Resistance to thrips in pepper

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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences

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Awang Maharijaya

Thesis

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 18 November 2013
at 4 p.m. in the Aula.

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Resistance to thrips in pepper,
109 pages.

PhD thesis, Wageningen University, Wageningen, NL (2013)
With references, with summaries in Dutch, English and Indonesian

ISBN: 978-94-6173-739-7

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Chapter 1

General Introduction

Pepper (*Capsicum*), one of the most important horticultural crops

Pepper belongs to the genus *Capsicum* in the *Solanaceae* family. The genus *Capsicum* itself consists of twenty-five distinct species (Baral & Bosland, 2002). Almost all Capsicum species are diploid with 12 chromosome pairs (Moscone et al., 1996). Five of these species are domesticated: *C. annuum, C. frutescens, C. chinense, C. baccatum and C. pubescens* (Pickersgill, 1997). *Capsicum annuum* is the most cultivated species worldwide. It is also the most important species from an economic and nutritional viewpoint (Djian-Caporalino et al., 2006).

Pepper is used in many forms, such as fresh or as cooked vegetables, as herbs or spices, and as various kinds of processed products. Because of its high nutritional value, for example carotenoids (provitamin A), ascorbic acid (vitamin C), tocopherols (vitamin E), phenolic compounds, flavonoids, and capsaicinoid (Topuz & Ozdemir, 2007), pepper had been used in health, pharmacology and the medicine industry (Cichewicz & Thorpe, 1996; Bosland & Votava, 2000; Takashi et al., 2001). Besides that, many varieties of pepper have been developed as ornamental plants such as pot, bedding, and garden plants because of their unique fruits and leaf color, shape and size (Stummel & Bosland, 2007). Capcaisin processed from pepper fruit has also been used as protective spray against captive wildlife (Miller, 2001).

As a result, it is not surprising that based on data released by the World Food and Agriculture Organization (FAO) (www.faostat.fao.org), pepper ranks as one of the most cultivated vegetables in the world today. In developing countries, pepper production challenges that of the tomato as leading vegetable crop (Djian-Caporalino et al., 2006). China, Mexico, Turkey, Indonesia and Spain are top five fresh pepper producers while India, China, Pakistan, Thailand and Peru are the largest dried pepper producers in the world today (FAOSTAT, 2011). The production of pepper for spices and as vegetable has increased year after year (Djian-Caporalino et al., 2006). From 1961 to 2009, the harvested area, yield and production of pepper both fresh and dried increased (Figure 1). The number of countries producing pepper also increased. For example, several European countries including the Netherlands started to produce peppers. The Netherlands started to produce fresh pepper around 1976 and now is the third biggest producer in Europe after Turkey and Spain (Figure 2).

Pepper production is constrained by abiotic factors such as drought, salinity, flooding and soil acidity and biotic factors such as pests and diseases. Abiotic stresses can directly inhibit plant growth and production. It can also cause some physiological fruit disorders such as uneven ripening, cracking, blossom end rot and malformation. However, constraints from biotic factors are even more severe (Table 1). In many places, especially in tropical and sub-tropical countries where the climate is favorable for many pests and diseases, biotic stresses are the dominant factor that reduces pepper production.

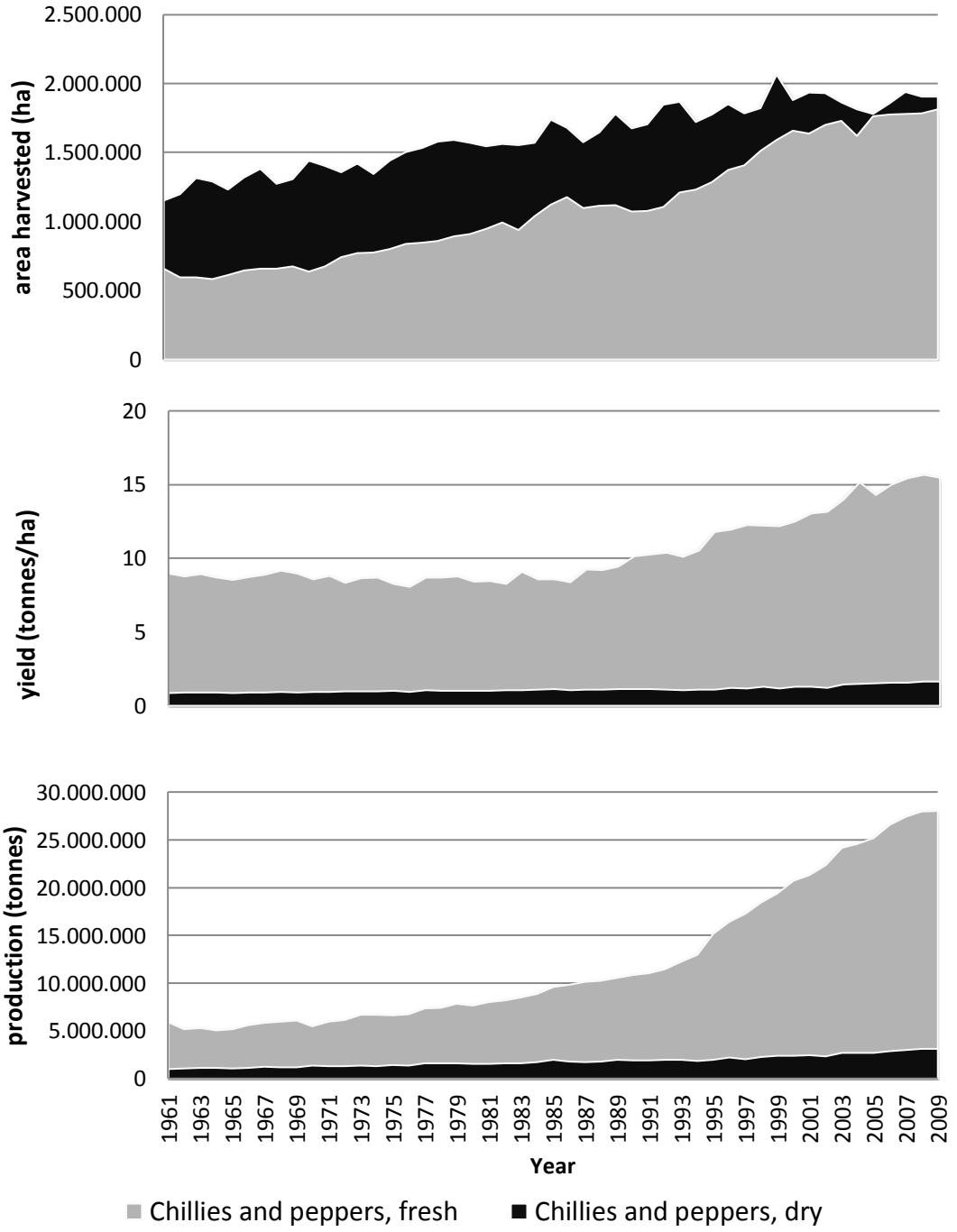

Figure 1. Total harvested area, yield and production of pepper in the world (source: faostat.fao.org)

Thrips as major pest in pepper production

Of the insect pests that attack pepper, thrips are among the most damaging, both in greenhouse and field cultivation (Siemonsma & Piluek, 1994). Thrips are small insects. Adults are about 1 mm long and the females are usually larger than the males. Thrips belong to the insect order Thysanoptera. At least 16 thrips species are reported to occur on pepper (Talekar, 1991; Capinera, 2001).

Among these, *Frankliniella occidentalis* (Figure 3a) is the most common thrips species on pepper in Europe (Tommasini & Maini, 1995), while *Thrips parvispinus* (Figure 3b) is the main species in Asian countries such as Indonesia, Malaysia, the Philippines, Thailand and Taiwan (Reyes, 1994; Vos & Frinking, 1998; Prabaningrum & Suhardjono, 2007). However *F. occidentalis* is also becoming a serious pepper pest in Asian countries, including Japan, Malaysia, Korea and China (Zhang et al., 2007), while *T. parvispinus* is also discovered in Europe (Mound & Collins, 2000). Thus both *F. occidentalis* and *T. parvispinus* are important pests in pepper.

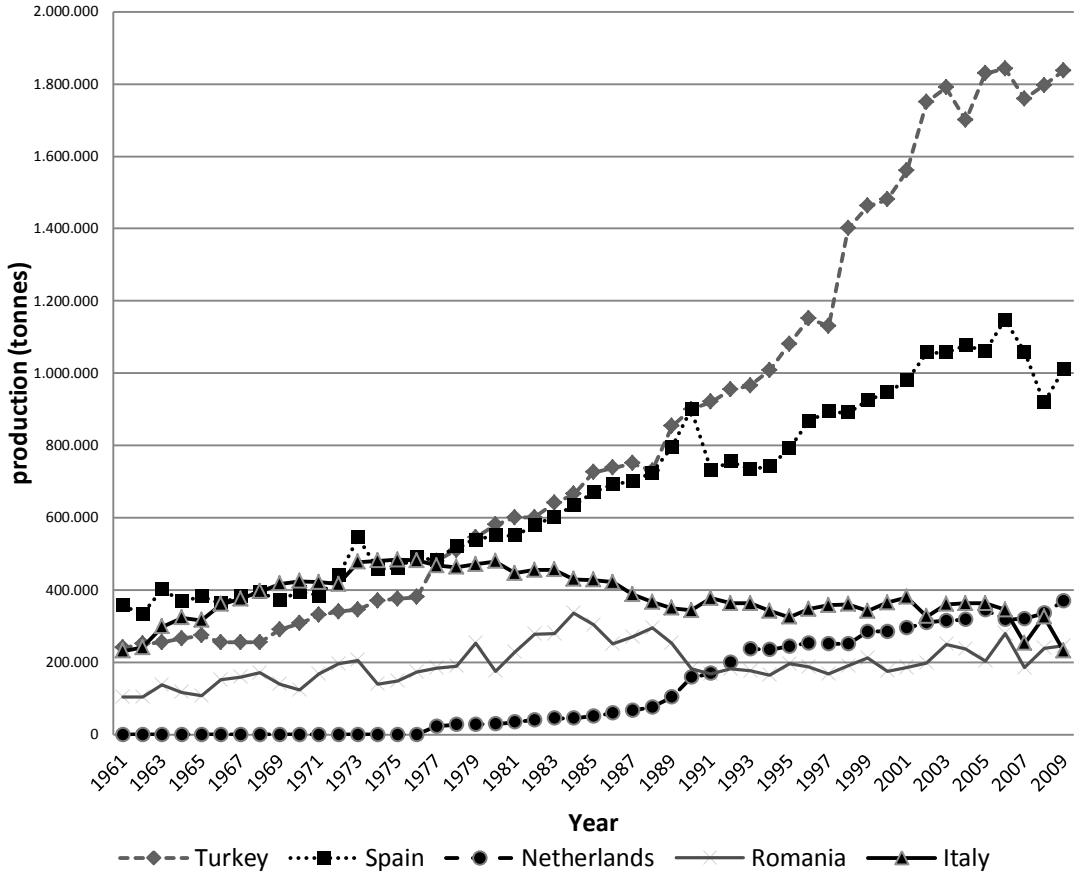

Figure 2. Total fresh pepper production by five top pepper producers in Europe

The developmental stages of different species are quite similar (Figure 4). Adult females lay eggs into the parenchymal tissue of leaves, flowers, or fruits. The eggs hatch into small and transparent first instar larvae which immediately begin to feed. To become an adult, a juvenile has to pass through two larval stages, the pre-pupa and the pupa stage. The transition from first to second larval stage can be detected by the skin tissue that remains on the leaf disc after moulting. Pre-pupae are recognized by their short wing sheaths. Pupae can be distinguished from pre-pupae by their longer wing sheaths which almost reach the end of the abdomen. Second instar larvae are active and feed abundantly, while the prepupal and pupal stages do not feed or move unless disturbed.

Adults can be recognized by the presence of wings (Tommasini & Maini, 1995; Vanrijn et al., 1995). The development can be influenced by environmental factors such as temperature and photoperiod (Ishida et al., 2003), and the quality of the host plant (Maris et al., 2004; Zhang et al., 2007).

Figure 3. Thrips species used in this study. A) *Frankliniella occidentalis* B) *Thrips parvispinus*

Thrips can cause damage on pepper directly by feeding on leaves, fruit or flowers. Feeding injury from thrips on leaves may affect leaf size, affect carbon allocation in the plant (Welter et al., 1990; Shipp et al., 1998), reduce photosynthetic capacity (Tommasini & Maini, 1995) and eventually reduce yield (Steiner, 1990; Welter et al., 1990). Thrips feeding on pepper fruit cause bronzing and silvering of the fruit skin reducing its market quality (Shipp et al., 1998). Thrips feed by penetrating the plant cells with their stylet-like mouth parts and sucking out the cell sap which can kill plant tissue around the feeding site (Kindt et al., 2003). Mechanical damage also occurs during oviposition when eggs are inserted into plant tissue.

Thrips can also cause indirect damage, by vectoring plant viruses. One of the most important viruses transmitted by thrips in pepper is Tomato Spotted Wilt Virus (TSWV) (Ulman et al., 1992). Tospovirus are the cause of a number of significant emerging diseases, such as capsicum chlorosis. Transmission to plant hosts occurs when thrips feed. The virus is acquired during the first and early second larval instar when there is a temporary association between mid-gut, visceral muscles and salivary glands (Moritz et al., 2004). After that, the virus is transferred into a plant with the saliva of a feeding adult (Jones, 2005).

Thrips management and control

Thrips management and control practices include chemical treatments, biological control, crop management, and integrated pest management (IPM).

Thrips are difficult to control, primarily due to their polyphagous nature. Host plants include most vegetables, fruit trees, cereals and ornamentals. Thrips are also difficult to control because of their high reproductive rate and their facultative parthenogenetic mode of reproduction, i.e. their ability to lay eggs without mating (Brodsgaard, 1989). At moderate temperatures, 20-25^{o}C, it usually takes 2-3 weeks for thrips to develop from egg to adult. Thrips are also difficult to control because of their cryptic habit: larvae hide in closed buds and pupate in soil (Jensen, 2000b; Herron & James, 2005).

Table 1. Yield loss estimations caused by several abiotic and biotic stresses on pepper

	Factors		Estimated yield loss (%)	References
Abiotic stress	Drought		35– 40	Figueiredo et al. (2008), Kulkarni & Phalke (2009)
	Salinity		14 – 38	De Pascale et al. (2000), Morales-Garcia & Stewart (2004), Kurunc et al. (2011)
	Acid soil		21 – 30	Choi et al. (2010)
	Flooding		45	Palada & Wu (2008)
Biotic stress	Insects	Aphids	56 -65	Fereres et al. (1996)
		Thrips	23 – 74	Vos & Duriat (1995a), Shipp et al. (1998), Patel et al. (2009)
		Mites	100	Jovicih et al. (2005)
	Fungi	*Colletotrichum* spp.	19 - 63	Vos & Duriat (1995a), Pakdeevaraporn (2005)
		Phytophthora capsici	70 - 100	Liu & Lu (2003)
	Bacteria	*Xanthomonas campestris*	23 - 44	Bashan et al. (1985)
	Nematodes	*Meloidogyne* spp	52	Vos & Duriat (1995a)
	Viruses		15 - 100	Agranovsky (1993), Vos & Duriat (1995a), Gitaitis et al. (1998)
	Weeds		18 - 45	Lanini & Strange (1994), Fereres et al. (1996)

Chemical control

Some pesticides with active ingredients such as malathion, chlorpyrifos, fenitrothion, quinalphos have been shown to cause thrips mortality (Helyer & Brobyn, 1992). However, because of the thrips' cryptic habit they are not directly exposed to pesticide sprays, which limits their effectiveness. This problem can be solved by application of systemic insecticides, for example the use of granular insecticide to control *F. occidentalis* on Daisy (Cloyd, 1998) and Verbena hybrids (Heungens & Buysse, 1996). However, the use of systemic insecticides may also be ineffective because the amount of active ingredient moving into plant parts may not be sufficient to kill thrips. For similar reasons drench application of systemic pesticides in pepper is also not effective (Kay & Herron, 2010).

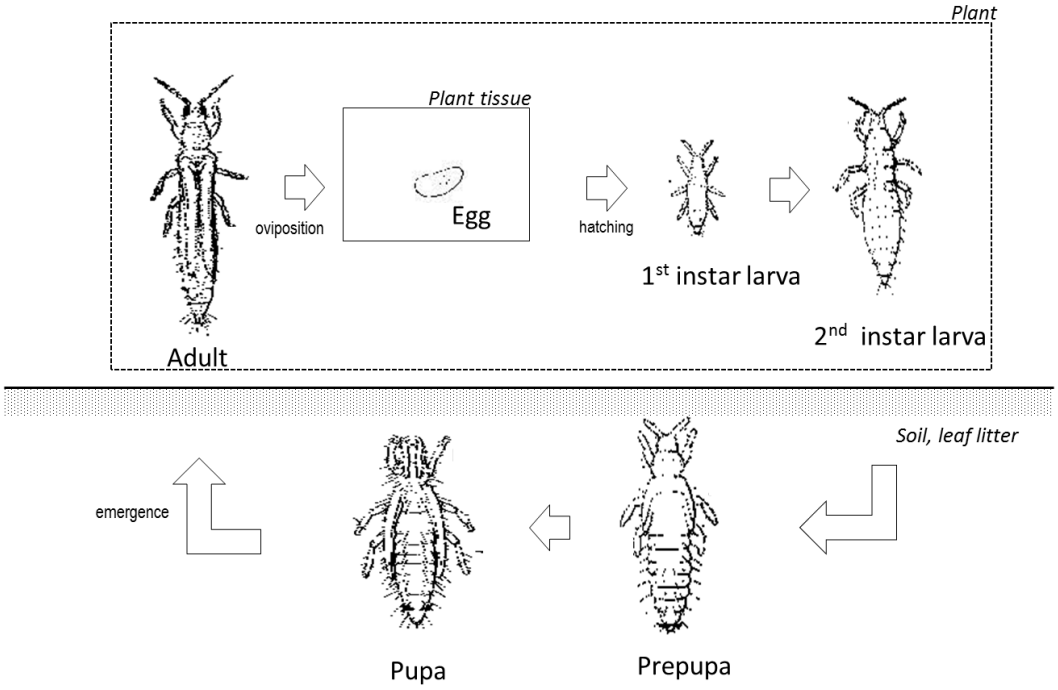

Figure 4. Developmental stages of thrips

Thrips also rapidly develop resistance to insecticides (Jensen, 2000a; Herron & James, 2005; Bielza, 2008). Moreover, there is an increasing public demand for reduction of pesticide uses and withdrawal of certain chemical compounds because of their harmful effects on growers, consumers, and the environment (Dik et al., 2000). Almost all pesticides are incompatible with natural enemies (Delbeke et al., 1997; Bielza, 2008), which limits their use in IPM. So, although pesticides are still widely used to control thrips by growers now, there is a clear need for other approaches.

