Riboflavin in solution degrades within a few hours under influence of ultraviolet light (Ahmad *et al.*, 2006; Xu *et al.*, 2015; Stanojević *et al.*, 2015). This rapid degradation makes it unlikely for riboflavin of plant origin to be transferred from whiteflies to the next trophic levels and accumulate to high concentrations in the environment. This contrasts with persistent synthetic pesticides, whose low (bio)degradability is a major contributor to their level of concern (Cousins *et al.*, 2019; Donley *et al.*, 2024). Yet it is worthwhile to draw parallels between synthetic pesticides and naturally occurring

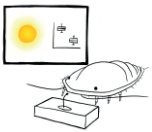


resistance mechanisms to learn from common pitfalls before making claims about sustainability.

*“I contend, furthermore, that we have allowed these chemicals to be used with little or no advance investigation of their effect on soil, water, wildlife, and man himself. Future generations are unlikely to condone our lack of prudent concern for the integrity of the natural world that supports all life. There is still very limited awareness of the nature of the threat. This is an era of specialists, each of whom sees his own problem and is unaware of or intolerant of the larger frame into which it fits. It is also an era dominated by industry, in which the right to make a dollar at whatever cost is seldom challenged.”*

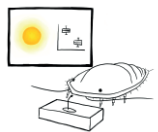
Rachel Carson – Silent Spring

Merely decades after Carson’s warnings against the intensive application of pesticides before thoroughly investigating their effects on non-target organisms, neonicotinoids were introduced as having no non-target effects without the evidence to back this claim up. By now, it is generally understood that neonicotinoids have devastating non-target effects, including increased mortality in invertebrates like pollinators, poisoning of birds, accumulation and sublethal effects in mammals and accumulation in soil, air, water and wild plants (Mamy *et al.*, 2023). Yet Carson’s words are still relevant today, and, although there are important differences between persistent synthetic chemicals and naturally occurring specialised metabolites, we too should be careful not to make the same mistakes and be careful with claims about new safe and sustainable crop protection methods without properly investigating non-target effects. Not only must the safety for human consumption be ascertained, but the biodegradability and



specificity for the target organism should also be assessed. I would like to encourage that this is not only done by testing the acute toxicity but also in long-term bioassays, because important chronic effects might otherwise be overlooked for synthetic pesticides (Laskowski, 2001), but is also for naturally occurring resistance mechanisms. For example, we saw this for *S. chmielewskii* LA1840, on which whitefly survival was previously found to be equal to control survival in short-term experiments (Kortbeek *et al.*, 2021), but the nymphal development was strongly hampered in long-term bioassays in **chapter 3**. Furthermore, to get a realistic idea of any non-target effects, we should realise that organisms will not encounter our metabolite of interest in a sterile condition, but in a complex environment in a mixture with many compounds. Despite the recent popularity of calling a research project ‘interdisciplinary’ or ‘transdisciplinary’, we are still in an ‘era of specialists’ in which molecular biology is seldomly discussed in the context of an ecosystem and the biological context for the function of a gene is more likely to be sought in a climate chamber than the complex interactions in a plant’s natural environment. For the purpose of a repeatable experiment, these standardised, controlled environments are absolutely necessary, but if we lose sight of the complexity of the real world, we can never be certain whether our work is part of the solution or helps to enable the next silent spring.