Biological control

Biological control of thrips in pepper has been based mainly on the use of thrips predators such as *Orius* spp. (Heteroptera: Anthocoridae) (Baez et al., 2004; Bosco et al., 2008) and *Neoseiulus cucumeris* (Castane et al., 1999) for *F. occidentalis* and *Menochilus sexmaculatus* and *Coccinella transversalis* for *T. parvispinus* (Prabaningrum et al., 2008). Some parasitoids has been used for controlling thrips population such as *Ceranisus menes* (Walker) (Hirose et al., 1992; Murai & Loomans, 2001) and *Ceranisus americensis* (Girault) (Loomans, 2006). Among fungi, *Neozygites parvispora* has been used to control *F. occidentalis* (Maniania et al., 2002) and *Verticillium lecanii* has been used to control *T. parvispinus* in pepper (Prabaningrum et al., 2008). *Bacillus thuringiensis* can also be applied to control thrips (Helyer & Brobyn, 1992).

However the use of biological control has its own problems. For instance, the thrips predator *N. cucumeris* is dependent on the presence of a specific developmental stage of thrips (i.e. first instar thrips), their reproduction rate

may be lower than that of thrips, or they may require different humidity and temperatures for optimal growth (Cloutier et al., 1995). Some predators such as *Orius* spp in pepper are pollenophagous and are only efficient in crops with abundant pollen production (Castane et al., 1999) which can cause ineffectiveness of using this predator to control thrips in vegetative stages.

Crop management practices

Some crop management practices may help to reduce thrips infestation such as soil sterilization between crops can kills thrips pupae in the soil, mass trapping with sticky traps or ribbons, and the use of silver colored plastic soil cover (Castane et al., 1999; Weintraub, 2007). However their success rate varied because of the high cost needed for soil sterilization and placing sticky traps, and heat accumulation by plastic mulches can decrease plant growth and fruit yield in pepper (Locher et al., 2005; Diaz-Perez, 2010b; a). The use of plastic mulches can reduce the number of thrips in pepper, but the reduction does not result in reduced incidence of virus (Reitz et al., 2003). Another disadvantage of using plastics is that it can also affect pollinators and predators negatively (Weintraub, 2007).

Integrated pest management

As an alternative to reduce the use of pesticides, IPM has been implemented in pepper (Reitz et al., 2003; Weintraub, 2007). IPM is also designed to cover the ineffective of the use biological control. IPM includes the combination of biology control, crop management practices, and chemical applications to control thrips with consideration of ecological requirements. This strategy is not easy to be implemented and adopted by farmers and it is not always effective (Vos et al., 1995a; Weintraub, 2007). Pepper growers are in desperate need of varieties resistant to thrips that fit in an IPM scheme.

Resistant varieties

As mentioned thrips control using chemical, biological, crop management, and even IPM do not solve the problem caused by thrips. An addition of thrips-resistant varieties would increase the effectiveness of thrips control. Resistance to thrips may also delay and reduce the transmission of viruses as shown by (Maris et al., 2003) for TSWV. Unfortunately, there is no commercial pepper variety with an adequate level of resistance to thrips today. Therefore, breeding programs toward thrips resistance should be implemented. However pepper breeding for resistance against thrips is difficult to achieve without good knowledge on putative sources of host plant resistance, mechanisms of resistance, and genetic information related to it.

Current knowledge regarding thrips resistant crops

Source of resistance to thrips in pepper

Although no commercial pepper cultivars are available with high levels of resistance to thrips, several wild accessions have been identified that show resistance to *F. Occidentalis* (Fery & Schalk, 1991; Maris et al., 2003) and *Scirtothrips dorsalis* (Kumar et al., 1996; Babu et al., 2002). However, the number of accessions which have been confirmed to have a high resistance level to thrips as well as the number of thrips species which have been tested is still limited. Also, little information regarding mechanisms of resistance against thrips in pepper is available.

Plant defense mechanisms against insects

Plant responses to insects can be classified as direct defense and indirect defense. Direct defense mechanisms affect the insect directly. Indirect defenses promote the attraction or effectiveness of natural enemies of the insect pest (Kessler & Baldwin, 2002).

Plant defense against insect can also be categorized as antixenosis, antibiosis, and tolerance. Antixenosis denotes the presence of morphological or chemical factors that alter insect behavior, resulting in low preference of insect for the crop (Kogan & Ortman, 1978). Antibiosis includes factors that increase insect mortality, increase developmental period and decrease reproduction. Tolerance is the ability of plants to produce offspring and/or marketable yield in spite of insect attack. Antixenosis and antibiosis can be mediated by morphological/structural characters, chemical substances or both; in principle a plant trait can have both antixenotic and antibiotic effects (Rosenthal & Kotanen, 1994). Plant tolerance can be mediated by plant traits that enable plants to grow and produce yield even under insect pressure, although they may be costly to the plant (Strauss & Agrawal, 1999), such as induced phytochemical response, changes to morphology and compensatory growth (Bailey & Schweitzer, 2010).

Both morphological and chemical factors can play a role in both direct and indirect defense. Plant morphology can function as direct defense by preventing insect settling, moving and feeding of the insect can be prevented by plant traits, such as epicuticular waxes and trichomes. Plant morphology can also function as indirect defense by providing shelter to natural enemies such as domantia structure (Schoonhoven et al., 2005). Similarly, chemicals in plants can also play in both direct and indirect defense. Direct defense metabolites can be toxic or repellent, thereby affecting insect behavior and physiology (Roda & Baldwin, 2003) while indirect defense can be triggered by releasing volatile compound to attract natural enemies of the insect pest. The chemicals causing direct and indirect defense seem to be different. This is not always true for chemicals involved in antixenosis and antibiosis. For instance piperitenone oxide can act as toxic and reproduction retardant (antibiosis) as well as repellent against *Anopheles stephensi* (Tripathi et al., 2004).

These defense mechanisms can be also categorized into two categories: pre-formed (constitutive) defenses and inducible defenses. Constitutive defense

include physical and chemical barriers that exist before insect attack, whereas inducible defenses include defensive mechanisms that become activated upon insect attack (Reitz et al., 2003).

Pepper defense mechanisms against insects

Although still very limited, especially for resistance to thrips, some mechanisms of defense in pepper have been reported. Tolerance to *F. occidentalis* has been reported by comparing the population of thrips in resistant and susceptible accessions (Fery & Schalk, 1991). According to Fery & Schalk (1991) resistant cultivars support larval and adult thrips populations as large as those in susceptible cultivars, but with significantly less damage done.

Trichomes were reported to play a role in reducing leaf curling damage caused by thrips (*S. dorsalis*) and mites (*Polyphagotarsonemus latus* Banks) feeding (Yadwad et al., 2008). Trichome density and cuticle thickness also affected the level of damage caused by whitefly (*Bemicia tabaci*) in pepper (Firdaus et al., 2011). However, there is no information from earlier studies that indicate if resistance was based on antixenosis or antibiosis. A trichome-based resistance mechanism is a complex system. The negative effects to insects can be caused by chemical and/or mechanical factors. For example, density, length and form of the trichomes can construct mechanical barriers to *Tuta absoluta* (Leite et al., 1999) in tomato. Trichomes can also release toxic exudates which may entrap, irritate, and potentially kill the pest (Fery & Kennedy, 1987).

Antixenosis was suggested to be the defense mechanism active in *C. pubescence* against *Myzus persicae* (Bosland & Ellington, 1996). The dense hairiness of *C. pubescence* leaves may be impregnable to aphid feeding, or at least not preferred by aphids. Another form of antixenosis has been found in which three compounds produced in *C. annuum* leafs, namely 4-aminobutanoic acid, (2S,4R)-4-hydroxy-1-methyl-2-pyrrolidine carboxylic acid and 4-amino-1-*β*-D-ribofuranosyl-2(1*H*)-pyrimidinone, show significant oviposition deterrence toward adult flies of *Liriomyza trifolii* (Burgess) (Dekebo et al., 2007).

Another antibiosis mechanism to *F. occidentalis* has been reported in pepper (Maris et al., 2004). Reproduction, studied by comparison of larval survival and oviposition on two pepper accessions differing for their resistance to thrips, was negatively affected by the thrips resistant phenotype. Thrips resistant plants do not affect pupal stages. Significantly fewer offspring were produced per adult on the thrips resistant plants compared to the thrips susceptible plants. Larval mortality rate was significantly higher on the thrips resistant plants than on the thrips susceptible plants. These two things resulted in the impeded population built-up on thrips resistant plants. However, the contributing factor to this antibiosis was not elucidated. Antibiotic compounds have also been found in leaf extracts of *C. annuum.* In an artificial diet setup it can inhibit larval growth and development, cause a delay in pupation period and dramatically reduce fecundity and fertility of *Helicoverpa armigera* (Tamhane et al., 2005). A proteinase inhibitor is suspected to play a role in this example of antibiosis.

Plant defense mechanisms in various crops against thrips

Mechanisms of plant defense in other plant species against thrips have been reported, including morphological characters that contribute to antixenosis such as color in *Gerbera jamesonii*, *calistephus chinensis*, and chrysanthemum (Blumthal et al., 2005). Thrips preference was determined by observation of the number of thrips on flowers with different color. Significantly, more adult thrips weres found on yellow flowers compared to red, magenta, orange, pink, purple, lavender and white flowers. Another morphological character related to antixenosis is the physical barrier caused by wax layer in gladiolus (Zeier & Wright, 1995). Antibiosis was found as one of resistance mechanism against thrips as larvae died, and population growth was reduced in resistance leaves of chrysanthemum (Ohta, 2002). Highly significant correlations between aromatic amino acid concentrations in leaf protein and damage caused by *F. occidentalis* larvae have been found in cucumber, lettuce, tomato and pepper, suggesting that higher concentrations of aromatic amino acid in plant proteins are an important factor in antibiosis (Mollema & Cole, 1996).

Less leaf silvering damage caused by *F. occidentalis* was found in tomato leaves containing acylsugars (Mirnezhad et al., 2010). A similar observation was made in chrysanthemum flowers in which chlorogenic acid was identified as a factor for defense against *F. Occidentalis* (Leiss et al., 2009b). Some compounds were also identified that correlate with reduced damage in senecio caused by *F. occidentalis* (Leiss et al., 2009a).

Tolerance has been reported as the mechanism in common bean (*Phasealus vulgaris*) that reduces damage by *Thrips palmi.* Under medium to high thrips infestation in field and greenhouse cultivation, tolerant genotypes of common bean show a tendency to have smaller yield losses although they suffer not significantly lesser damage (Frei et al., 2004). Tolerance has also been reported in some cowpea landraces (*Vigna unguiculata*) resistant to *Megalurothrips sjostedti.* Tolerant genotypes of cowpea support the development and survival of *M. sjostedti* similarly to that of susceptible genotypes (Alabi et al., 2004).

Effectiveness comparison of mechanisms against thrips

In general, it is hard to say which the most effective mechanism against thrips is. However, due to the importance of preventing virus transfer, it is clear that tolerance is a less preferable mechanism against thrips. Tolerance mechanisms allow thrips to visit, to attack the plant and even it accommodates thrips to reproduce in the plant tissue.

Antibiosis mechanism will affect the biology of thrips and their reproduction in plants. Since viruses are acquired during larval stages and will be transmitted by the adults and larvae, it sounds reasonably that interruption of thrips life-cycle might significantly reduce the acquiring and transmission of viruses to other pepper plants and other thrips host plants. Due to a possibly lower population of thrips the direct damage in antibiosis mechanism might also significantly decrease.

Antixenosis mechanisms which can strongly protect the plant from thrips landing, feeding and oviposition seem to be the perfect mechanisms for thrips resistance in pepper. With strong antixenotic factors it may be possible to reduce direct damage, virus acquisition and transmission (Mutschler et al., 2006). However, this may not always true when antixenosis is incomplete. Antixenosis might potentially also increase thrips probing and movements which can enhance the spread of viruses within a pepper crop or to other crops since the thrips are polyphagous pest (Joost & Riley, 2005).

Compared to indirect defense, direct defense seems to be more simple as it does not need another agent i.e. thrips predators or parasitoids. In fact, introducing natural enemies is not always effective because of their lower reproduction rate compared to thrips, their different requirements for growing, etc. (Cloutier et al., 1995). Indirect defense also require thrips landing and probing the plant first to activate the indirect defense which might already cause virus transmission. Thus in relation to thrips transmission, constitutive defense mechanisms seems more effective.

Indirect selection for thrips resistance

Breeding programs towards thrips resistant pepper varieties should be conducted since they will be very advantageous for thrips control. Information about thrips resistance mechanisms will help pepper breeders to conduct breeding programs in more effective and efficient ways when indirect selection methods are present.

Indirect selection would be very helpful for several reasons. First, it can avoid bias caused by large variability in the environment which might influence thrips damage scoring. Such variability in the environment may be caused by a mix of disease and other insect damage in field and prevent reliable comparisons. A selection based phenotype may be misleading due to systematic or random environmental effects; for instance a plant might not show symptoms of insect feeding because it was accidentally not visited by insects rather than because of a high resistance level. This may cause the selection of plants that do not contain the target gene at all (Yencho et al., 2000). Second, insect bioassays to select desired accessions for breeding programs are also posing the risk of contamination to research facilities. Therefore, breeders dislike insect bioassays and prefer to use indirect selection in their breeding program.

Indirect selection could be done by using plant traits that are associated with thrips damage such as morphological characters or the presence of compounds correlated with resistance. High correlation of morphological characters or compounds with thrips damage might provide pepper breeders with promising tools for indirect selection instead of using undesirable insect bioassays.

Molecular markers and detection of Quantitative Trait Loci (QTLs) in pepper

Breeding programs can be accelerated using molecular approaches, for example the use of molecular markers for indirect selection, in a process called marker-aided selection (MAS). Molecular markers are based on differences in the DNA nucleotide sequences, on different alleles. These differences are referred to as

DNA polymorphisms, and they arise as a result of insertions, deletions, duplications, and substitutions of nucleotides (Liu, 1997). Molecular markers can be applied to identify genes of interest and to track their alleles in a MAS breeding program including insect resistance (Yencho et al., 2000).

The use of MAS is very promising because it allows breeders to select on the basis of genetic composition instead of, or in addition to selection based on phenotypes. If a phenotypic trait is tightly linked to molecular markers, the genetic segregation of the gene can be determined by marker genotyping instead of phenotyping (Staub et al., 1996), avoiding the risk of selection based on phenotype.

Molecular markers have been used in pepper genetics and breeding to construct linkage map, detect QTLs and markers for genes involved in several traits of interest. Several pepper linkage maps have been constructed using intraspecific crosses and different kind of markers such as RFLP, SSR, CAPS, AFLP have been used (Minamiyama et al., 2006; Yi et al., 2006; Barchi et al., 2007; Lee et al., 2009; Wu et al., 2009). This allows the detection of QTLs in pepper, and many QTLs have been reported for traits such as plant development (Barchi et al., 2009), fruit characteristics (Zygier et al., 2005; Ben-Chaim et al., 2006; Barchi et al., 2009), male sterility (Wang et al., 2004), and resistance against pathogens e.g. antrachnose (Voorrips et al., 2004), *Phytophthora capcisi* (Thabuis et al., 2004), and powdery mildew (Lefebvre et al., 2003). Up to now detection of QTLs and candidate genes for thrips resistance in pepper is still missing. Also in other crops only a few reports have been published on QTL mapping resistance against thrips: in common bean against *Thrips palmi karny* (Frei et al., 2005) and in cowpea (*Vigna unguiculata*) against *Frankliniella schultzei* (Muchero et al., 2010).

Scope of the investigation

Although pepper accessions with high resistance levels to thrips have been found, the number is still limited. Also, information regarding mechanisms of resistance against thrips in pepper is still scarse. Therefore, this thesis is aimed at obtaining more knowledge regarding thrips resistance in pepper, especially concerning the exploration of resistant accessions, the resistance mechanisms, the identification of factors that contribute to the resistance, and the identification of QTLs.

In **Chapter 2,** the level of resistance to thrips of several pepper accessions known from literature and CGN (Centre for Genetic Resources, the Netherlands) collections were evaluated using *in vitro* methods i.e. leaf disc, detached leaf, which were compared to field and greenhouse tests. Two thrips species: *F. occidentalis* and *T. parvispinus* were used as representatives of common thrips species in Europe and Asia, respectively.

In **Chapter 3**, factors that contribute to thrips resistance in pepper were investigated. The effect of resistance in pepper to thrips reproduction and development was studied using three highly resistant, three medium resistant and three susceptible accessions. Adult and pre-adult survival, developmental time and reproduction rate were assessed. Secondary metabolites which

correlate to thrips resistance in pepper were identified using GC-MS (Gass Chromatography – Mass Spectrometry).

In **Chapter 4,** QTLs conferring resistance to thrips in pepper were identified using damage caused by larvae and the survival of first and second instar larval stages observed in a non-choice test as resistance parameters in an intraspecific crossing between two accessions with contrasting levels of resistance against thrips. **Chapter 5** describes the identification of QTLs for several metabolites related to thrips resistance in pepper.

Finally, **Chapter 6** provides a general discussion in which the most important results of this thesis as well as their potential use in further research programmes and future perspectives are discussed.

Chapter 2

Screening of pepper accessions for resistance against two thrips species (*Frankliniella occidentalis* and *Thrips parvispinus*)

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Published as Maharijaya A, Vosman B, Steenhuis-Broers G, Harpenas A, Purwito A, Visser RGF & Voorrips RE (2011) Screening of pepper accessions for resistance against two thrips species (*Frankliniella occidentalis* and *Thrips parvispinus*). Euphytica 177: 401-410.

Abstract

Thrips are damaging pests in pepper worldwide. They can cause damage directly by feeding on leaves, fruits or flowers, and also indirectly by transferring viruses, especially Tomato Spotted Wilt Virus (TSWV). Although thrips are among the most damaging pests in pepper, until now there is no commercial variety with a useful level of resistance to thrips. This is at least partly due to the lack of knowledge on resistance levels in pepper germplasm, of QTLs and/or genes for resistance, and of information about resistance mechanisms to thrips in pepper.

This paper describes our research aimed at developing practical and reliable screening methods for thrips resistance in pepper and at identifying pepper accessions showing a strong resistance to thrips. Thirty-two pepper accessions from four species of pepper (*Capsicum annuum, C. baccatum, C. chinense and C. frutescens*) and two species of thrips (*Frankliniella occidentalis* and *Thrips parvispinus*) were used in this study. Our results indicate that the laboratory based leaf disc test and the detached leaf test can be used as reliable screening methods for thrips resistance in pepper. We observed a large variation for resistance to thrips in *Capsicum* that can be exploited in breeding programs.

Keywords: *Capsicum*, *in-vitro* test, multiple resistance, insect resistance

Introduction

Pepper (*Capsicum spp.*) is one of the most widely grown vegetables in the world and faces problems from thrips attack (Siemonsma & Piluek, 1994; Grubben & Denton, 2004). Thrips can cause damage on pepper directly by feeding on leaves, fruits or flowers. Feeding injury from thrips on leaves may affect leaf size, affect carbon allocation in the plant (Welter et al., 1990; Shipp et al., 1998) and reduce photosynthetic capacity (Tommasini & Maini, 1995). Thrips also cause indirect damage by transmitting plant viruses of the *Tospovirus, Ilarvirus, Carmovirus, Sobemovirus,* and *Machlomovirus* genera (Jones, 2005). One of the most important viruses transmitted by thrips in pepper is Tomato Spotted Wilt Virus (TSWV) (Ulman et al., 1992).

At least 16 thrips species have been reported to occur on *Capsicum* (Talekar, 1991; Capinera, 2001). *Frankliniella occidentalis* is the most common thrips species on *Capsicum* in Europe (Tommasini & Maini, 1995), while *Thrips parvispinus* is the main species in Indonesia, Malaysia, the Philippines, Thailand and Taiwan (Reyes, 1994). On Java, Indonesia, *T. parvispinus* has been reported as a major pest of *Capsicum* (Vos & Frinking, 1998; Prabaningrum & Suhardjono, 2007).

Thrips are difficult to control because of their polyphagous nature and their high reproduction rate (Weintraub, 2007). At moderate temperatures (20-25 ^{o}C), *F. occidentalis* takes about 2-3 weeks to complete its life cycle, but at 30^{o}C it may take less than 10 days (Tommasini & Maini, 1995). Another factor that contributes to a rapid development of thrips is that their reproduction is facultatively parthenogenic (Brodsgaard, 1989).

Controlling thrips using pesticides is difficult and not very effective because of their cryptic habit. They prefer enclosed areas such as buds, flowers, under the calyx of the fruits and in newly opening leaves (Jensen, 2000a; Weintraub, 2007). In addition, they develop resistance to insecticides rapidly. Resistance to insecticides of three major classes: organophosphates, carbamates and pyrethroids has been reported (Jensen, 2000a; Herron & James, 2005; Bielza, 2008). Nevertheless, pesticides are still widely used to control thrips. However, there is an increasing public demand for reduction of pesticide use and withdrawal of certain chemical compounds because of their harmful effects on growers, consumers and the environment (Dik et al., 2000).

As an alternative to the use of insecticides, integrated pest management (IPM) has been implemented in pepper (Weintraub, 2007). However, solely relying on IPM is difficult when no varieties are available that are at least moderately resistant to thrips. In fact, the most effective way to eliminate the thrips problem would be the use of highly resistant varieties. Resistance to thrips may also delay and reduce the transmission of viruses as shown by (Maris et al., 2003) for TSWV. However, resistant pepper varieties do not exist and are unlikely to become available soon.

Studies on thrips resistance in pepper are needed to support breeding programs aimed at developing thrips resistant varieties. As a first step pepper accessions with an effective level of resistance to thrips need to be identified. This requires reliable and efficient methods to assess the resistance of accessions. Our study therefore has two objectives. The first objective is to develop and evaluate

efficient phenotyping methods, which are needed for the screening of pepper lines and accessions. If such methods are to be of use in research and breeding they must be easy to conduct, accurate, reproducible, requiring little space, time, and energy. Several test methods have been described in the past including a leaf disc assay for thrips resistance in cucumber (Kogel et al., 1997), a detached leaf test for *Helicoverpa armigera* resistance in pea (Sharma et al., 2005) and a screen cage test for aphids resistance in sweet pepper (Pineda et al., 2007).

The second objective of our study is to identify accessions with different levels of thrips resistance (including highly resistant accessions) that can be used for studies aimed at elucidating the genetics of resistance against thrips.

Materials and Methods

Plant Material

Pepper accessions with possible resistance to thrips were selected on the basis of available literature (Fery and Schalk, 1991; Maris et al, 2003) and supplemented with other accessions of various species and geographic origins; they were obtained from the Center of Genetic Resources, the Netherlands at Wageningen, the Netherlands; from Plant Research International, Wageningen and from PT East West Seed Indonesia (EWINDO), Purwakarta, Indonesia. In total, 32 pepper accessions from four species: *C. annuum, C. chinense, C. baccatum* and *C. frutescens* were used (Table 1).

Thrips Species

Two species of thrips were used, *Frankliniella occidentalis* and *Thrips parvispinus*. *Frankliniella occidentalis* was selected as it is the most prevalent thrips species in European pepper cultivation (Tommasini & Maini, 1995), while *T. parvispinus* was selected as representative of Asian thrips (Reyes, 1994; Vos & Frinking, 1998; Prabaningrum & Suhardjono, 2007).

Screening Methods

a. Greenhouse Tests

Pepper accessions were grown on raised beds in a screenhouse of EWINDO at Purwakarta, West Java, Indonesia. Seedlings were raised under insect free conditions in a seedling bed and transplanted six weeks after germination. Six plants per accession were planted in a plot, with two replications in a randomized block design. Plants were spaced 75 cm between rows and 45 cm between plants in a row. Pepper plants were grown according to standard screenhouse pepper cultivation techniques (Rossel & Ferguson, 1979). Thrips infestation was spontaneous as expected, starting from two weeks after transplantation. Thrips were identified as *T. parvispinus*. Four weeks after the first symptoms occurred (when the most susceptible accessions were very severely affected), peppers were rated for damage using a relative scale from 0 (no damage) to 3 (severe damage, i.e. strongly curled leaves, silvering and

black spots). In the Netherlands the plant material was grown at 25°C, 16/8 h day/night cycle under standard glasshouse conditions at Wageningen University and Research Centre, WageningenFour plants per accession were planted in a plot, with two replications in a randomized block design. After a natural thrips (*F. occidentalis*) infestation, plant were rated using a relative scale from 0 (no damage) to 3 (severely curled leaves) seven weeks after transplantation.

b. Leaf Disc Tests

T. parvispinus were collected from a pepper field at Purwakarta, Indonesia, while *F. occidentalis* were reared on susceptible Chrysanthemum cultivar Spoetnik (Fides, De Lier, the Netherlands) in an insect greenhouse at 25°C and 70% relative humidity (Koschier et al., 2000). Adult female thrips were starved for 24 hours in a cage with only water (Murai & Loomans, 2001). Leaf discs (4 cm in diameter) were taken from fully opened leaves using a leaf punch and placed in Petri dishes on water agar (15g/l agar) with the lower (abaxial) side upward. Ten starved female adult thrips were placed on each leaf disc using a wet brush. Dishes were closed using either silk-like textile (in Indonesia) or air-permeable plastic (in the Netherlands) to prevent thrips from escaping and placed in a climate room at 24°C, 16 h light, 70% RH. There were six replicates for each accession. The extent of 'silver damage' and destruction by thrips feeding, oviposition and secretion were rated together using a relative scale from 0 (no damage) to 3 (severe damage) two days after inoculation.

c. Detached Leaf Tests

The detached leaf tests were performed as the leaf disc test, except that intact leaves from each accession were placed with their petioles in wet Oasis® (2cm x 5cm x 4cm) and were put in a jar. Jars were closed using silk-like textile (in Indonesia) or air-permeable plastic (in the Netherlands) and placed in a climate room at 24°C, 16 h light, 70% RH. There were six replicates for each accession. The extent of 'silver damage' and destruction by thrips feeding, oviposition and secretion were rated together using a relative scale from 0 (no damage) to 3 (severe damage) two days after inoculation.

Heritability Estimation

Heritability values of each test were calculated using variance components estimated from analysis of variance using the following formulas: Genetic variance (σ^2_g) = (Accession mean square – Residual mean square)/r; Phenotypic variance (σ^2_p) = $\sigma^2_g + \sigma^2_{e;}$ Heritability (h^2) = σ^2_g / σ^2_p; where r is the number of replicates.

Statistical Analysis

Accession effects were tested using Kruskal-Wallis tests; for pairwise comparisons between accessions Wilcoxon tests were used. Spearman rank correlations were calculated to compare the different test methods.

Grouping accessions with a similar pattern of resistance

Accessions were clustered based on the results of the three test methods for each thrips species, using hierarchical clustering according to the minimum variance method (Ward, 1963) and multiscale bootstrap resampling analysis (Suzuki & Shimodaira, 2006). Calculations and construction of the dendrograms were performed using the R software package Pvclust (http://www.r-project.org/).

Results

Greenhouse tests

In the screenhouse and greenhouse tests we observed leaf deformation, curling and silvering mostly at the abaxial side of the leaves (Figure 1a and 1b). Those symptoms occurred together, i.e. accessions with much leaf deformation also showed much curling and silvering, and vice versa. Thrips were also found inside the flowers and in young leaf buds.

All symptoms started to occur three weeks after transplanting. The damage scores were recorded seven weeks after transplanting, when the most susceptible accessions were very severely affected. In the screenhouse test with *T. parvispinus*, the seven most severely damaged accessions did not differ significantly from each other, while *C. annuum* 'AC 1979' and 'Bisbas' were the most resistant in this test (Table 1). In the greenhouse test with *F. occidentalis,* the seven most damaged accessions did not differ significantly from each other, nor did the nine least damaged accessions (Table 1).

Leaf disc tests

Both *T. parvispinus and F. occidentalis* produced silvering damage and black spots (Figure 1c). Symptoms appeared two days after inoculation on the abaxial side. Based on the microscopic observation (100x), we could not find any differences between the type of damage caused by *T. parvispinus* and *F. occidentalis* in our leaf disc experiments.

The mean damage scores observed in leaf disc test with *T. parvispinus* ranged from 0.0 to 2.7. The twelve most damaged accessions did not differ significantly from each other, nor did the five least affected accessions (Table 1). In the tests with *F. occidentalis* the mean damage scores ranged from 0.2 to 3.0. In this case within the seven most damaged and the eight least damaged accessions no significant differences were observed (Table 1).

Detached leaf test

The damage in the detached leaf tests at two days after inoculation was very similar to that in the leaf disc tests (Figure 1c and 1d). All damage was found at the abaxial side of the leaves. Also in this test *T. parvispinus and F. occidentalis* produced identical symptoms.

The mean damage scores in the detached leaf test with *T. parvispinus* ranged from 0.0 to 3.0. The ten most damaged accessions did not differ significantly from each other, nor did the six least damaged accessions (Table 1). In the tests with *F. occidentalis* the mean damage scores ranged from 0.2 to 3.0. In this case within the six most damaged and the six least damaged accessions no significant differences were observed (Table 1).

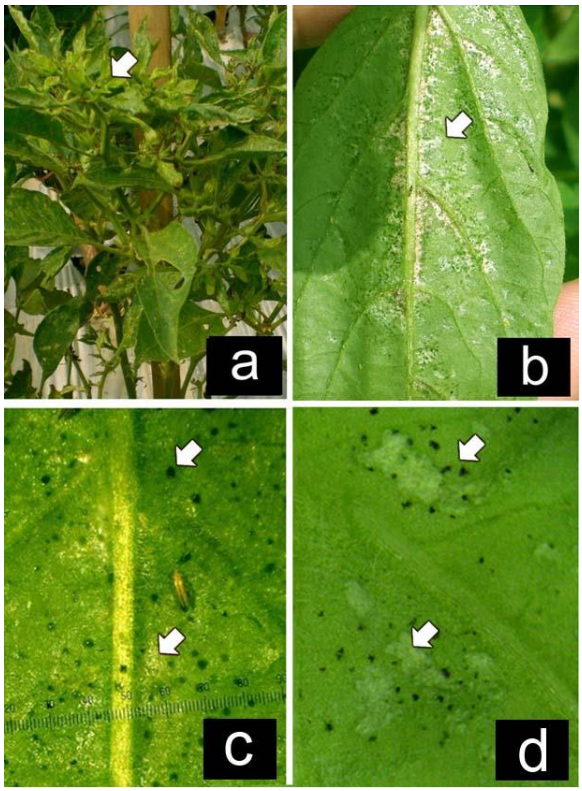

Figure 1. Damage caused by thrips in different screening methods. (a) leaf curling and deformation in the greenhouse test (indicated by arrow), (b) silvering damage caused by thrips feeding and black spots caused by fecal material in the greenhouse test (indicated by arrow), (c) silver damage caused by thrips feeding and black spots caused by fecal material in the leaf disc test (indicated by arrows), (d) idem, in the detached leaf test.

Comparison between tests

We observed several different types of damage: leaf deformation, leaf curling, black spots, and silvering on the abaxial side of the leaf. There were no differences between the symptoms caused by *T. parvispinus and F. occidentalis* in the leaf disc and detached leaf tests. The symptoms in the greenhouse for both *T. parvispinus* and *F. occidentalis* were also identical.

The observed symptoms differed between the tests with whole plants (greenhouse test) and those with leaf discs or detached leaves. In the greenhouse test the observed symptoms included silvering, curling and deformation of leaves, while in the leaf disc and detached leaf tests the

symptoms were silvering and the presence of black spots. Heritability of damage scores in all screening methods was calculated and is shown in Table 2. The heritability varied from 0.68 to 0.92.

Table 1. Damage scores in screening methods of thrips resistance in pepper

		Thrips parvispinus						***Frankliniella occidentalis***					
Acc Code	**Acession name**	**Green house***		**Leaf disc***		**Detached leaf***		**Green house***		**Leaf Disc***		**Detached Leaf***	
CGN16975	*C. annuum* AC 1979	0.2	a	0.3	ab	0.0	a	0.3	a	0.2	a	0.6	abcd
CGN20503	*C. annuum* Bisbas	0.5	ab	0.3	ab	0.2	a	1.1	bc	0.5	ab	0.2	a
CGN23765	*C. annuum* CM 331	1.0	c	0.7	abc	0.7	ab	0.3	a	0.8	abcd	0.5	abc
CGN21534	*C. annuum* Chili de Arbol							0.8	ab	1.0	bcd	1.0	bcde
CGN17042	*C. baccatum* no. 1553	1.0	c	0.0	a	0.6	ab	0.3	a	0.7	abcd	0.7	abcd
CGN21469	*C. chinense* AC 2212							0.8	ab	1.0	bcd	1.0	cde
CGN23222	*C. annuum* Keystone Resistant Giant	0.8	bc	1.0	bc	0.5	a	0.5	ab	1.0	bcd	0.7	bcde
PRI1994048	*C. annuum* Tit Super	1.5	d	2.0	de	2.0	cd	1.0	bc	1.0	abcd	0.5	abc
PRI1996236	*C. annuum* Laris	2.0	e	2.0	de	2.0	cd	1.8	def	1.5	cde	1.0	bcde
CGN21470	*C. baccatum* Aji Blanco Christal	1.0	c	0.3	ab	0.5	a	1.1	bc	1.0	bcd	0.3	ab
CGN23098	*C. annuum* Yolo Wonder	3.0	g	2.0	de	2.5	de	1.5	cde	1.2	bcd	1.5	def
CGN16922	*C. annuum* Sweet Chocolate							1.1	abcd	1.2	bcd	1.3	cdef
PRI1999049	*C. annuum* Jatilaba	1.5	d	1.3	cd	1.5	bc	0.5	ab	1.0	abcd	1.7	efg
CGN17028	*C. baccatum*							0.5	ab	1.1	bcde	2.0	fgi
PRI2007008	*C. annuum* PBC 535-IR cayene	2.5	f	2.7	e	2.5	de	1.0	bc	0.5	abc	1.0	bcde
PRI2007007	*C. annuum* PBC 473 cayene	3.0	g	2.3	e	2.5	de	1.8	def	2.0	ef	1.0	bcde
CGN22173	*C. annuum* Sweet Banana	3.0	g	2.0	e	2.5	de	1.5	cde	1.0	bcd	1.0	bcde
CGN22817	*C. frutescens* L. Lombok							1.3	efgh	1.5	bcdef	1.5	defg
CGN23206	*C. baccatum* RU 72-51							2.0	efg	1.5	cde	1.8	fg
CGN19189	*C. annuum* California Wonder 300	2.0	e	1.0	bc	2.5	de	1.5	cde	1.0	bcd	1.5	defg
PRI2004001	*C. annuum* Bruinsma Wonder	2.5	f	1.3	cd	1.5	bc	1.5	cde	0.7	abc	2.3	gijk
CGN22830	*C. annuum* Chili Serrano							1.8	cdefg	1.7	def	2.0	fgi
CGN22862	*C. chinense* no. 1720							2.5	gh	1.5	bcde	2.0	fgij
CGN21546	*C. frutescens* L. Tabasco							2.5	gh	1.5	bcdef	2.0	fgi
CGN16994	*C. chinense* RU 72-194							2.5	gh	1.5	cde	2.0	fgi
CGN23289	*C. annuum* Long Sweet	3.0	g	2.7	e	3.0	e	1.8	def	2.5	fg	2.0	fgi
CGN22829	*C. chinense* Miscucho Colorado	2.3	ef	2.3	e	2.5	de	1.2	bcd	2.5	fg	2.5	ijk
CGN16995	*C. chinense* RU 72-241							2.5	gh	2.5	fg	2.0	fgij
CGN17219	*C. chinense* no.4661 selection	3.0	g	2.7	e	3.0	e	2.0	efg	3.0	g	2.7	ijk
CGN21557	*C. chinense* no. 4661	2.0	c	2.7	e	3.0	e	2.4	fgh	3.0	g	2.8	jk
PRI1996112	*C. chinense* PI315023 (Mishme Black)	3.0	g	2.3	e	2.0	cd	2.0	efg	3.0	g	3.0	k
PRI1996108	*C. chinense* PI 281428	3.0	g	2.3	e	2.5	de	3.0	h	2.7	g	2.7	ijk

Within the same column scores followed by the same letter are not significantly different (P>0.05) according to the Wilcoxon test

* 0 = no damage, 3 = severe damage; presented data are averages over replicates within a test

Table 2. Genetic variance (σ^2_g), environment variance (σ^2_e), phenotypic variance (σ^2_p) and heritability (h^2) of score in screening methods of thrips resistance in pepper

Thrips species	Test method	σ^2_g	σ^2_e	σ^2_p	h^2
T. parvispinus	Greenhouse	0.947	0.082	1.030	0.92
	Leaf disc	0.835	0.189	1.024	0.82
	Detached leaf	0.964	0.184	1.149	0.85
F. occidentalis	Greenhouse	0.555	0.262	0.817	0.68
	Leaf disc	0.610	0.255	0.864	0.71
	Detached leaf	0.571	0.159	0.730	0.78

All correlations among the tests with *T. parvispinus* (greenhouse, leaf disc, detached leaf tests) were high ($0.77<R<0.87$) and significant ($P<0.001$). The correlations were slightly lower between the tests with *F. occidentalis* (greenhouse, leaf disc, and detached leaf: $0.73<R<0.77$, $P<0.01$) (Table 3). The correlation across species with the same test methods were also significantly correlated (Greenhouse: $R=0.76$, $P<0.001$; leaf disc: $R=0.71$, $P<0.001$; detached leaf: $R=0.69$, $P<0.001$).