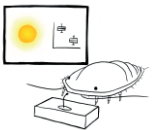
Another important parallel between synthetic pesticides and specialised metabolites is the accumulation of substances in the pollen and nectar, and the effect thereof on pollinators. The accumulation of neonicotinoids from the phloem into pollen and nectar is one of the reasons for the non-target effects of neonicotinoids (Zhang *et al.*, 2023) and the possibility and consequences of this happening with phloem-based specialised metabolites involved in insect resistance should therefore be well understood before



implementation in the field. A direction for future phloem research I therefore want to suggest is the possible transport of phloem-based specialised metabolites to pollen and nectar and the effect on pollinators. Like other plant parts, the pollen and nectar contain a variety of specialised metabolites (Palmer-Young *et al.*, 2019a; Barberis *et al.*, 2023). These floral specialised metabolites can have multiple functions, including the attraction of pollinators, inhibition of pollen consumption and defence against pathogens (Kessler & Baldwin, 2007; Huang *et al.*, 2012; Palmer-Young *et al.*, 2019b; Wang *et al.*, 2019b). The effects of floral metabolites on pollinators can be various, ranging from negative effects like decreasing the activity of bees (Hurst *et al.*, 2014) to positive effects like increasing the resistance of bumblebees to parasites (Richardson *et al.*, 2016). Neither the composition of the specialised metabolite profile in pollen of tomato (tomato flowers do not produce nectar), nor the origin of pollen and nectar specialised metabolites in general is known. Specialised metabolites in pollen and nectar are presumed to originate from the phloem (Adler, 2000) and alterations in the phloem specialised metabolite profile might therefore be reflected in the pollen or nectar.

#### Phloem research outside the box

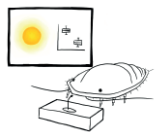
If we widen our perspective a little further, we can find even more opportunities for phloem research to help answer ecologically relevant questions. Considering that most plants do not grow in a greenhouse or climate chamber, it is necessary to understand the effect of environmental variables on phloem-based specialised metabolites before we can understand their effects on plant-insect interactions outside of a controlled environment. These environmental variables include prior or combined insect and pathogen infections, temperature changes,



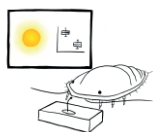
flooding, drought, and other abiotic stresses, but also soil contaminants. Among the contaminants of high concern for both human health and ecological risks is the class of per- and polyfluoroalkyl substances (PFAS; Bline *et al.*, 2024). PFAS are present in most (if not all) habitats around the world (Vendl *et al.*, 2024) and are introduced into agricultural land in high quantities as multiple PFAS containing pesticides are regularly applied (Donley *et al.*, 2024). Plants growing on contaminated soil can take up PFAS and transfer them to different parts of the shoot (Greger & Landberg, 2024; Battisti *et al.*, 2024; Xu *et al.*, 2024). In tomato plants, for example, the transported PFAS accumulate in leaves and fruit (Battisti *et al.*, 2024). The transport of PFAS is assumed to happen via the phloem and xylem, but the actual presence of PFAS in phloem and xylem samples has never been shown. In recent years, PFAS have been found to affect many molecular mechanisms in plants (Karamat *et al.*, 2024), but little is known about the effect of PFAS on the specialised metabolism. Our understanding of the effect of PFAS on ecosystems and crop plants would greatly benefit from studying PFAS in the vasculature, and their effect on specialised metabolites and thereby plant-insect interactions.

### Linking phloem-mobile resistance to phloem-based specialised metabolites

After we found evidence for a phloem-mobile defence mechanism that caused hampered nymphal development in *S. chmielewskii* in **chapter 3**, we decided to screen more wild tomato species and analyse their phloem composition in **chapter 5** to learn more about the occurrence of this type of defence in wild tomato, and the involvement of specialised metabolites in



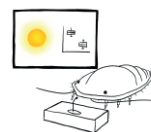
the phloem in particular. In previous research on the natural variation in trichome-based resistance in wild tomato, a set of 17 accessions from nine wild species and two cultivars was used (Kortbeek *et al.*, 2021). The high biochemical diversity in this set of plants led to sparsity, reducing the power of calculations on the relation between metabolites and phenotypes. As a solution for future research, Kortbeek suggested to increase the number of accessions per species and limit the number of wild species, potentially to one species (Kortbeek, 2022). We used a set of genotypes that was partially based on those used by Kortbeek *et al.* (2021) but we investigated less species and more accessions per species. The plants we selected, and performed experiments with, in **chapter 5** were 12 accessions from three wild species and one cultivar. In future research on the mechanisms, biochemical pathways and genetic background underlying the resistance, the selection of genotypes should be narrowed down even further to one species, such as *S. chmielewskii*, to eliminate an again larger part of the biochemical diversity unrelated to the phenotype of interest. Of this species, a collection of 29 accessions is available in the lab, that could, for example, be used as part of a population for a metabolome-based Genome Wide Association Study (GWAS) (Wen *et al.*, 2014; Levina *et al.*, 2021). In other wild tomato species, such metabolome-based GWAS has successfully been applied to analyse the genetic background of the fruit metabolome (Perez-Fons *et al.*, 2014; Sauvage *et al.*, 2014; Zhao *et al.*, 2019).



To analyse the phloem composition of the selected plants, we first needed to collect phloem samples, which can be challenging as explained in **chapter 2** and the first paragraph of this discussion. The existing methods to collect these phloem exudates all have their own limitations (Killiny, 2019). Although the EDTA mediated phloem collection method used here allows for some contamination from other tissue, it is currently the best

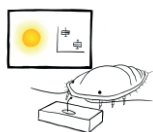
method for tomato available. Aphid stylectomy would yield purer phloem samples but requires tomato infesting aphids which, to our knowledge, do not exist. Using whiteflies instead of aphids is not feasible, due to the tiny size of whiteflies (aphid species used for stylectomy are roughly twice as large as the largest *B. tabaci* adults). Moreover, stylectomy is a tedious approach and the methodology is not trivial, while it only yields very low quantities. A drawback of the EDTA mediated collection method is the necessity to remove the high concentration of EDTA from the samples to prevent it from overshadowing many metabolites. The removal of EDTA requires an elaborate solid phase extraction method with filtering steps that might also remove interesting metabolites and exposes a sample to light and oxygen. Although the extraction method we used in **chapter 5** was optimised for our samples, we most likely lost metabolites during the extraction, especially those sensitive to light or oxygen, like riboflavin.

By analysing phloem samples of the selected plants in **chapter 5** we showed that the phloem exudates of wild and cultivated tomato contain a wide variety of metabolites. As I mentioned in the introduction of this thesis, the increasing sensitivity of the machinery involved in untargeted metabolomics allows for the detection of an increasingly larger part of the compounds present in a sample. Although this can be a great advantage, it also leads to larger datasets from which it can be difficult to filter the background noise and select relevant metabolites. In general, these large metabolomics datasets contain many more metabolites than samples (high dimensionality), metabolites can occur in only a small subset of samples (sparsity), and there can be a strong correlation between groups of metabolites, for example because they are part of the same metabolic pathway or share a precursor (multicollinearity). In **chapter 3** we successfully used a Random Forest algorithm to select riboflavin as the most promising candidate metabolite

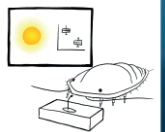


linked to the resistance phenotype. However, this was an older dataset from 2011. When we attempted to apply the same algorithm to analyse a pilot dataset that was acquired with the current machines, the dimensionality and multicollinearity of the data were too high to successfully analyse the data with this method. The multicollinearity in the data made it impossible to compute feature importances, the measure used to select features (in this case metabolites) that are important to categorize samples as resistant or susceptible. The feature importances were computed using permutations, a method to estimate the contribution of a feature to a model by testing the power of the model after randomisation (Altmann *et al.*, 2010). In case of high multicollinearity, the permutations do not lead to any reduced power of the model, because the features correlated to permuted features remain unaltered, resulting in variable importances of zero for all features. To solve this issue, we used a Principal Component Analysis (PCA) to cluster the features into Principal Components (PCs). These PCs were then used as input for the Machine Learning and feature selection.