Table 3. Spearman rank correlation coefficients and significance between damage score in screening methods of thrips resistance in pepper

		T. parvispinus		*F. occidentalis*		
		Leaf disc	Detached leaf	Greenhouse	Leaf disc	Detached leaf
T. parvispinus	Greenhouse	0.77 **	0.80 **	0.76 **	0.65 *	0.70 **
	Leaf disc		0.87 **	0.71 **	0.71 **	0.71 **
	Detached leaf			0.73 **	0.70 **	0.69 **
F. occidentalis	Greenhouse				0.77 **	0.73 *
	Leaf disc					0.77 **

* and ** indicate significance $P<0.01$ and $P<0.001$ respectively

Grouping accessions with a similar level of resistance

A hierarchical clustering of pepper accessions based on the test results with both thrips species produced dendrograms where all branchings have a high confidence level as based on bootstrap analysis (Figure 2). Grouping the accessions into three clusters in both cases produced groups with low, intermediate and high resistance. All six accessions in the cluster resistant to *T. parvispinus* were also resistant to *F. occidentalis*, while only one (*C. annuum* PBC535 IR Cayenne) of the accessions that were resistant to *F. occidentalis* was susceptible to *T. parvispinus*. Conversely, all seven accessions in the cluster susceptible to *F. occidentalis* were also susceptible to *T. parvispinus*, and all 10 accessions susceptible to *T. parvispinus* were also susceptible or intermediate to *F. occidentalis* with the one exception mentioned above.

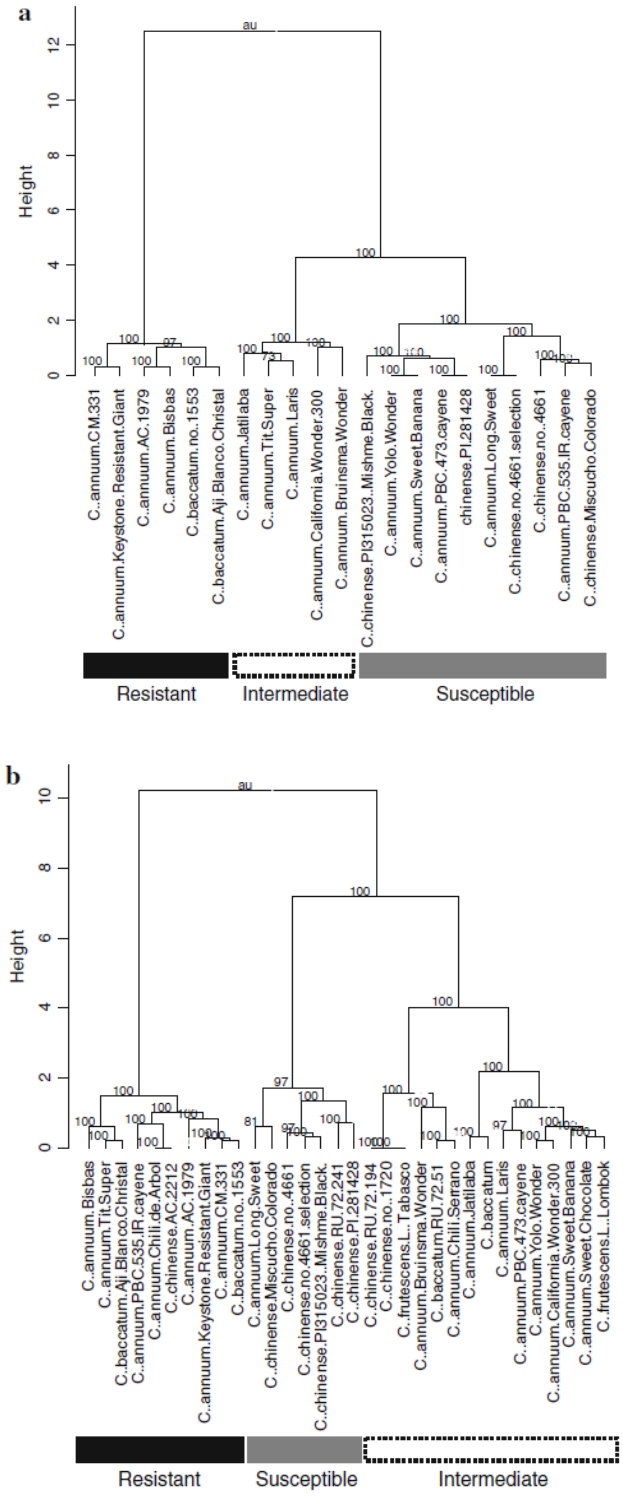

Figure 2. Cluster analysis of pepper accessions based on their resistance level in three different tests against (a) *Thrips parvispinus* and (b) *Frankliniella occidentalis*. Values at branches are approximately unbiased (AU) *p*-values as percentages (Suzuki & Shimodaira, 2006). Although all branchings are strongly supported we have indicated three clusters in both dendrograms that represent resistant, intermediate and susceptible accessions.

Discussion

Different resistance tests for thrips show highly similar results

High and very significant correlations between tests using one thrips species (Table 3) indicate that it is possible to use either the leaf disc or detached leaf test to screen pepper accessions for resistance against thrips, thus avoiding the problematic tests with whole plants. Compared to the greenhouse tests, leaf disc and detached leaf tests are relatively easy to conduct. A small climate room is sufficient to test many accessions. They also require less time: two days after inoculation the damage can be scored, compared with up to seven weeks after transplantation for screenhouse and greenhouse tests. An additional advantage is that the plants from which leaves are tested remain uninfested by thrips. Finally, environmental factors during these tests can be better controlled than in greenhouse tests. The high heritability of thrips resistance (Table 2) in the leaf disc and detached leaf tests with both *T. parvispinus* and *F. occidentalis* indicate that the observed parameter in these tests (damage score) is strongly determined by genetic factors. The higher heritability in the greenhouse test with *T. parvispinus* in Indonesia compared with the other tests may be caused by the large amounts and uniform distribution of thrips in the test after a few weeks, and the fact that they developed under natural conditions from insects healthy enough to reach and enter the greenhouse on their own account. This contrasts with the smaller number of thrips (10) used in the laboratory tests, which were reared under artificial, perhaps non-optimal conditions and which were not selected for vigour.

It has been reported that more adult thrips were found on unwounded plants than on wounded plants (Delphia et al., 2007). However, we did not observe any difference in the type of symptoms on leaf discs versus whole leaves, nor in the general amount of damage. Furthermore the correlation between leaf disc and detached leaf tests was high and significant. As the leaf disc test allows a more standardized comparison than the detached leaf test and the leaf discs are more convenient to handle than whole leaves, the leaf disc test is the most suitable for assessing a large number of pepper accessions for resistance to thrips.

Different thrips species show similar results in pepper

We observed high correlations between the tests with both thrips species (Table 3). Furthermore, the damage caused by *F. occidentalis* and *T. parvispinus* was very similar in all the tests and on all accessions in our study. In the literature we found no reports of differences in damage caused by different thrips species on pepper. For onion, one report mentions that feeding injury caused by *F. occidentalis* is similar to that caused by *T. tabaci* Lindeman (Capinera, 2001). These similarities in damage type and the high correlations between the amount of damage caused by different thrips species suggest that thrips resistance, at least in pepper, may not be very species-specific. We are aware of only one earlier report of resistance against multiple thrips species. Babu et al. (2002) mentioned a high degree of resistance to *Scirtothrips dorsalis* and *Polyphagotarsonemus latus* in pepper accessions. Resistance to multiple thrips species is interesting as at least 16 species of thrips have been reported to occur on *Capsicum* (Talekar, 1991; Capinera, 2001). A wide-range resistance would

be very useful in the many regions where pepper is grown and attacked by multiple thrips species such as some Asian countries where both *T. parvispinus* (Reyes, 1994) and *F. occidentalis* (Zhang et al., 2007) occur.

A large variation in resistance to thrips is found in pepper germplasm

We observed large differences in thrips damage between pepper accessions in our collection (Table 1). Earlier studies also reported a considerable variability within pepper germplasm for the response to thrips (Fery & Schalk, 1991; Kumar et al., 1996; Babu et al., 2002). Unfortunately, we were not able to obtain the accessions studied by Kumar et al. (1996) and Babu et al. (2002), but some accessions used by Fery and Schalk (1991) were included in our experiments. Using *F. occidentalis* in a greenhouse test with damage scored on a scale from 1 to 5, Fery and Schalk (1991) rated Keystone Resistant Giant, Yolo Wonder, Sweet Banana, and California Wonder as 2.0, 2.1, 2.2 and 2.3 respectively. In our greenhouse test with *F. occidentalis*, these accessions were rated at 0.5, 1.5, 1.5 and 1.5 on a scale from 0 to 3 (Table 1). Keystone Resistant Giant is the most resistant accession in Fery and Schalk's study (1991). Our study supports this by ranking Keystone Resistant Giant as resistant, and Yolo Wonder, Sweet Banana, and California Wonder as intermediate (Table 1, Figure 2b). However, among our accessions we observed a wider range of damage scores and accessions more resistant than Keystone Resistant Giant.

Six pepper accessions (*C. annuum* AC 1979, *C. annuum* Bisbas, *C. annuum* Keystone Resistant Giant, *C. annuum* CM 331, *C. baccatum* no. 1553, and *C. baccatum* Aji Blanco Christal) are identified as good sources for resistance against *T. parvispinus* and *F. occidentalis*. Six accessions are identified as susceptible accessions to both *T. parvispinus* and *F. occidentalis* (*C. annuum* Long Sweet, *C. chinense* Miscucho Colorado, *C. chinense* PI 281428, *C. chinense* no. 4661, *C. chinense* no.4661 selection and *C. chinense* PI315023).

These result show that there is considerable variation for resistance to thrips in *Capsicum* that can be exploited in breeding programs and also further genetic studies related to thrips resistance in pepper.

Acknowledgements

The research was financially supported by the Royal Netherlands Academy of Arts and Sciences in the framework of the Scientific Programme Indonesia—The Netherlands (SPIN) named IndoSol. We thank P.T. East West Seed Indonesia for providing the necessary facilities in conducting the Indonesian experiments.

Chapter 3

Resistance factors in pepper inhibit larval development of thrips (*Frankliniella occidentalis*)

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Published as Maharijaya A, Vosman B, Verstappen F, Steenhuis-Broers G, Mumm R, Purwito A, Visser RGF & Voorrips RE (2012) Resistance factors in pepper inhibit larval development of thrips (*Frankliniella occidentalis*). Entomologia Experimentalis Et Applicata 145: 67-72.

Abstract

The western flower thrips [*Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae)] is a major pest in pepper cultivation. Therefore, host plant resistance to thrips is a desirable trait. The objectives of this study were to determine the effect of resistance on the development of thrips and to identify metabolite compounds related to the resistance. Three highly resistant, three medium resistant, and three susceptible pepper accessions were used in this study. Adult and pre-adult survival, developmental time, and oviposition rate were assessed. Gas chromatography – mass spectrometry was used to identify compounds that correlate with the level of resistance to thrips. Our results show that resistance of pepper accessions has a significant effect on oviposition rate and larval mortality. Seven compounds were identified that correlate with resistance to thrips and six compounds were identified that correlate with susceptibility to thrips. Some of these compounds, such as tocopherols, were previously shown to have an effect on insects in general. Also, some specific secondary metabolites (alkanes) seem to be more abundant in susceptible accessions and were induced by thrips infestation.

Key words: Capsicum annuum, Solanaceae, larval mortality, oviposition rate, metabolomics, Thysanoptera, Thripidae, tocopherol, alkanes, secondary metabolites

Introduction

Thrips are a major pest in pepper [*Capsicum* spp. (Solanaceae)] cultivation worldwide, causing dramatic yield losses (Siemonsma & Piluek, 1994). They can cause direct damage by feeding and oviposition on the leaves and developing fruits, resulting in their deformation. Consequently, photosynthetic capacity of the plant is reduced (Shipp et al., 1998). Besides direct damage, thrips can also cause indirect damage by transmitting viruses, of which Tomato spotted wilt virus (TSWV) is the most important (Ulman et al., 1992). Of the 16 thrips species attacking pepper (Talekar, 1991; Capinera, 2001), *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is the most important in Europe (Tommasini & Maini, 1995) and it is becoming a common pest insect in Asian countries, including Japan, Malaysia, Korea, and China as well (Zhang et al., 2007).

Host plant resistance against insect pests is a much desired trait (Broekgaarden et al., 2011). Several studies have identified thrips-resistant pepper accessions (Fery & Schalk, 1991; Maris et al., 2004; Chapter 2). However, information about the nature of the resistance is lacking in all these studies. Differences in observed resistance may be due to antixenosis (reduced preference causing lower oviposition and/or feeding) and/or to antibiosis (reduced survival and/or reproduction) (Smith, 2005). The influence of pepper host plant resistance on *F. occidentalis* life-cycle parameters has been reported by Maris et al. (2004) using one resistant and one susceptible accession only. They found that the thrips-resistant plant can decrease thrips oviposition rate and increase larva mortality rate.

Secondary metabolites may affect resistance to thrips by influencing their growth, development, reproduction, and survival. Examples are acylsugars in tomato (Blauth et al., 1998), a specific cysteine protease inhibitor in potato (Outchkourov et al., 2004), jasmonate acid in *Arabidopsis thaliana* (L.) Heynh. (Abe et al., 2008), chlorogenic acid in chrysanthemum (Leiss et al., 2009b), and pyrrolizzidine alkaloids, jacobine, jaconine, and kaempferol glucosides in *Senecio* spp. (Leiss et al., 2009a). Genes that are involved in secondary metabolite accumulation may be utilized in breeding programs to enhance insect resistance (Linda, 2004). However, no information is available on metabolites in pepper in relation to thrips resistance.

The objective of this study was to characterize in detail the response of *F. occidentalis* towards pepper accessions differing in resistance level and to identify secondary metabolites potentially related to thrips resistance.

Materials and methods

Plant material

Nine pepper accessions were chosen based on the results of a previous screening for thrips resistance (Chapter 2). Briefly, three groups of accessions were identified: susceptible, moderately resistant, and fully resistant accessions based on injury to the leaves after being infested with female adults of *F. occidentalis*. The resistance level of the plants was rated using a relative scale from 0 (no injury) to 3 (severe injury). The accessions used, resistance level,

and injury scores are shown in Table 1.

Pepper plants were grown from seed on rockwool in a glasshouse at Wageningen University and Research Centrum, Wageningen, The Netherlands (at 25 °C and a photoperiod of L16:D8). Plants were irrigated daily with nutrient solution (EC = 2.1 dS m^{-1}; pH = 5-6) containing (in mM): 0.5 NH_4, 6.75 K, 5.0 Ca, 1.5 Mg, 15.5 NO_3, 1.75 SO_4, 1.25 P, 0.015 Fe, 0.01 Mn, 0.005 Zn, 0.03 B, 0.00075 Cu, and 0.0005 Mo. All pepper accessions were obtained from the Center of Genetic Resources, Wageningen, The Netherlands. The plants were kept free from insect pests without application of insecticides. The plants were 12 weeks old at the start of the thrips bioassay.

Thrips

A population of *F. occidentalis* was reared on the susceptible chrysanthemum cultivar Spoetnik® (Fides, De Lier, The Netherlands) in a growth chamber at 25 °C, L16:D8, and 70% r.h.. For experiments, female adults of *F. occidentalis* were randomly collected using an aspirator. They were anaesthetized with carbon dioxide (CO_2) and then placed in a Perspex ring cage without any food source except water (Murai & Loomans, 2001) for 24 h before being used.

Thrips larvae (L1 stage) were obtained by allowing female thrips to lay eggs in small cucumber fruits for 1 day, after which the adult thrips were brushed off and fruits were kept at 25 °C for 4 days, after which the new larvae emerged (Mollema et al., 1993).

Effect of resistance on thrips development- Adult survival.

Adult survival was studied by placing 10 females on a single leaf disc taken from a new, fully-opened leaf using a leaf punch (∅ 4cm) that was placed with the abaxial side downwards on 1.5 % (wt/vol) agar in a Petri dish, covered with air permeable plastic (Fresh Cling®, Essef, Ledegem, Belgium). Each accession was replicated six times. Leaf discs were incubated in a climate room at 25 °C, L16:D8, and 70% r.h.. After 4 days, the numbers of living and dead females were counted under a stereo microscope.

Developmental study.

Thrips development was studied by placing one individual synchronized L1 (first instar) larva on a leaf disc (∅ 4cm) as in the adult survival experiment. Sixty leaf discs were used for each accession. The number of individuals developing through successive developmental stages was determined by daily observation until adult emergence or until the larva died. Leaf discs were replaced by fresh ones once a week during this experiment.

The transition from larval stage L1 to L2 was distinguished by the skin tissue that remained on the leaf disc after moulting, which is easily recognized under a stereo microscope. Pre-pupae are recognized by their short wings sheaths. Pupae can be distinguished from pre-pupae by their longer wing sheaths which almost reach the end of the abdomen. Both the prepupal and the pupal stages

do not feed or move unless disturbed. Adults can be recognized by the presence of wings (Vanrijn et al., 1995)

Oviposition rate.

Ten females were placed on a single leaf disc (∅ 4cm) as in the adult survival experiment. Each accession was replicated six times. In this experiment, one resistant (*Capsicum annuum* L. Ac. 1979), one intermediate (*C. annuum* Bruinsma Wonder), and one susceptible (*Capsicum chinense* Jacq. no. 4661) accession were used. After allowing 24 h for oviposition, all females were removed. Every day, the newly-emerged larvae were counted under a stereo microscope and removed, until no further larvae emerged. At the end of the experiment, the unhatched eggs were counted after boiling the leaf discs in a microwave oven (180 watt) in 2 ml water for 60 s. Unhatched thrips eggs appear as white, kidney-shaped, and about 0.2 mm-long structures.

Statistical analysis.