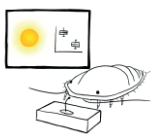
As setting up this method is not trivial for biologists without a bioinformatics background or affinity, we created a software package in Python in **chapter 4** to streamline the analysis for ourselves as well as other researchers. The package clusters the features into PCs and uses Automated Machine Learning to build the best fitting pipeline for a dataset by selecting a Random Forest, Gradient Boosting or Extreme Gradient Boosting classifier, potentially preceded by one or multiple preprocessing steps. The Python packages scikit-learn (Pedregosa *et al.*, 2011) and TPOT (Olson *et al.*, 2016) that we used to develop the Machine Learning functionalities in our package require extensive knowledge of Machine Learning and coding in Python. With our package we aimed to make this method accessible to a wider audience, i.e. the aforementioned biologists without a bioinformatics



background or affinity. Furthermore, we added options to the package to analyse the developmental data from the bioassays and to filter the metabolomics data, since there were no packages available in Python to conveniently perform these tasks. The package performed well with our bioassay and metabolomics data from **chapter 5** as well as on the data used for the examples in **chapter 4**, indicating that the package might successfully be used for data on other topics if they suffer from the same ‘limitations’ like multicollinearity. For example, the functionalities to analyse insect development could also be used for disease development over time, as long as the severity is recorded as stages. Also, the metabolomics functionalities could be applied for classification questions with other datasets with many features per sample. Prerequisites for application are high dimensionality and multicollinearity, since the clustering step is not optional in the current version of the package. This means that in more balanced datasets, like the data from **chapter 3** in which riboflavin was identified, the relevant metabolites can get hidden in a PC cluster that, as a whole cluster, does not have a strong influence on the classification. The Machine Learning pipeline might in such case only have a very low accuracy. To improve the package as presented in **chapter 4**, the clustering step could be made optional and alternative methods to retrieve feature importances could be added, like recursive feature elimination (Gregorutti *et al.*, 2017), greedy forward feature selection (Drobnič *et al.*, 2020) or area under the curve variable importance (Bradter *et al.*, 2022), to make the package more broadly applicable. Furthermore, the package currently only contains classification options for the Machine Learning, so the addition of regression options could make the package useful for data with continuous phenotypes as well.

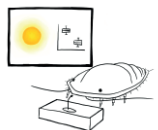


Despite the developments in bioinformatics tools and their improving ability to handle unbalanced datasets, the most important step to successfully select metabolic features (or other types of candidates) from a large screen like that in **chapter 5** is still a proper experimental design. For the experimental design, I fully agree with the recommendations of Kortbeek, as mentioned earlier (Kortbeek, 2022). Although it might be more likely to encounter an interesting phenotype that is worth further investigating in a large screen that includes different species, the chances of identifying variables (metabolic features, protein, genes etc.) that are actually linked to the phenotype, instead of to a species, are likely higher in a large collection of accessions from a single species. These could for example be the 29 above-mentioned *S. chmielewskii* accessions or genetic populations like introgression libraries. Yet, even when it is possible to select metabolic candidates from a large untargeted metabolomics experiment, the most important bottleneck, at this point, remains the annotation of metabolites, as also became clear in **chapter 5**. There, we selected two metabolic features that appeared to be linked to the resistance phenotype but were not able to add an annotation. An attempt to annotate the candidates based on a prediction of their chemical structures from the spectral data using a neural networking tool (a form of artificial intelligence) tool resulted in a putative annotation of one of the candidates. However, this annotation had to be rejected after analysis of analytical standards. Artificial intelligence is a rapidly developing field and, although the annotation attempt in **chapter 5** was not successful, can be an increasingly important aid in the assignment of annotation that would otherwise not have been possible (Coler *et al.*, 2024). With the help of a next generation of annotation tools, it might very well be possible to accurately annotate the candidates from **chapter 5**, but until that time further analyses of phloem fractions enriched in the candidate metabolites will hopefully shed some light on their identity.

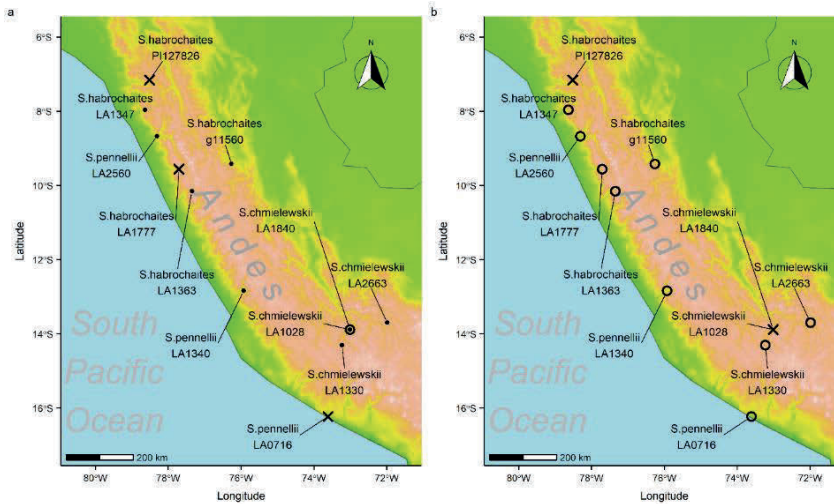


You can't judge a book by looking at the cover – you can't judge phloem by looking at trichomes

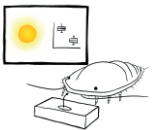
Of the four wild accessions used in both studies, only one was labelled 'resistant' in both the adult survival assay of Kortbeek *et al.* (2021) and our nymph development assay in **chapter 5** (Fig. 5.3). The other three accessions were resistant according to one of the assays but susceptible in the other. From these findings we can conclude that adult survival and nymph development are affected by two separate defence mechanisms in the host plant and that the trichome- and phloem-based defence mechanisms are not connected. Wild tomato species are endemic to the Andean region, and some accessions with and without a trichome-based defence mechanism originate from locations near each other (Fig. 1.2; Kortbeek *et al.*, 2021). In such case, it is likely that these accessions would have encounter the same insect species. I would argue that a complete absence of defence mechanisms against these insects in some accessions is therefore improbable, as this would have severely reduced their fitness compared to nearby located resistant relatives. Although the phloem is a logical site for defences against phloem-feeding insects, as it is the site where the plant and the insect most intimately interact, the evolution of such a phloem-based defence mechanism in wild tomato is not straightforward to elucidate. Not much is known about phloem-feeding insects native to the Andean region. Yet, even less is known about the co-evolution of phloem-feeding insects with wild solanaceous species as possible host plant and the resulting selection pressure towards resistance mechanisms. The only phloem-feeding insects native to the Andes described in literature are 16 species of psyllids, some of which were found to have solanaceous host plants (Serbina & Burckhardt, 2017). Although this interaction would not inherently result in a resistance to whitefly, one could speculate that it could have caused a



selection pressure towards plants that are better adapted to psyllid infestations. One of these adaptations could be a phloem-based resistance mechanism based on specialised metabolites in the phloem that inadvertently also provides resistance to other phloem-feeding insects, including whiteflies.

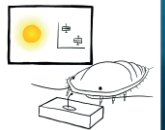


**Figure 6.3** Geographical locations of wild tomato genotypes used in chapter 5 with their whitefly resistance phenotype as (a) adult survival according to Kortbeek *et al.* (2021), and (b) nymph development as determined in chapter 5. Phenotypes are indicated with X (resistant), O (susceptible) and ● (unknown). There are different costs and benefits involved in deploying a phloem-based resistance mechanism instead of trichome-based resistance. On one hand, the complete dependence on a phloem-based defence mechanism against phloem-feeding insects makes a plant more vulnerable to diseases transmitted by those insects, because the defences will only be encountered upon feeding. Yet, on the other hand, the absence of trichomes also makes the leaf surface more hospitable for pollinators and natural enemies of herbivorous insects. Furthermore, specialised metabolites produced in the trichomes are stored or secreted locally, the energy invested in specialised metabolites in the trichomes is “lost” to those trichomes. Metabolites in the