Analysis of variance (ANOVA) was used to test for significant differences in adult survival, developmental period, and oviposition rate among all accessions that were tested in this study. Mean values were compared using Duncan's Multiple Range Test when significant F-values were obtained ($P<0.05$). Spearman rank correlations were calculated to test the correlation between survival rates of immature stages with the duration of immature stages. These statistical calculation were done using SAS statistical software (SAS Institute, 2004).

Metabolomics

Two cuttings of each of the nine pepper accessions used in the bioassay were grown together in one pot (25 cm in diameter) with potting compost at 25 ^{o}C, L16:D8 in a greenhouse for 3 weeks. For each accession, two pots were infested by releasing 20 L1 larvae per cutting, and two pots were not infested. Five days after infestation, pepper leaves were collected from the two plants in each pot together as one sample, carefully cleaned with a soft brush, and immediately frozen in liquid nitrogen, after which they were stored at -80 ^{o}C until use.

Each leaf sample was ground under liquid nitrogen to a fine powder. Five hundred mg of leaf powder was put in a reaction tube with 3 ml dichloromethane (DCM) as solvent using carvone (5μg/ml, 96%; Sigma-Aldrich, Zwijndrecht, the Netherlands) as internal standard. Tubes were then placed in a ultrasonic bath at room temperature for 10 min and centrifuged for 5 min at 1 515 ***g***. The supernatant was passed over a bed of sodium sulphate (Na_2SO_4) powder to remove water. The DCM extracts were analyzed by gas chromatography- mass spectrometry using an Agilent 7890A (Agilent Technologies, Amstelveen, The Netherlands) equipped with a 30-m Zebron ZB-5 ms column with 5 m retention gap (0.25 mm i.d., 0.25-μm film thickness; Phenomenex, Torrance, CA, USA) and an Agilent 5975C quadrupol mass analyzer (Agilent Technologies, Amstelveen, The Netherlands). The GC was programmed from 45 °C for 1 min, raised to 300 °C at 10 °C min^{-1}, and held at 300 °C for 7 min. One microliter of

sample was injected in splitless mode. The injection port and interface temperature were 250 °C and 280 °C, respectively, and the helium inlet pressure was controlled electronically to achieve a constant column flow of 1.0 ml min^{-1}. The column effluent was ionized using electron impact at 70 eV, and scanning was performed from 45 to 450 atomic mass units.

An untargeted metabolomics approach was applied to process the raw GC-MS data (Tikunov et al., 2005). MetAlign software (Lommen, 2009) was used to extract and align all mass signals (s/n≥ 3). Absent mass signals were randomized between 0.1 and 3 times the noise. Mass signals that were present in ≤ 4 samples were discarded, signal redundancy per metabolite was removed by means of clustering and mass spectra were reconstructed using MsClust software (Tikunov et al., 2012). Metabolites were putatively identified by matching the mass spectra of obtained metabolites to authentic reference standards, to the commercial libraries NIST08 (http://www.nist.gov/index.html) and Wiley (version 138 (http://www.wiley.com/WileyCDA/Section/index.html), to the Wageningen Natural compounds spectral libraries (a custom made library of authentic reference standards) and by comparison with retention indices of the literature calculated using a series of alkanes and fitted with a third order polynomial function (Strehmel et al., 2008).

To select candidate metabolite compounds related to thrips resistance, for each metabolite the (two-tailed) significance of the Pearson correlation of injury scores of the accessions to *F. occidentalis* that were observed from a previous experiment (Chapter 2; Table 1), versus $\log_{10}$ of the peak heights for the 18 non-challenged samples was calculated. For the identification of metabolites whose abundance responds to thrips infestation, only the samples of the three resistant and three susceptible accessions were used. Within both resistance groups, for each metabolite two-tailed t-tests were applied on the $\log_{10}$ values of the peak heights of the non-challenged vs. the challenged samples.

Results

Adult and pre-adult survival rate

No significant genotypic effect on adult survival rates of *F. occidentalis* was found (Table 1). However, significant differences between resistant, intermediate, and susceptible accessions were found for survival rates from the L1 to L2 larval stage and from the L2 to pre-pupal stage. The survival from the L1 to L2 stage varied from 0 to 100%. High percentages (80 - 100%) were found for the three susceptible accessions. In contrast, all resistant accessions completely suppressed the development of L1 to L2 larvae. On the leaves of moderately resistant accessions, development of L1 to L2 was observed but survival rates were low (15 to 55%). Significant differences were also found within the groups of susceptible and intermediate accessions.

Large differences in survival were also observed for the transition from the L2 larval stage to the pre-pupal stage. Survival on the three susceptible accessions was high (78 - 88%) but there was no significant variation within this group. No pre-pupae developed on two of the moderately resistant accessions (*Capsicum*

frutescens L. Lombok and *C. annuum* Laris), while 54 of the L2 larvae survived on the third moderately resistant *C. annuum* Bruinsma Wonder.

The development from pre-pupae to pupae and adults could only be studied in the three susceptible accessions and in the moderately resistant accession Bruinsma Wonder, as in the other accessions no pre-pupae were formed. No significant genotypic differences were found for survival in these stages. The survival rate from pre-pupa to pupa varied from 77 to 83% and almost all (92 to 100%) pupae developed into adults.

Table 1 Survival rates (%) of immature stages of *Frankliniella occidentalis* reared on nine pepper genotypes

Accessions	Level of resistance[1]	Survival rate (%)[2]				
		Adult	L1 to L2	L2 to pre-pupa	Pre-pupa to pupa	Pupa to adult
C. *chinense* PI 281428	S (2.9)	93	85b (51)	88a (45)	77 (35)	94 (33)
C. *chinense* PI 315023	S (2.8)	90	80b (48)	87a (42)	81 (34)	100 (34)
C. *chinense* no. 4661	S (2.6)	90	100a (60)	78a (47)	83 (39)	92 (36)
C. annuum Bruinsma Wonder	M (1.8)	88	55c (33)	54 b(18)	83 (15)	100 (15)
C. *frutescens* Lombok	M (1.4)	80	15e (9)	0c (0)	-	-
C. *annuum* Laris	M (1.1)	85	30d (18)	0c (0)	-	-
C. *baccatum* no. 1553	R (0.8)	78	0f (0)	-[3]	-	-
C. *annuum* Bisbas	R (0.6)	88	0f (0)	-	-	-
C. *annuum* Ac. 1979	R (0.3)	80	0f (0)	-	-	-

[1]Based on the result of previous screening for thrips resistance in which injury scores were given (injury score; 0 = no injury, 3 = severe injury, S = susceptible, M = medium resistant, R = resistant; Chapter 2)
[2]Number in parentheses are live insects at each developmental stage. The starting number was 60 first instar (L1).
Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test: P = 0.05).
[3]Indicates that no individuals reached the developmental stage.

Developmental period

Significant genotypic effects were found at all developmental stages from L1 larvae to pupae for the time needed to complete each stage. The mean duration of the L1 stage varied from 4.2 to 5.6 days. The mean duration of the pre-pupal stage varied from 1.8 to 2.8 days, while the mean duration of the pupal stage varied from 2.9 to 3.5 days (Table 2). There was no relation between the resistance level and the duration of the L1 stage (P = 0.36). As mentioned above, there were no data for the resistant accessions as no L1 larvae developed to the L2 stage. The mean duration of the L2 stage varied from 3.8 to 7.7 days. Again, significant differences were observed but without relation to the resistance level. The same was true for the pre-pupal and the pupal stages.

Oviposition

We studied oviposition and the percentage of eggs hatched in three accessions. Total oviposition was 15.8, 6.5, and 3.3 eggs/female for susceptible *C. chinense* no. 4661, moderately *C. annuum* Bruinsma Wonder, and resistant *C. annuum* Ac. 1979, respectively; the accession effect was significant (P<0.0001). There

was no significant accession effect on the percentage of eggs hatched, which varied from 94 to 97%. Among the nine accessions, significant differences were observed for the number of larvae (hatched eggs) produced per female, with significantly more larvae being produced on the three susceptible than on the six moderately and resistant accessions (Figure 1).

Table 2 Duration (in days) of immature stages (mean ± SE) of *Frankliniella occidentalis* reared on nine pepper accessions

Accession	Level of resistance[1]	Duration (days)			
		L1 to L2 larva	L2 larva to pre-pupa	Pre-pupa to pupa	Pupa to adult
C. *chinense* PI 281428	S (2.9)	5.6 ± 0.8a	5.2 ±1.3 b	2.7±0.4 ab	3.5 ±0.5 a
C. *chinense* PI 315023	S (2.8)	4.6 ±0.5 bc	7.1 ±0.7 a	2.3 ±0.5 b	2.9 ±0.4b
C. *chinense* no. 4661	S (2.6)	4.2 ±0.4 c	7.7 ±1.0 a	2.8 ±0.4 a	3.1 ±0.4 ab
C. annuum Bruinsma Wonder	M (1.8)	5.1 ±0.3 ab	3.8 ±1.1 b	1.8 ±0.5 c	3.3 ±0.5 a
C. *frutescens* Lombok	M (1.4)	5.0 ±0.0 ab	-	-	-
C. *annuum* Laris	M (1.1)	5.5 ±0.5 a	-	-	-
C. *baccatum* no. 1553	R (0.8)	-[2)]	-	-	-
C. *annuum* Bisbas	R (0.6)	-	-	-	-
C. *annuum* Ac. 1979	R (0.3)	-	-	-	-

[1]Based on the result of previous screening for thrips resistance in which injury scores were given (injury score; 0 = no injury, 3 = severe injury, S = susceptible, M = medium resistant, R = resistant; Chapter 2).
[2]Indicates that no individuals reached the developmental stage.
Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test: P = 0.05).

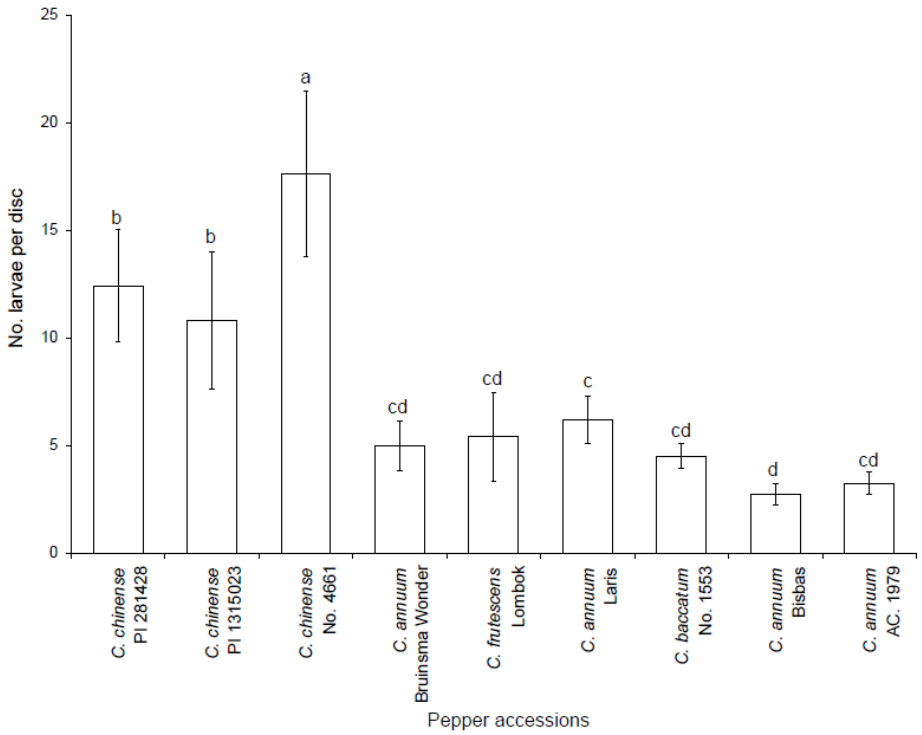

Figure 1 Mean (± SD) number of larvae (hatched eggs) produced by 10 randomly collected female *Frankliniella occidentalis* on leaf discs of nine pepper accessions at 5 days after oviposition.

Metabolites related to thrips resistance

Seventy-nine metabolites were detected over all samples. Based on the (two-tailed) significance of the Pearson correlation between the $\log_{10}$ (peak heights) of the non-challenged samples and the level of resistance against thrips, 13 metabolites were selected for further study. Seven metabolites were positively, and six metabolites negatively correlated to thrips resistance. Eight of these metabolites had a mass spectrum and retention time allowing a tentative (partial) identification of the compound. Of these eight identified compounds, δ-tocopherol, β-tocopherol or γ-tocopherol, an unknown sesquiterpene, and an unknown phytosterol were more abundant in the resistant accessions, while heptacosane, hexacosane, nonacosane, and octacosane were more abundant in the susceptible accessions (Table 3). Thrips feeding induced different compounds in the resistant, susceptible, and moderately resistant accessions. In the resistant accessions, seven compounds were induced after thrips infestation: 4,18,12,16-tetramethylheptadecan-4-olide, α-tocopherol, and five unknown compounds. In moderately resistant accessions, four unknown compounds were induced that were identical to four of the five unknown compounds induced in resistant accessions. In susceptible accessions, six compounds were induced after thrips infestation: pentacosane, docosane, tricosane, linolenic acid, n-hexadecanoid acid, and a C-18 fatty acid.

Table 3 Thrips resistance–related compounds identified in leaves of pepper accessions

No.	Putative compound name	Abundant in	Retention time (min)*	Mass (*m/z*)**	Class of compound
1	δ-Tocopherol	Resistant	26.90	402	Tocopherols
2	β-Tocopherol or γ-tocopherol	Resistant	27.72	416	Tocopherols
3	Unknown sesquiterpenes	Resistant	19.54	69	Terpenes
4	Unknown phytosterol	Resistant	30.54	271	Sterols
5	Heptacosane	Susceptible	25.14	57	Alkanes
6	Hexacosane	Susceptible	24.39	57	Alkanes
7	Nonacosane	Susceptible	26.54	57	Alkanes
8	Octacosane	Susceptible	25.85	57	Alkanes
9	Unknown	Resistant	33.09	57	
10	Unknown	Resistant	23.88	149	
11	Unknown	Resistant	26.35	419	
12	Unknown	Susceptible	26.29	57	
13	Unknown	Susceptible	23.42	130	

*Amount of time that the compound was retained in the GC column.
**Mass to charge ratio.

Discussion

Resistance factors in pepper suppress larval development of thrips

In the present study, we showed in leaf bioassays that resistance factors present in the intermediate and resistant pepper accessions have no effect on adult mortality but increase pre-adult mortality of thrips. It cannot be excluded that there might be differences in thrips behavior on a whole plant compared to a leaf disc. (Chitturi et al., 2006) have shown that female *F. occidentalis* prefer to

feed on whole plant compared to excised leaves in a choice test. However, a previous study showed high correlation between thrips injury observed in leaf bioassays and in whole plant tests (Chapter 2), which indicates that it is possible to use leaf bioassays to rate the level of resistance of pepper against thrips.

Strong and significant effects occur during larval development, especially during the transition from L1 to L2 stages which is completely suppressed in the resistant accessions and partially in the moderately resistant accessions. No significant effects were found on egg mortality or pre-pupal and pupal survival. Also for cucumber it has been reported that adult mortality of *F. occidentalis* was not affected by resistance factors (Soria & Mollema, 1995).

Apart from differences in larval survival we also observed significant differences in the duration of developmental stages of thrips. However, these differences were not correlated with the level of resistance. This was perhaps to be expected, as the level of resistance was assessed based on leaf injury (Chapter 2) and not on the duration of the developmental stages. Also in other studies, no effect of resistance on the duration of developmental stages of thrips was observed (Trichillo & Leigh, 1988; Soria & Mollema, 1995; Alabi et al., 2004; Maris et al., 2004). Obviously, a longer developmental period would further delay thrips population development from reaching the threshold level for economic damage. As mortality and developmental period apparently are not correlated, there may be opportunity to combine the two traits to further increase the resistance level.

Resistance factors in pepper prevent oviposition

Resistance has a negative effect on oviposition as indicated by the lower number of larvae found after oviposition on resistant compared to susceptible accessions (Figure 1). As egg hatch (between 94 and 97%) was not significantly different for resistant and susceptible accessions, the difference in number of larvae must have been due to the differences in number of eggs deposited by the females. Reduction in oviposition may have several causes, such as the presence of toxins, deterrents, or antifeedants as well as low levels of nutrients in the leaves of resistant plants, reducing food intake and thereby affecting egg production (Soria & Mollema, 1995; Leather et al., 1998; Awmack & Leather, 2002). It is also possible that females spend more time looking for appropriate feeding or oviposition sites on resistant plants. It has been reported that *F. occidentalis* feeding behavior is disturbed on resistant cucumber, on which *F. occidentalis* spend less time on feeding and more time moving around (Harrewijn et al., 1996).

The role of secondary metabolites in resistance

In our study, several compounds have been identified that correlate with resistance to thrips. Seven compounds were positively, and six negatively correlated to thrips resistance. Here we discuss the possible role of the compounds that could be identified, which does not mean that the unidentified compounds are less important. Tocopherols, including β-, γ-, and δ-tocopherol were found to be correlated with thrips resistance. Tocopherols have been linked

to insect resistance in other studies as well. Especially γ-tocopherol is known for its negative effect on insect development (Mohamed et al., 1997; Shepherd et al., 1999). Moreover, vitamin E has been reported to have negative effects on *Trichoplusia ni* Huebner larval growth in soybean (Neupane & Norris, 1991) and to inhibit the development of *Spodoptera frugiperda* (J.E. Smith),in *Roldana barba-johannis* (DC.) H.Rob. & Brettell (Asteraceae) (Cespedes et al., 2004). To the best of our knowledge, our report is the first to that also links δ-tocopherol and β-tocopherol to insect development.

We found a correlation of an unidentified sesquiterpene with resistance. Sesquiterpenes are (semi-) volatile compounds, many of which have toxic or deterrent characteristics. They can therefore play a role in plant communication, pollinator attraction (Pichersky & Gershenzon, 2002; Gershenzon & Dudareva, 2007; Mumm & Dicke, 2010), and in resistance towards insects (Burnett Jr et al., 1974; Lin et al., 1987; Carter et al., 1989; Eigenbrode et al., 1994; Gonzalez-Coloma et al., 1995; Koschier et al., 2000; Beale et al., 2006; Bleeker et al., 2009). However, further study is needed to determine the type of sesquiterpene related to thrips resistance in pepper and the role it plays in the interaction.