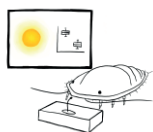
phloem, on the contrary, can be transported and deployed throughout the plant. Alternatively, a plant can also have both defence mechanisms, as we found for *S. habrochaites* PI127826, the labs' favourite wild tomato accession, in **chapter 5**. On this super-resistant plant, the trichomes form a first layer of defence by repelling most whiteflies using a volatile sesquiterpene and a second defensive layer by killing almost all whiteflies that nonetheless encounter the trichomes with an epoxy-alcohol derivative of the sesquiterpene (Bleeker *et al.*, 2011; Zabel *et al.*, 2021). For whiteflies that still manage to lay eggs on PI127826, the phloem appeared to form a third line of defence by preventing the normal development of the offspring. Like PI127826, *S. habrochaites* LA1777, an accession with a very similar appearance, also has a high sesquiterpene production in the trichomes which leads to a strong trichome-based resistance (Kortbeek *et al.*, 2021, 2023). However, LA1777 did not exhibit the phloem-based resistance we observed in PI127826.

To rule out the influence of trichomes and other physical barriers on the whitefly performance during bioassays, in **chapter 3** and **chapter 5** we performed bioassays on grafts, where we used a susceptible cultivar grafted onto the different genotypes as rootstocks, with multiple true leaves and axillary meristems still attached to the rootstocks. By doing so, we could perform bioassays on leaves of the same susceptible cultivar with the only difference being the vasculature-mobile compounds originating from the rootstocks. Grafting of crop plants is widely used in agriculture, to combine the high yield of elite cultivars with the resistance of rootstock cultivars against soil borne pathogens and pests (Thies, 2021). In such case, grafting is applied to make use of a resistance mechanism of the rootstock locally in the roots, e.g. against nematodes and fungi, but molecules can also pass the graft junction and get transported from rootstock to scion or vice versa via



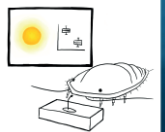
the phloem and xylem. These molecules include RNAs and proteins (Lu *et al.*, 2020; Li *et al.*, 2022), with perhaps the most famous example being the FLOWERING LOCUS T protein (Corbesier *et al.*, 2007). Moreover, factors involved in resistance to whiteflies can also be transferred this way, as was previously found for whitefly resistant tomato rootstocks which conferred their resistance to otherwise susceptible scions (Žanić *et al.*, 2017; Mandušić *et al.*, 2019). We were now able to also link a graft mobile resistance mechanism to metabolites in the phloem of resistant genotypes used as rootstock.

An experimental drawback of using grafted plants when studying the rootstock phloem, is that the phloem from the rootstock mixes with the phloem from the scion. This results in a dilution of the compounds originating from the rootstock and, consequently, a possibly weaker phenotype. We indeed saw this reflected in the weaker resistance phenotype in the grafting experiment in **chapter 3** compared to the bioassays on complete plants. Also, we were not able to predict whether the compounds responsible for the resistance originate from the roots or leaves. As solution, we used rootstocks with multiple leaves and axial meristems, whereas rootstocks for agricultural applications usually have no leaves. During pilot experiments we noticed that when the cultivar scions grew much faster than the shoots of the rootstock, the resistance phenotypes indeed became weaker over time, confirming the dilution hypothesis. Nevertheless, the detailed long-term bioassays in which we followed the nymphal development from eggs to the last instar allowed us to quantify these diluted resistance phenotypes. This type of long-term bioassay is much more suitable for the analysis of mild/diluted toxicity than short-term acute toxicity bioassays (Laskowski, 2001). Whilst short-term bioassays are effective for the analysis of acute effects of highly toxic compounds on mortality or



fecundity, they do not give any insights on the effects of long-term exposure, the accumulation of damage over time, or on the development of juveniles. However, these effects can be perceived by a long-term bioassay spanning (most of) an entire lifecycle, which takes roughly a month for whiteflies, depending on environmental conditions.

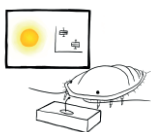
Consequently, this approach drastically increased the duration (and with that, the dilution effect of the phloem as the scion keeps growing and producing more phloem) and labour intensity of the experiments. The bioassays of **chapter 5** therefore had to be divided into multiple, consecutively performed experiments. The different seasons in which the bioassays were performed had a clear effect on the whitefly performance and nymphal development, as the number of eggs, hatching rate, developmental speed and percentage of nymphs reaching the fourth instar stage on control plants could vary between different bioassay experiments. These seasonal effects on whitefly performance have previously been observed and might be caused by seasonal changes in plant and/or whitefly metabolism (Santegoets *et al.*, 2021). We could thus not directly compare the development between bioassay experiments. However, we could compare genotypes within bioassay experiments, because the whitefly performance on one genotype relative to the performance on another genotype remained similar throughout all bioassay experiments (e.g. whitefly nymphs on grafts with a cultivar rootstock always performed better than nymphs on grafts with a resistant *S. chmielewskii* rootstock). With the goal of labelling genotypes as “containing a phloem-based resistance” instead of assigning a precise value to the amount of resistance, this comparison within a subset of genotypes was sufficient. Interestingly, we also saw a direct weather effect on whitefly behaviour, as there was little to no oviposition on any of the plant genotypes during thunder- or snowstorms,

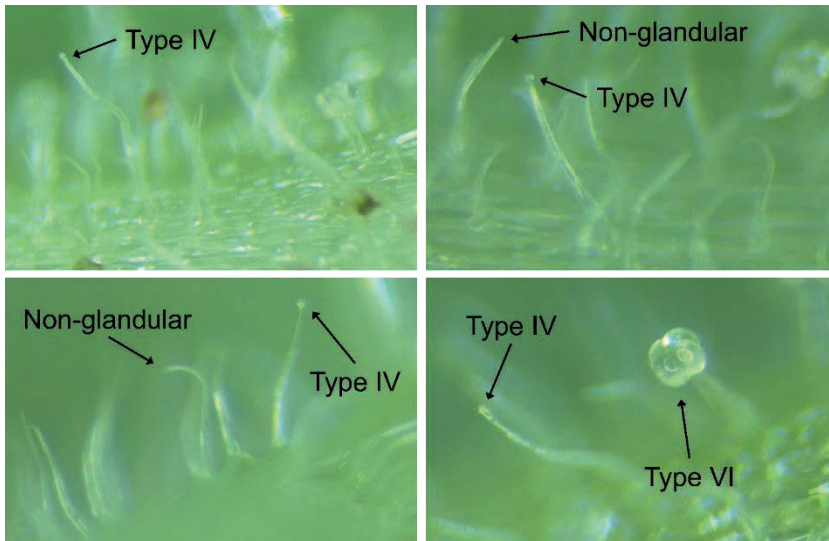


and the usually fast moving first instars, or crawlers, were mostly stationary. Blizzards and thunderstorms are often accompanied by rapid changes in atmospheric pressure, which can alter insect behaviour (Marchand & McNeil, 2000; Fournier *et al.*, 2005; Pellegrino *et al.*, 2013; Austin *et al.*, 2014; McFarlane *et al.*, 2015). Although no such effect on *B. tabaci* behaviour has been described in literature, changes in atmospheric pressure can be presumed to have played a role in the behavioural changes we observed.