We also found a significant correlation of the abundance of an unknown phytosterol with resistance. Phytosterols have been reported to affect insects in different ways. Stigmasterol in *Cacalia tangutica* (Maxim.) Hand.-Mazz.has insecticidal effects on *Musca domestica* L. and *Aedes albopictus* Skuse (Xu et al., 2009). In contrast (Behmer et al., 2011) reported that high contents of stigmasterol and sitosterol in tobacco increase aphid survival and reproduction. Sitosterol stimulates the southwestern corn borer (*Diatraea grandiosella* Dyar) to feed on its host plants and may be a feeding stimulants for many plant feeding insects (Beck, 1965).

In our study, some alkanes were correlated with susceptibility to thrips. This fits with other reports of high amounts of alkanes, including hexacosane, octacosane, and nonacosane correlating with susceptibility to various insects, including aphids in raspberry (Shepherd et al., 1999), *Tuta absoluta* Meyrick in tomato (Oliveira et al., 2009), *Plutella xylostella* L. in cabbage (Eigenbrode et al., 1991; Eigenbrode & Pillai, 1998), and *Ostrinia nubilalis* Hubner in *Zea mays* L. (Udayagiri & Mason, 1997).

Induction of secondary compounds by thrips

Our results showed that peppers respond to thrips infestation by induction or suppression of several compounds. There are differences in the compounds that are induced or suppressed in resistant and susceptible accessions. The induction of α-tocopherol in resistant accessions after thrips infestation is interesting. As described earlier, tocopherols correlate to thrips injury and development. Thus, the induction of these compounds may also play a role in plant defense against thrips. It is known that δ-tocopherol and γ-tocopherol are converted into α-tocopherol through the shikimate pathway (DellaPenna, 2005; DellaPenna & Pogson, 2006). For another compound induced in resistant peppers by thrips infestation, 4,18,12,16-tetramethylheptadecan-4-olide, no information is available concerning its function in plant- insect interactions.

In susceptible accessions, the induction of some alkanes is interesting as some reports show that alkanes have a positive relation to insect development as described earlier. Effects of tricosane and pentacosane, two of the alkanes induced in susceptible plants, on insect behavior have also reported for their relationship with insect behavior such as repelling bumblebees from *Melilotus* spec. flowers visited earlier (Goulson et al., 2000), and avoidance of *Coccinella septempunctata L.* by *Aphidius ervi* Haliday in *Vicia faba* L. (Nakashima et al., 2004).

The induction of linolenic acid by thrips feeding in susceptible accessions in our study might be related to its role in the production of octadecanoids, i.e., jasmonic acid and related compounds that are involved in plant defense responses against pathogens, herbivores, or mechanical injury (Schaller, 2001). The non-detection of induction of linolenic acid in resistant and moderate accessions in our study might be due to the fact that leaves were harvested 5 days after thrips infestation: at that moment larvae were completely eliminated in resistant accessions and the induction of linolenic acid might have disappeared by then, while in susceptible accessions the continued presence of thrips sustained the induction.

Some compounds related with resistance or induced by thrips feeding could not be identified in our experiment as no matches were found in the available metabolite libraries. The lack of adequate metabolite libraries is still a major challenge in metabolomics studies (Allwood et al., 2008).

We have shown that feeding on leaves of resistant pepper plants inhibits the development of larvae. *Frankliniella occidentalis* adults are naturally attracted to flowers and also feed on pollen. However, they usually return to the leaves to deposit eggs (Hake et al., 1996; Lewis, 1997), and therefore especially the early larval stages need to be able to feed on leaves in order to reach maturity. The inhibition of larval survival and development on pepper leaves is therefore an important factor contributing to effective crop resistance to thrips.

Acknowledgments

This research was financially supported by the Royal Netherlands Academy of Arts and Sciences in the framework of the Scientific Programme Indonesia Netherlands (SPIN). RM acknowledges additional financial support from the Netherlands Genomics Initiative via the Centre for BioSystems Genomics.

Chapter 4

QTL mapping of thrips resistance in pepper in an interspecific cross between *Capsicum annuum* and *C. chinense*

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Abstract

This study was aimed at the elucidation of the genetic background of the resistance to thrips (*Frankliniella occidentalis*), one of the most damaging pests in pepper (*Capsicum*), through a QTL mapping approach. The QTL analysis for *F. occidentalis* resistance in pepper was performed in an F2 population consisting of 196 plants derived from an interspecific cross between the highly resistant *Capsicum annuum*AC 1979 as female parent and the highly susceptible *C. chinense* 4661 as male parent. Fifty-six SSR, 108 AFLP and 7 SNP markers were used to construct a genetic map with a total length of 1,630cM. Damage caused by larvae and the survival of first and second instar larval stages observed in a non-choice test were used as parameters of resistance in this study. Interval mapping detected one QTL for each of these parameters, all co-localizing near the same marker on chromosome 6. Use of this marker as co-factor in MQM analysis failed to uncover any additional QTLs. This QTL explained about 50% of the genetic variation, and the resistance allele of this QTL was inherited from the resistant parent. Thrips resistance was not linked to trichome density.

Keywords: *Frankliniella occidentalis,* damage, larval mortality, in vitro test, insect resistance

Introduction

Pepper (*Capsicum*) production worldwide is constrained by thrips as one of the most damaging pests (Siemonsma & Piluek, 1994). There are at least 16 species of thrips that attack *Capsicum* (Talekar, 1991; Capinera, 2001). Among those, *Frankliniella occidentalis* is the major species found in Europe (Tommasini & Maini, 1995) and it has also been found in Asia recently (Zhang et al., 2007). Thrips cause direct damage by feeding on pepper fruits, flowers and leaves (Welter et al., 1990; Tommasini & Maini, 1995; Shipp et al., 1998) and also indirect damage by spreading viruses, especially Tomato Spotted Wilt Virus (TSWV).

Thrips-resistant varieties would increase the effectiveness of thrips control. Resistance to thrips may also delay and reduce the transmission of viruses as shown by Maris et al. (2003) for TSWV. Several pepper accessions have been found to carry resistance to thrips which could be exploited further to breed thrips-resistant varieties Fery & Schalk, 1991; Maris et al., 2003; Chapter 2).

Molecular marker linkage maps have been constructed for several Capsicum populations (Minamiyama et al., 2006; Yi et al., 2006; Barchi et al., 2007; Lee et al., 2009; Wu et al., 2009). These have been used to detect QTLs for plant development and fruit characteristics (Palloix et al., 2009; Borovsky & Paran, 2011) and for resistance against pathogens such as anthracnose (*Colletotrichum spp*) (Voorrips et al., 2004), *Phytophthora capsici* (Thabuis et al., 2004) and powdery mildew (Lefebvre et al., 2003). For resistance to thrips in pepper, a QTL has been identified by Syngenta Biotechnology Inc. on chromosome 5 (Linders et al., 2010). In other crops, QTL for resistance to thrips were previously detected in cowpea (Muchero et al., 2010), potato (Galvez et al., 2005), and common bean (Frei et al., 2005).

Our study was aimed at the elucidation of the genetic background of the resistance to thrips that we identified earlier in *C. annuum* AC 1979 (Chapter 2) through a QTL mapping approach. Since the resistant parent of our population was the same as used by Linders et al., (2010) we were also interested to compare our results with theirs. Since the presence of trichomes has been implicated in pepper resistance against the thrips *Scirtotrips dorsalis* (Yadwad et al., 2008) we also included this trait in our study.

Material and Method

Plant Material

A mapping population consisting of 196 F_2 plants was developed from a cross between *C. annuum* AC 1979 as female parent and *C. chinense* 4661 as male parent. The two parents were chosen based on screening result for resistance against two thrips species, *F. occidentalis* and *T. parvispinus* using several different resistance tests (Chapter 2). *Capsicum annuum* AC 1979 was highly resistant while *C. chinense* 4661 was very susceptible in these tests. Both accessions were obtained from the Center of Genetic Resources, the Netherlands. The F_2 population was grown together with two first-generation inbred lines obtained by self-pollinating the two parental plants and with cuttings

of the F_1 in a glasshouse at Wageningen University and Research Centrum, the Netherlands. The plants were maintained in a glasshouse at 25°C, 16/8 h day/night without any pesticide application. Pests were controlled biologically using predator organisms according to standard Dutch pepper cultivation practices.

Thrips

A *F. occidentalis* population was collected from thrips-infested *Arabidopsis thaliana* plants in a greenhouse of Wageningen UR (Wageningen, the Netherlands). After confirmation of the collected thrips as *F. occidentalis* a population was developed and maintained by rearing female thrips on small cucumber fruits in a climate chamber at 25°C, 16/8 h day/night. Thrips larvae (L1 stage) were obtained by allowing female thrips to lay eggs in small cucumber fruits for one day, after which the adult thrips were brushed off and fruits were kept at 25°C for four days, when the new larvae emerged (Mollema et al., 1993). The size of the synchronized larvae population was sufficient to infest a complete replication of the resistance test in one day.

Resistance test

Five newly emerged *F. occidentalis* L1 larvae were placed on a single fresh fully opened leaf that was placed with the abaxial side downwards in a sterile 50 x 9 mm petri dish with lid (BD Falcon®). Leaves and larvae were incubated in a climate chamber at 25°C, 16 h light, 70% RH.

Damage caused by larvae was scored after two days using a visual scale ranging from 0 (no damage) to 3 (severe damage) as described in Chapter 2. Development of L1 larvae into the L2 stage was assessed by counting the number of L2 larvae and dividing this by the total number of larvae placed on the leaf. The transition from larval stage L1 to L2 was determined by the presence of skin tissue that remained on the leaf disc after molting, which can be seen under a stereo microscope. Development of L2 larvae was assessed by counting the number of pre-pupae divided by the original number of L1 larvae. Pre-pupae can be recognized by the presence of short wing sheaths. Leaves were replaced by fresh ones every three days until all larvae had died or reached the pre-pupa stage; this required incubation and observation up to 8 days. These two quantities are henceforth designated by "survival to L2" and "survival to pre-pupa" respectively.

Each replication of the resistance test consisted of one petri dish per F2 plant, three dishes for each parental inbred and two dishes of the F1. The complete test consisted of five replications, each started on a single day with approximately one week intervals.

Trichome density

Trichome density was scored according to the descriptors for *Capsicum* published by the International Plant Genetic Resources Institute (IPGRI et al., 1995), based on a visual scale: 0 (< 50·cm^{-2}), 1 (50 to 100·cm^{-2}), 2 (100 to

200·cm^{-2}) and 3 (>200·cm^{-2}) at the region near to the veins and midrib on the abaxial leaf surface of fully developed leaves. Observation for trichome density was done at three different plant stages: early vegetative stage (three weeks after planting), vegetative stage (six weeks after planting), and reproduction stage (nine weeks after planting).

Statistical analysis

Means for each F2 plant, the parental inbreds and the F1 were obtained by ANOVA analysis with the five replications of the resistance test as blocks, after transforming the fraction survival to L2 and pre-pupa stages as y = arcsine (sqrt(x)) in order to stabilize variances. Pearson correlation coefficients were calculated for the three parameters observed in the resistance test and leaf trichome densities, based on the means of both parents, F1 and F2 individuals.

Heritabilities in broad sense for all test were estimated according to Allard (1999) using the following formula: Heritability (h^2) = (σ^2F_2 - (σ^2F_{1+} σ^2P_{R+} σ^2P_S)/3)/(σ^2F_2); where σ^2F_2 is variance of the F_2, σ^2F_1 the variance of the F_1, σ^2P_R the variance of the resistant parent and σ^2P_S the variance of the susceptible parent.

Molecular markers and linkage map

The KingFisher® (www.thermo.com) device was used with AGOWA mag® Maxi DNA Isolation Kit (www.agowa.de) to isolate genomic DNA of the F2 individuals, F1 and parents. AFLP (Amplified Fragment Length Polymorphism) markers as described by (Vos et al., 1995b) were detected using combinations of *Eco*RI and *Mse*I or *Pst*I and *Mse*I primers with two selective nucleotides for *Pst*I and three selective nucleotides for *Eco*RI. The pre-amplification primers were E01, P00, and M02. Fifteen primers combination were used: P17-M39, P17-M32, P14-M50, P14-M49, P14-M48, P14-M41, P11-M61, P11-M48, E38-M49, E36-M48, E35-M58, E35-M49, E35-M48, E34-M48, and E32-M49 (primer sequences as in Keygene (2004)). The *Pst*I and *Eco*RI primers were labeled with fluorescent dyes IRD700 and IRD 800 (Li-Cor, Lincoln, USA). The AFLP products were separated and visualized on 6% denaturing polyacrylamide gel using a Li-Cor® sequencer. AFLP data then were scored using Quantar software (Keygene®). Polymorphic bands were scored co-dominantly when there was a distinct difference between homozygous and heterozygous band intensities.

Fifty-six simple sequence repeat (SSR) primers were used to amplify microsatellite markers. These were used to assign linkage groups to pepper chromosomes based on published maps (Yi et al., 2006; Lee et al., 2009; Wu et al, 2009) and an unpublished map from INRA (Institut National de La Recherche Agronomique, France; personal communication, Dr A. Palloix) (Table 1). The PCR mix for SSR primer contained 5 µl of 50 ng genomic DNA, 0.25 µl 1M each of forward and reverse primer, 0.4 µl dNTP, 1 µl LC Green® (Idaho Technology), 0.1 µL Phire^{TM} Hot Start DNA Polymerase (Finnzymes®), 2 µL buffer, and 5 µl MQ. The solution was overlaid with 20 µL of mineral oil. The thermal cycling condition were set as follows: incubation at 94^o for 2 min, 40 cycles of 94^o for 60 seconds, 60^{o}C for 60 seconds, 72^{o}C for 60 seconds, followed by 5 minutes 72^{o}C

extension and hold at 4°C. The PCR products were analyzed with the LightScanner® system (Idaho Technology) using melting temperature from 60°C to 95°C at the default melting rate (0.1°C^{S-1}). LightScanner® analysis software was used to normalize the curves and to score them as heterozygote or one of the two homozygotes. In cases where the heterozygote patterns could not be well discriminated from one of the homozygotes the marker was scored dominantly.

Four SNP primer combinations from (Linders et al., 2010) were used (forward + reserve primer, both 5'-3'): LM_2001: CTTTGGAGGTAGCGGTATG + CAACAAACGAACCACAATG, LM_2002: CCCGTTTACAAGCAAAGAG + GACCCCTGAAGAACCTCTC, LM_2004: TGTAGGATTACAAGAACATTATCG + GCGAGCTATTACACCGAAG, and LM_2006: TCGGCCTGACTAGTATTGAC + CGGGTACCAGATGTAGGG. These primers were used to confirm the position of a QTL for thrips resistance (Linders et al., 2010) on chromosome 5. The PCR protocol, visualization and scoring methods for these primers were the same as those for SSR primers.

Three SNP primer combinations were used in order to amplify SNPs in the pepper gene corresponding to Unigene37909 (www.solgenomics.net) (forward + reserve primer, both 5'-3'): Unigene37909_960: GCTGGATGTTCCCTCTTGAC + TAGCTCGGGTTAGACGGT, Unigene37909_1470: GGAAGATGTGGACATGAAGG + CACACTCTTCTGCCAGC, and Unigene37909_1575: GCCATCTTCTGCACCATTT+TCTCACCCATATCAATCTCTTCG.

A linkage map was constructed using JoinMap 4.1 software (Van Ooijen, 2006). Markers with more than about 40 missing values were discarded. Groups of markers of a more or less constant composition over a range of LOD values were used as a starting point to create linkage groups. Where multiple linkage groups were found with SSR markers known to reside on the same pepper chromosome an attempt was made to combine the markers into one linkage group. Mapping within linkage groups was carried out with the regression algorithm and a maximum jump level of 5. The final result was obtained by deleting markers that did not fit well as judged by the nearest neighbour stress or the mean chi-square contribution.

QTL mapping

Potential QTLs for damage, larval survival and trichome density were identified using the MapQTL 6.0 package (Van Ooijen, 2009). Firstly, interval mapping analysis was performed to find regions with potential QTL effects. Secondly, co-dominant markers in these regions were used as co-factors in multiple-QTL mapping (MQM). Significance thresholds of log of odds (LOD) corresponding to a genome-wide confidence level of $P<0.05$ were determined for each trait using the permutation test of MapQTL 6.0 with 1,000 iterations. The QTL graphs were prepared with MAPCHART 2.2 (Voorrips, 2002).

Table 1. List of SSR primers used to assign linkage groups in to possibly chromosome locations in pepper

	Markers	Chr.)*	Forward primer (5'-3')**	Reverse primer (5'-3')**
1	Epms 725	1	TTGAATCGTTGAAGCCCATT	ATCTGAAGCTGGGCTCCTTT
2	Hpms 1-41	1	GGGTATCATCCGTTGAAAGTTAGG	CAAGAGGTATCACAACATGAGAGG
3	Hpms 1-281	1	TGAGGCAGTGGTATGGTCTGC	CCCGAGTTCGTCTGCCAATAG
4	Gpms 169	2	TCGAACAAATGGGTCATGTG	GATGAGGGTCCTGTGCTACC
5	Gpms 37	2	ATTTGTATATTATTTCTTGGCCTTG	TGAACTACCCAATTCCAGCC
6	Hpms E073	3	TTATTCAGGCCCACTTATCGAA	CAGCAGCCAAATTCTTGATTTC
7	Hpms E008	3	CCCCTTAACTTTTAATTCTAGATCTGC	TCGTTGTTCCTCCATCACCTCA
8	Gpms 198	3	AGCTTTAGACAGTGTCTGCGTG	TGATGATAAATTGCCTTCCG
9	Epms 386	3	ACGCCAAGAAAATCATCTCC	CCATTGCTGAAGAAAATGGG
10	Hpms E122	3	GCAATGGCTCAGGTCTCCATCT	TGTCGCCCTTTAATGCAAAACC
11	Gpms 93	3	ATCCTTGGCGTATTTTGCAC	TTCACTTTGCACACAGGCTT
12	HpmsAT2 14	4	TTTAGGGTTTCCAACTCTTCTTCC	CTAACCCCACCAAGCAAAACAC
13	Hpms 1-165	4	GGCTATTTCCGACAAACCCTCAG	CCATTGGTGTTTTCACTGTTGTG
14	Hpms E099	4	CAATCATTGCCACCTTATTTTTGC	TCACAAGGGGTTGATGGAAATG
15	Hpms E055	4	GGCCGCTTAAAGTTGTTCAAGG	TGTGGCTAGCGGTGTTATGCAC
16	Hpms E049	4	CACTCCAACAGCAGCAGCAAAC	CCTTGCCGATGTTGAAGCTTTT
17	Hpms E085	4	TGCCCAAATATCAGTCAAGCTCA	TGGTTGTTGTTCTCATGGTGGTG
18	Hpms E111	4	CCATCATTTCTCCCCAATTCCA	GAGAGCAGAAGAAGGGGTGGTG
19	Hpms E116	5	CATCTCTCCGTTGAATCTATTTCC	ACGGTCATCCATTAGAACCGTA
20	Hpms 2-45	5	CGAAAGGTAGTTTTGGGCCTTTG	TGGGCCCAATATGCTTAAGAGC
21	Gpms 165	5	TGAACAATAATAATTGACAGGACAG	AGCCTCGCAGTTTGTTCTTAC
22	Hpms 2-23	5	CCCTCGGCTCAGGATAAATACC	CCCAGACTCCCACTTTGTG
23	Hpms E015	5	TTGTGAGGGTTTGACACTGGGA	CCGAGCTCGATGAGGATGAACT
24	Hpms E014	6	CTTTGGAACATTTCTTTGGGGG	GCGGACGTAGCAGTAGGTTTGG
25	Hpms E088	6	GCAAATGGTTCCCTAAACTGCTT	GCTCTCCGTTTCCGATGTGATT
26	Hpms E078	6	TTTGTGAAGAAGCAACCGGTGA	TGTGAGGAAGAAAGTGCGAAGG
27	Hpms 1-5	6	CCAAACGAACCGATGAACACTC	GACAATGTTGAAAAAGGTGGAAGAC
28	HpmsAT2-20	6	TGCACTGTCTTGTGTTAAAATGACG	AAAATTGCACAAATATGGCTGCTG
29	HpmsE113	6	CCCTAAAGCTCGAGAAATTGAAGC	GAATGCTGTTGCTGGGGTTGTT
30	Epms 376	6	ACCCACCTTCATCAACAACC	ATTTGTGGCTTTTCGAAACG
31	Hpms E068	7	TGTTCCTTTTGTTGTTACCTTTTG	CGTCTAGGAATGGAAGAAGAGC
32	Hpms E057	7	ACCCACTCCCTCTCCTCTTTGG	GCAGTGGAAAAACAGTCCTGTGG
33	Hpms 1-227	7	CGTGGCTTCAAGTATGGACTGC	GGGGCGGAACTTTTCTTATCC
34	Epms 342	8	CTGGTAGTTGCAAGAGTAGATCG	ATGATCTTTGACGACGAGGG
35	Hpms E115	1/8	TCATCTCATAGCCTGCCCCCTA	CCACTTGAAGAAGCCATGACCA
36	Hpms 1-148	1/8	GGCGGAGAAGAACTAGACGATTAGC	CCACCCATTCCACATAGACG
37	Hpms E004	1/8	TGGGAAGAGAAATTGTGAAAGCA	CAATGCCAACAATGGCATCCTA
38	Epms 310	8	TGGGAAGAGAAATTGTGAAAGC	AGGAAACATGGTTCAATGCC
39	Gpms 194	9	AGGTGGCAGTTGAGGCTAAG	GTTCTAGGTCTTTGCCCTGG
40	Hpms 1-3	9	TGGGAAATAGGATGCGCTAAACC	AACTTTAAGACTCAAAATCCATAACC
41	Hpms E051	9	TGGCCAGCTTCACACAGAGGTA	TGTCACAATATTGGAGGCCAGAA
42	Epms 419	9	TTCAGGTGCAGGTATCATCG	GGGTACTTGTCCATTTATCCAG
43	Hpms E143	9	CCATTCAGCTAGGGTTCAGTCCA	CGACCAAATCGAATCTTCGTGA
44	Hpms E013	10	GCGCCAAGTGAGTTGAATTGAT	CACCAATCCGCTTGCTGTTGTA
45	Hpms E059	10	GCAAGGACGCAGTCGTTAGACA	CCGCCTGTGCTGAATTGTTTAG
46	Hpms 2-21	10	TTTTTCAATTGATGCATGACCGATA	CATGTCATTTTGTCATTGATTTGG
47	Hpms E065	10	TGAAATAGGCCAATCCCTTTGC	ATTCCCTGGGATTCCTGCATTA
48	Hpms E031	10	CCCTAAATCAACCCCAAATTCAA	CCCCCATTACCTGACTGCAAAA
49	Hpms E096	10	CGGGTCAAACAAAAACCGAAGT	GCTTGTGGTTGAGCTCGCTCTT
50	Gpms 159	10	AAGAACATGAGGAACTTTAACCATG	TTCACCCTTCTCCGACTCC
51	Epms 561	11	ATTGGACTTCAAATTTGGCC	AAACCAAAATCAGCATTAAAATATAAAC
52	Epms 410	11	GGAAACTAAACACACTTTCTCTCTC	ACTGGACGCCAGTTTGATTC
53	Epms 391	11	TTTCTTCTCTGGCCCTTTTG	ACGCCTATTGCGAATTTCAG
54	Hpms 2-2	11	GCAAGGATGCTTAGTTGGGTGTC	TCCCAAAATTACCTTGCAGCAC
55	Hpms E094	12	CCAGTTGAGAGCTGCTGCAAAA	CACCAACAAAACAAAGGCCACA
56	Hpms E128	12	TGGATCCCAAAAGACTCAGAACA	TATTTCCCTCAGTCGAGGTCGT
57	Hpms E064	12	CCCTCCTTTTACCTCGTCAAAAA	ATGCCAAGGAGCAATGAGAACC

)*putative chromosome position of Hpms markers arebased on Lee et al. (2009)and Yi et al.(2006), while putative chromosome position of Gpms and Epms markers are based onunpublished INRA (Institut National de La RechercheAgronomique, France) map (personal communication) and Wu et al.(2009).

)** primer sequences for Hpms markers are based on Lee et al. (2009) and Yi et al.(2006), while the primer sequences for Gpms and Epms markers are based on Nagy et al. (2007) andBarchi et al. (2007).

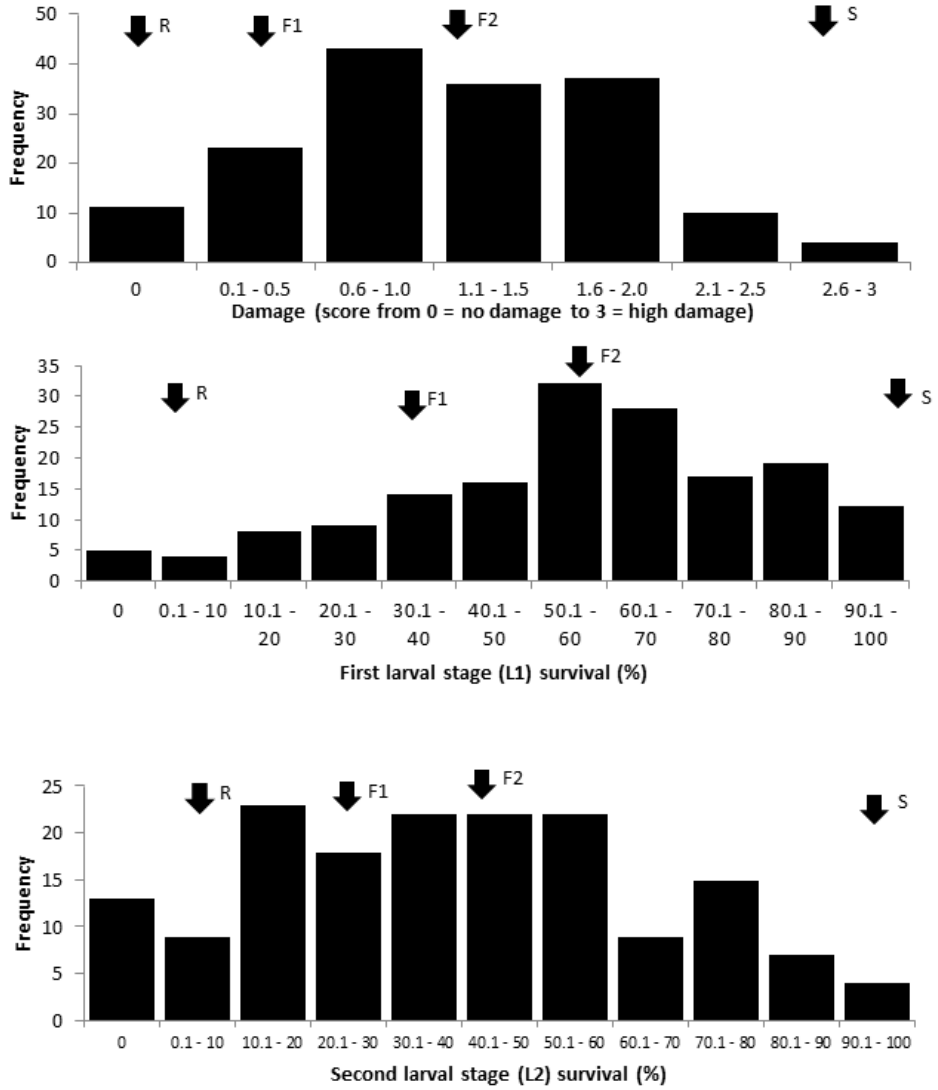

Figure 1. Frequency distributions for overall damage caused by first instar larva, survival to L2 (second larval stage), and survival to pre-pupa in F2 population from a cross between resistant and susceptible accessions of pepper. Arrows indicate the approximate means of the resistant parent (R), susceptible parent (S), F1 and F2 population

Results

Resistance test

The F2 population showed a continuous variation for damage level caused by larvae and for survival to L2 and survival to pre-pupa. Frequency distributions of phenotypic data were skewed towards the resistant parent for damage and survival to pre-pupa, while for survival to L2 it was skewed toward the susceptible parent (Figure 1). In all replicates of the resistant parent the damage was 0 and the survival to L2 and survival to pre-pupa was very low, while all

replicates of the susceptible parent exhibited significant feeding damage and very high survival rate both for survival to L2 and survival to pre-pupa. The wide-sense heritability of all parameters scored in the laboratory tests with *F. occidentalis* was high (Table 2).

Damage caused by larvae, survival to L2 and survival to pre-pupa were highly correlated with coefficients 0.68 to 0.80 and P<0.001. However, none of the parameters scored in the resistance tests were significantly correlated with trichome density (Table 3).

Table 2. Values of resistance related traits for parents, F1 and F2 plants after infestation with newly emerging L1 larvae of *Frankliniella occidentalis*

	Damage[b]	Survival to L2[c]	Survival to pre-pupa[d]
Resistant parent	0 ± 0.00[a]	0.20 ± 0.12	0.20 ± 0.12
Susceptible parent	2.73 ± 0.04	1.57 ± 0.00	1.36 ± 0.2
F_1	0.4 ± 0.28	0.60 ± 0.06	0.55 ± 0.12
F_2	1.16 ± 0.69	0.88 ± 0.38	0.66 ± 0.38
Heritability [e]	0.94	0.96	0.93

[a] Mean ± standard deviation
[b] Score of relative damage caused byL1 larvae of *F. occidentalis* at two days after infestation: 0 (no damage) to 3 (severe damage)
[c] arcsine(sqrt(x)) of fraction L1 larvae that survived to L2 stage
[d] arcsine(sqrt(x)) of fraction of L1larvae that survived to pre-pupa stage
[e] Broad sense heritability calculated according to Allard (1999)

Linkage map

Briefly, a linkage map was constructed consisting of 22 linkage groups. The linkage groups were varied from 16.5 to 197.4 cM, with a total length of 1630 cM. The total map included 171 markers (56 SSR, 108 AFLP, and 7 SNP), of which 86 (57.3%) were scored co-dominantly.

Linkage groups were assigned to pepper chromosomes based on SSR anchor markers (see M&M). Seven chromosomes (1, 2, 6, 7, 8, 9, 11) had only one linkage group assigned, while the other five had two or in one case (chromosome 3) three linkage groups assigned. Four linkage groups consisting of a total of 20 AFLPs and spanning 205 cM could not be assigned to chromosomes. Four markers (LM_2001, LM_2002, LM_2004 and LM_2006) described by Linders et al. (2010) as mapping to chromosome 5 were confirmed to map on that chromosome. Three SNP markers for the pepper gene corresponding to Unigene 37909 (www.solgenomics.net) were mapped within 1.5 cM on chromosome 8.

QTL mapping

Interval mapping of damage, survival to L2 and survival to pre-pupa all resulted in the detection of the same QTL on chromosome six (P06, Figure 2). MQM mapping using the marker nearest the top of the three LOD profiles (Hpms078) as cofactor failed to reveal any additional QTLs. In particular no QTL signal was found on chromosome five at the three markers mentioned by Linders et al

(2010) to target a QTL for thrips resistance. The peaks of our QTLs were located between 0 and 5 cM below marker HpmsE078. The LOD scores at this marker were 21.5, 24.3 and 19.7, with an explained phenotypic variance of 45.3%, 49.5% and 42.5% for damage, survival to L2 and survival to pre-pupa, respectively (Table 4). Since the heritabilities of damage, survival to L2 and survival to pre-pupa were 0.94, 0.96 and 0.93 (Table 2), the QTL explained 48.2%, 51.6% and 47.0% of the genetic variance in the F2 for the three traits. The resistance allele of this QTL was inherited from the resistant parent. The dominance effect of the QTL was small in comparison with the additive affect, with susceptibility being partially dominant over resistance (Table 4).

Table 3. Spearman rank correlation coefficients and significance between score in all tests

		Survival to L2	Survival to pre-pupa	Leaf trichomes density		
				Early vegetative	Late vegetative	Reproductive
Damage caused by larva		0.68*	0.72*	0.13	0.11	0.12
Survival to L2			0.78*	0.10	0.09	0.09
Survival to pre-pupa				0.08	0.09	0.09
Leaf trichome density	Early vegetative				0.86*	0.71*
	Late vegetative					0.83*

* indicate significance P<0.001

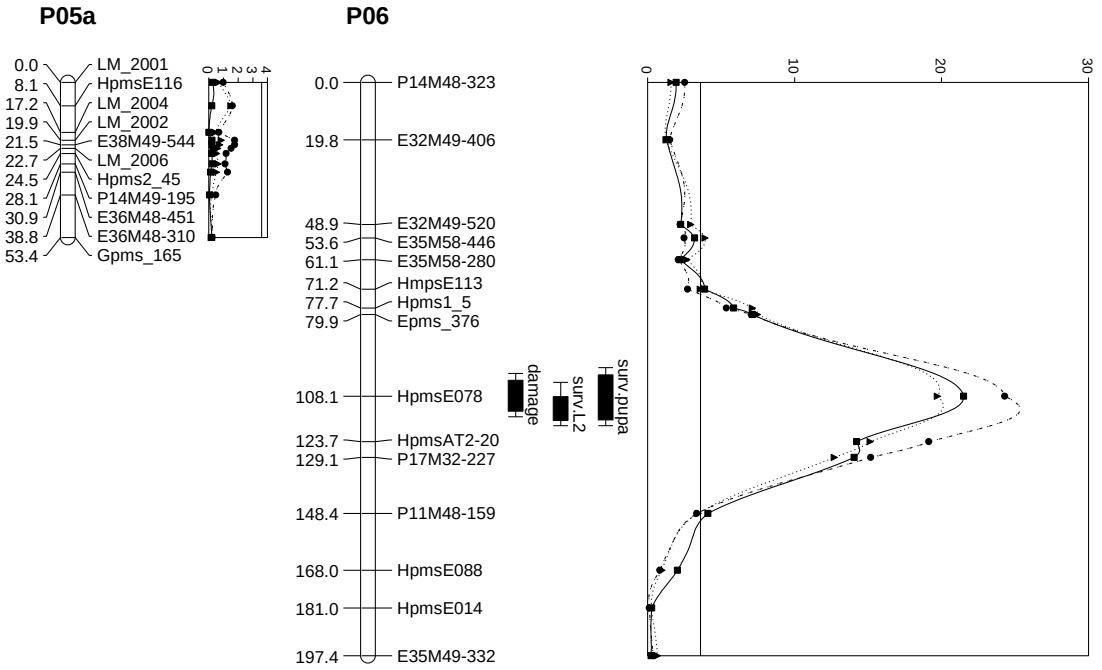

Figure 2. LOD profiles and 1-LOD and 2-LOD support intervals for resistance QTLs on chromosomes5 and 6. Solid, dashes and dotted lines represent the profiles for damage, survival to L2 and survival to pre-pupa respectively, after inoculation with newly emerged L1 larvae of *F. occidentalis.* The line at LOD 3.6 represents the LOD threshold. On chromosome 5 no QTLs were detected for these traits.

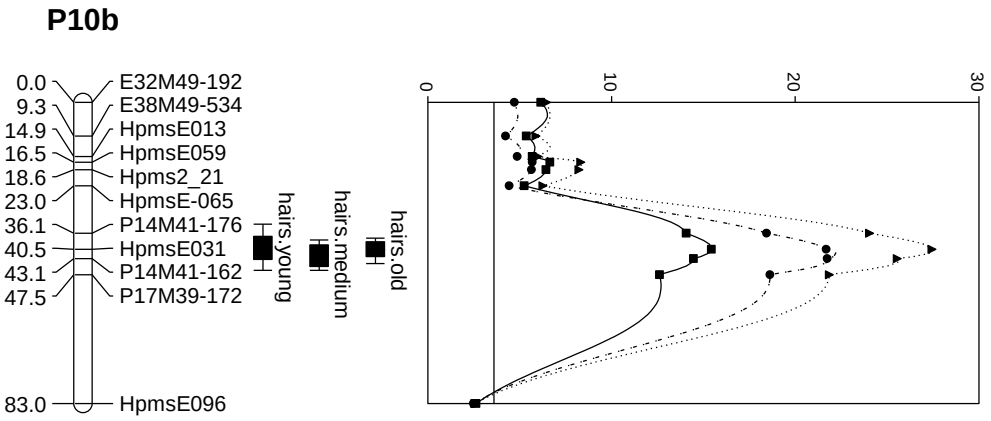

Figure 3. LOD profiles for QTL for trichome density on chromosome 10. Solid, dashes and dotted lines are the trichome density at early vegetative, late vegetative and reproductive stage, respectively.