### The effect of grafting on trichomes

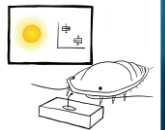
Since I used grafts to eliminate the trichome-based resistance, it was important to ensure that the trichomes on the scion were not influenced by the rootstock, especially for the *S. pennellii* and *S. habrochaites* accessions with a strong trichome-based resistance. The glandular trichomes on the two wild species are different from those on *S. lycopersicum* and can therefore easily be distinguished while studying the leaf surface through a binocular microscope during a bioassay (Glas *et al.*, 2012). The type VI trichomes, that morphologically differ between wild species and the cultivar, were not affected on the cultivar scions by any of the rootstock accessions. Morphologically, the trichomes were indistinguishable from type VI trichomes on control *S. lycopersicum* plants, but also the number of trichomes was unaffected. After the grafts with a *S. habrochaites* PI127826 rootstock appeared to be resistant, I picked type VI trichomes from those grafts which were kindly taken along in a GC-MS experiment by Rodrigo Therezan de Freitas to analyse their terpene content. This, again, showed that the type VI trichomes on cultivar scions were not affected by the rootstock.





**Figure 6.4** Glandular (type IV and VI) and non-glandular trichomes on *Solanum lycopersicum* leaves of grafts with *S. habrochaites* PI127826 rootstocks.

The other type of trichomes that is linked to whitefly resistance, type IV, is not present at the mature leaves (above the sixth leaf from the cotyledons) of *S. lycopersicum*, while abundant on all *S. pennellii* and *S. habrochaites* leaves. During the first bioassays with *S. pennellii* LA716 and *S. habrochaites* PI127826 as rootstocks, I found a small number of type IV trichomes on all leaves of the scions on those rootstocks that developed after grafting (Fig. 6.4). Interestingly, this did not seem to correlate with the resistance phenotype, as grafts with a PI127826 rootstock were resistant in those bioassays but grafts with an LA716 rootstock were not. I hypothesized that the development of type IV trichomes on those grafts could be the result of phloem-mobile microRNA156. It was previously found that microRNA156, linked to juvenility in plants, is able to pass the graft junction and affect leaf morphology but also trichome elongation (Bhogale *et al.*, 2014). However, the trichome phenotype I observed was not reliable enough in follow-up grafting experiments, with many type IV trichomes on *S. lycopersicum* scions with LA716 rootstock in some experiments but none

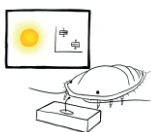


in other experiments. Because the development of type IV trichomes on the scions did not appear to influence the resistance phenotype, I did not test my hypothesis any further.

### Concluding remarks

The most important conclusion we can draw from my thesis is that there are phloem-based resistance mechanisms in wild tomato. These mechanisms are not universally shared among tomato species or even by accessions within a species. Instead, we found there is natural variation in phloem-based resistance, which can function as a wonderful source to learn more about phloem-based resistance. This variation was also visible in the phloem composition. The phloem of both wild and cultivated tomato contains a great diversity of specialised metabolites, some of which could be linked to the resistance phenotypes we studied. We found that the presence of a phloem-based resistance in a plant was unrelated to the presence of a trichome-based resistance, indicating that these types of resistance are based on distinct underlying mechanisms. The lack of an impressive exterior defence mechanism does thus not justify underestimating a plants potential to harbour interesting defence mechanisms.

Although it is too early to make claims about sustainability, phloem-based resistance in crops has the potential to become a more sustainable alternative to the use of insecticides provided that the mechanisms are properly investigated. The active compounds would be contained in the phloem and should therefore only target and affect phloem feeders. Yet, the long-term and non-target effects are still unknown and should be well understood before inadvertently making false claims as happened at the introduction of the synthetic, persistent and toxic neonicotinoids. I therefore have by no means the intention to declare that I found the holy grail for a pesticide free



future, but rather to share my exploration of the potential of the wild tomato phloem. My main goal is to make you, the reader, curious about what else the phloem is capable of by giving you an insight on the inside.