Table 4. QTL effects for resistance-related traits after inoculation with *F. occidentalis* and leaf trichome density in pepper

Traits	Marker at QTL peak	Chromosome	Position[a]	LOD	LOD threshold[b]	Additive effect[c]	Dominance effect	% Exp.[d]
Damage	HpmsE078	P06	108.1	21.5	3.6	-0.68	0.06	45.3
Survival to L2[e]	HpmsE078	P06	108.1	24.3	3.6	-0.37	0.09	49.5
Survival to pre-pupa[f]	HpmsE078	P06	108.1	19.7	3.6	-0.35	0.09	42.5
Trichome density early vegetative[g]	HpmsE031	P10b	40.5	15.4	3.6	-0.63	0.14	30.4
Trichome density late vegetative[g]	HpmsE031	P10b	40.5	21.7	3.6	-0.69	0.26	39.9
Trichome density reproductive[g]	HpmsE031	P10b	40.5	27.5	3.6	-0.74	0.30	47.5

[a] Position of the QTL, in cM, referred to the linkage group
[b] Logarithm of the odds (LOD) threshold corresponding to agenome wide confidence level of 0.05, estimated from permutation tests with 1,000 iterations
[c] Negative values indicate that *C. annuum*alleles have lower phenotypic values than *C. chinense* alleles
[d] Percentage of phenotypic variance explained by each QTL
[e] arcsine(sqrt(x)) of fraction L1 larvae that survived to L2 stage
[f] arcsine(sqrt(x)) of fraction of L1larvae that survived to pre-pupa stage
[g] based on a visual scale: 0 (< 50·cm^{-2}), 1 (50 to 100/cm^{-2}), 2 (100 to 200·cm^{-2}) and 3 (>200·cm^{-2}) at the region near to the veins and midrib on the abaxial leaf surface of fully developed leaves at three different plant stages: early vegetative stage (three weeks after planting), vegetative stage (six weeks after planting), and reproduction stage (nine weeks after planting).

One significant QTL was detected for leaf trichome density for all three observed leaf ages on chromosome 10 (Figure 3). The LOD scores for the detected QTL at all leaf ages were above the LOD score corresponding to a genome-wide confidence level of 95% which was 3.6 as determined by permutation test with 1,000 iterations. The peak of the LOD profile for early vegetative and reproductive stage was near marker HpmsE031; at this marker 30.4%, 39.9% and 47.5% of the variance of the F2 plant means was explained by the QTL for

early vegetative, vegetative and reproductive stage, respectively. Use of HpmsE031 as co-factor in MQM analysis failed to uncover any additional QTLs.

Discussion

Resistance test

The high heritabilities found for damage, survival to L2 and survival to pre-pupa in the resistance test indicate that variation due to environmental factors was minor relative to genetic effects. This was achieved by using a climate room with controlled environmental conditions and a thrips rearing that supplied us with large quantities of uniform and synchronized larvae. This is an important advantage for genetic studies in comparison with greenhouse or field tests. In previous work (Chapter 2) we have shown that the resistance estimated from the laboratory test corresponds well with that estimated from greenhouse and field tests.

The high correlations between damage caused by larvae and survival to L2 and survival to pre-pupa indicate that differences in tolerance (i.e. the development of symptoms in response to the presence and activities of the pest) do not play an important role in this case. The low number of larvae that survive on resistant plants shows that the mechanism of pepper defense against thrips larvae is based on antibiosis (Horber, 1980). It had been reported before that resistance in pepper blocks larval development of *F. occidentalis* in pepper (Maris et al., 2004; Chapter 3).

Trichome density is not related to thrips resistance in pepper

No correlation was found between any of the resistance parameters and trichome density in our study with *F. occidentalis.* This contrasts with an earlier finding that trichomes are associated with resistance to a different thrips species (*Scirtothrips dorsalis*) in pepper (Yadwad et al., 2008). This difference might be caused by the difference in thrips species, but also by the fact that Yadwad et al. (2008) rated the resistance based on damage caused by adult thrips in a preference test, whereas we used a non-choice test with larvae. Further, the significant correlations of thrips resistance and trichome density found by Yadwad et al. (2008) were F2 population specific. For only four out of seven F2 populations, each consisting of 60 plants, they found a significant correlation of resistance against thrips with trichome density at the mature pepper stage (R = 0.27 – 0.48) and no correlation was found for any of those seven populations at flowering stage.

Linkage map

Twenty-two linkage groups were constructed, for twelve chromosomes in the haploid pepper genome. The mapping of SSR markers in our linkage map was consistent with that in previous populations (Minamiyama et al., 2006; Yi et al., 2006; Barchi et al., 2007; Wu et al., 2009). The total length of our linkage map

was 1630 cM which is comparable to the maps published by these authors. Although in several cases we still have more than one linkage group per chromosome it is likely that our map covers most of the pepper genome.

QTL mapping

Since the three parameters of resistance in our test: damage, survival to L2 and survival to pre-pupa were highly correlated (Table 3) it is not surprising that the QTLs found for those three parameters co-localize near the same marker (HpmsE078 on chromosome 6). Only one QTL was detected for all three parameters, even when using this marker as co-factor in a multiple-QTL mapping (MQM) approach. This QTL explained about 50% of the genetic variation for the three parameters, leaving the other half unexplained. Since most of the genome is covered by our linkage map the missing genetic effect cannot be caused by other major QTLs, as these would have been detected by the MQM mapping. Therefore it is likely that several QTLs with small effects are segregating in this population as well. While the QTL has a small dominance effect with susceptibility partially dominant over resistance, the mean of the F2 population is near to the midparent value and the F1 is more resistant than the midparent, which suggests that the residual genetic effects are (partially) dominant for resistance.

The major QTL described by Linders et al. (2010) on chromosome 5 was not detected in our study, in spite of the fact that we included several markers linked to it. Likewise they gave no hint of a possible resistance QTL on chromosome 6. As they used the same resistant parent as we did (*C. annuum* AC 1979), but a different susceptible parent, this suggests that at least two major factors are involved in the resistance present in the shared parent, but that in both mapping populations only one of these segregated. When this is true, our susceptible parent contains the resistant allele of the QTL on chromosome 5. As this parent is indeed highly susceptible (Chapter 2; Chapter 3) the chromosome 5 QTL then does not provide any resistance in absence of the resistance allele on chromosome 6 QTL, and the reverse this is also likely to be the case.

Another possibility would be that the chromosome 6 QTL is effective exclusively against larvae, since the chromosome 5 QTL was detected in bioassays using a mix of adults and juveniles (Linders et al., 2010). It is less likely that the two QTL are specific to certain subpopulations of *F. occidentalis* since the resistance donor was even resistant to two different thrips species (*F. occidentalis* and *T. parvispinus*). Further experiments are needed to resolve this issue.

A highly significant QTL for trichome density was detected on chromosome 11. In accordance with the absence of correlation between trichome density and resistance parameters, this QTL was unlinked with the QTL for resistance. Our QTL for trichome density was found at the same position as the QTL found by Kim et al (2010).

The QTL detected on chromosome 6 is an important factor affecting thrips resistance in pepper, which implies that pepper breeders can get benefit through the introgression of this QTL. As the source of resistance belongs to *C. annuum,* which is the dominant pepper crop species, it may be assumed that the

introgression of this region to other *C. annuum* will be straightforward. Markers closely linked to HpmsE078 can be generated and used in marker assisted breeding for thrips resistance in pepper and in a further elucidation of the genes involved in this resistance.

Acknowledgements

The research was financially supported by the Royal Netherlands Academy of Arts and Sciences in the framework of the Scientific Programme Indonesia (SPIN).

Chapter 5

QTL analysis to identify metabolites potentially related to thrips resistance in pepper (*Capsicum*)

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Abstract

In earlier studies we have shown that resistance to trips is expressed in leaves of certain *Capsicum* accessions. The current study was aimed at the identification of metabolites in pepper leaves that might be responsible for the thrips resistance, for which we used GC-MS and LC-MS analysis in combination with mQTL (metabolite quantitative trait loci) mapping. We could detect 55 metabolites by GC-MS and 674 by LC-MS. Of these, 242 could be mapped on the *Capsicum* genome and were shown to be unequally distributed, resulting in 'hotspots' and 'coldspots' of mQTLs. Of the metabolites, eighteen were significantly correlated with larval survival of thrips. Unfortunately, for only two of the correlated compounds the chemical identity could be determined from available libraries. The QTL mapping showed that mQTLs for two metabolites overlap with those for resistance parameters, which may indicate a relation between these metabolites and resistance against thrips.

Keywords: *untargeted analysis, larval mortality, GC-MS, LC-MS, metabolomics*

Introduction

The importance of plant metabolites in the protection against insects has been reported before (Wink, 1988; Bennett & Wallsgrove, 1994; Pichersky & Gershenzon, 2002) and is a basis to develop strategies to reduce losses caused by insects in various crops. This development is called metabolomics-assisted breeding (Fernie & Schauer, 2009).

Gas-chromatography-mass-spectrometry (GC-MS) and liquid-chromatography-mass-spectrometry (LC-MS) are currently the standard mass-spectrometry methods for metabolite analysis (Villas-Bôas et al., 2005; Fernie & Schauer, 2009; Okazaki & Saito, 2012). The exploitation of GC-MS and LC-MS data in an untargeted metabolomics approach allows the detection of hundreds of metabolites, without prior knowledge on their identity (Tikunov et al., 2005). This is very suitable for metabolite profiling and therefore might be useful in detecting metabolites related to insect resistance in plants, such as thrips (*Frankliniella occidentalis*) resistance in pepper (*Capsicum* spp.).

Frankliniella occidentalis can cause large losses in pepper production through direct damage by feeding on leaves and fruits and indirect damage by transferring viruses (Shipp et al., 1998; Jones, 2005). Thrips control is difficult because of their polyphagous nature, high reproductive rate and their facultative parthenogenic mode of reproduction, i.e. their ability to lay eggs without mating (Brodsgaard, 1989; Weintraub, 2007), and therefore resistant varieties are urgently needed. Breeding of pepper varieties resistant to thrips can benefit from the exploration and exploitation of metabolites related to resistance. Unfortunately, no information about these kind of metabolites is available.

Wahyuni et al., (2012) showed that pepper accessions can be grouped by species based on the metabolite profiles of the fruits. We reported earlier on the correlation between presence or absence of metabolites detected using GC-MS analysis and the level of resistance in nine accessions of different *Capsicum* species (Chapter 3). Since that report was based on a small number of accessions of highly different origin, these findings needed to be validated, preferably in a segregating population resulting from a cross between a thrips resistant and a susceptible accession.

The current study was aimed at the identification of metabolites in pepper leaves that might be related to thrips resistance, using GC-MS and LC-MS in combination with mQTL (metabolomic quantitative trait locus) mapping in an F2 population resulting from a cross between *Capsicum* accessions with contrasting levels of resistance to thrips (Chapter 2 & Chapter 3). The identification of metabolites correlating with and/or mapping at the same positions as resistance may provide further clues for the elucidation of the resistance mechanism and more efficient ways of breeding thrips-resistant pepper varieties.

Materials and Methods

Plant materials

An F_2 population consisting of 196 plants was developed from a cross between *C. annuum* AC 1979 as female parent and *C. chinense* 4661 as male parent

(Chapter 4). The two parents differ in laboratory and field tests for their resistance level against thrips (Chapter 2). The maternal parent, *C. annuum* AC 1979 was very resistant to thrips and suppressed the development of L1-larvae while the paternal parent, *C. chinense* 4661 was very susceptible to thrips and supported the development of larvae (Chapter 3). Both accessions were obtained from the Center of Genetic Resources, the Netherlands. The F_2 population was grown together with two first-generation inbred lines obtained by self-pollination of the two parental plants and with cuttings of the F_1 plant in a glasshouse at Wageningen University and Research Center, the Netherlands. The plants were maintained in standard glasshouse cultivation for pepper at 25°C, 16/8 h day/night. Pests insects were controlled biologically using predator organisms according to standard Dutch pepper cultivation practices.

Chemical analysis of apolar and semi-polar pepper metabolites

a. Gas Chromatography – Mass Spectrometry analysis

We analyzed the apolar fraction of secondary metabolites using an organic solvent extract of leaf material. For this, fully opened pepper plant leaves were ground under liquid nitrogen to a fine powder. Five hundred mg of leaf powder was transferred in a reaction tube and 3 ml dichloromethane (Sigma-Aldrich) was added as solvent containing carvone as internal standard (5μg/ml; 96%, Sigma-Aldrich,Zwijndrecht, The Netherlands). Tubes were placed in an ultrasonic bath at room temperature for ten minutes and centrifuged for five min at 1515 g. The supernatant was dried by passing it through a glass column (Pasteur capillary pipette) filled with sodium sulphate (Na_2SO_4) powder and with a plug of silanized glass wool. Samples were injected using a 7683 series B injector (Agilent®) into a 7890 A gas chromatograph (Agilent®) coupled to a 5975 GC/MSD (Agilent®). Column: ZB-5MS 30 meter x 0.25 mm. x 0.25 μm, with 5 meter retention gap. Injection temperature was 250 °C, temperature of column was programmed at 45 °C for 1 min, 10 °C min^{-1} to 300 °C and 7 min at 300 °C. Column flow rate was 1 ml min^{-1}.

b. Liquid Chromatography – Mass Spectrometry analysis

Fully opened leaves of pepper plants were ground under liquid nitrogen to fine powder. Five hundred mg of the powder was put in a reaction tube with addition of 1.5 ml 99.9% methanol acidified with 0.125% formic acid. Tubes were sonicated for 15 min and centrifuged for five min at 1515 ***g***. Next, the supernatant was filtered through 0.2 um polytetrafluoroethylene filter. All the extracts were analyzed on a reversed phase liquid chromatograph coupled to a photodiode array detector and a high-resolution mass spectrometry (LC-PDA-QTOF-MS) system (Waters®), using negative electrospray ionization as described by De Vos et al. (2007).

c. GC-MS and LC-MS data analysis and putative metabolite annotation

An untargeted metabolomics approach was applied to process the raw GC-MS and LC-MS data (Tikunov et al., 2005). Datasets of GC-MS and LC-MS were processed separately by the MetAlign software package (Lommen, 2009) for baseline correction, noise estimation, and ion-wise mass spectral alignment.

Mass signals originating from the same metabolite were grouped into a so-called centrotype, based on corresponding retention time and intensity pattern over the samples using MsClust software (Tikunov et al., 2012). Since each centrotype represents a metabolite, in the following sections, these centrotypes are referred to as metabolites.

Metabolites detected by GC-MS were putatively identified by matching their mass spectra to authentic reference standards, available in the commercial libraries NIST08 (http://www.nist.gov/index.html) and Wiley (version 138, http://www.wiley.com.ezproxy.library.wur.nl/wileyCDA/section/index.html), to the Wageningen natural compounds spectral libraries (a custom made library of authentic reference standards), and by comparison with retention indices from the literature calculated using a series of alkanes and fitted using a third-order polynomial function (Strehmel et al., 2008).

Metabolites detected by LC-MS were putatively identified by comparing the retention times and mass value of detected compounds with that of two databases: Dictionary of Natural Products (http://dnp.chemnetbase.com/), KNApSAck (http://kanaya.naist.jp/KNApSAcK) and results of Marin et al. (2004) and Wahyuni et al. (2011).

Correlation analysis of metabolites with thrips resistance parameters

For all metabolites the two-sided significance was calculated of the Pearson correlation of $\log_{10}$ of peak height versus larval survival (as asin(sqrt(x)) transformed data, Chapter 5) over all non-challenged samples. A False Discovery Rate (FDR) correction according to Benjamini & Hochberg (1995) was applied to these significance values.

QTL mapping of metabolites detected by GC-MS and LC-MS

QTL mapping of metabolites detected by GC-MS and LC-MS was performed in an F2 population (see Plant material) for which a linkage map composed of SSR and AFLP markers was constructed previously (Chapter 4). Potential QTLs for metabolites were identified using the MapQTL 6.0 package (Van Ooijen, 2009) and the MQ^2 utility (Chibon et al., Submitted) through interval mapping analysis. A general LOD threshold for mQTL significance was determined using a genome wide permutation test with 1000 iterations for 10 different metabolites.

Results

Clustering of GC-MS mass signals based on their retention time and abundance profile across samples resulted in fifty-five centrotypes. For LC-MS this resulted in 674 metabolites. From the total number of detected metabolites, only 275 metabolites (38%) segregated in our F2 population.

Metabolites significantly correlating with larval survival of thrips

After applying FDR (α = 0.30) correction, twenty-one metabolites were significantly correlated with larval survival. The correlation was negative in seven cases and positive in fourteen cases. Two of the seven negatively correlated metabolites were tentatively identified as capsinoside III and *p*-hydroxybenzoic acid while four of the fourteen positively correlated metabolites were identified as octacosane, quercetin-dihexose-deoxyhexose-pentose, phloretin-C-diglycoside, and naringenin calcone-hexose (Table 1).

QTL mapping of untargeted metabolites detected by GC-MS and LC-MS

For 242 of 275 segregating metabolites in the current study (88%), at least one QTL with a maximum LOD score above 3.6 was detected by interval mapping. This LOD threshold was obtained by performing permutation tests for 10 randomly chosen metabolites, which resulted in LOD thresholds between 3.5 and 3.7 for a genome-wide confidence of 0.95. For most metabolites one single QTL was detected; the maximum number of QTLs for one metabolite was four.

The mQTLs for these metabolites were spread unequally over the chromosomes. There were "hotspots" on several linkage groups where multiple mQTLs co-located, e.g. a region at linkage group P03c where more than 40 mQTLs were found. In contrast there were some "empty" linkage groups such as linkage group P04b without any mQTL (Figure 1).

Co-localization of mQTLs with QTLs for thrips resistance in pepper

In the same F2 population used in this study we earlier mapped QTLs for three thrips resistance parameters, all on chromosome 6 near marker HpmsE078 at position 108 cM (Chapter 4). On the same linkage group we detected mQTLs for 44 different metabolites, five detected by GC-MS and 39 by LC-MS.

Ten of the twenty-one metabolites which were significantly correlated with larval survival of thrips (Chapter 4) had mQTLs on chromosome 6, while all mQTLs for the other eleven were located on different linkage groups (Table 1). Among these ten metabolites, the 2-LOD intervals of mQTLs for capsianoside-III and LC-5046 overlapped with those of the resistance QTLs (Figure 2).

Additionally, for some other metabolites there were mQTLs relatively close to the resistance QTLs although those metabolites were not significantly correlated with larval survival. The 2-LOD intervals of three of those mQTLs (for LC-2097, LC-2672, LC-2809) overlapped with those of the resistance QTLs (Figure 2). The first two of these had a second mQTL elsewhere on the genome (Supplementary Table 1).

Table 1. QTL effects of metabolites correlating with larval survival of thrips detected by GC-MS and LC-MS on F2 population of pepper from a cross between *Capsicum annuum* AC. 1979 X *C. chinense* no. 4661

Metabolite code[a]	Metabolite putative identification	Correlation with larva survival [b]	P value[b]	Linkage group	QTL position[c]	Colocalizing with resistance QTL[d]	LOD[e]	Additive[f]	Dominance	% Expl.[g]
GC-1428	Octacosane	0.14162	<0.001	P06	151.4	no	4.45	-0.15	0.16	11.5
				P08	68.2	no	3.84	0.12	0.00	10.0
				P10b	16.5	no	6.84	-0.15	-0.06	17.1
GC-1607	Unknown	0.24229	<0.001	P06	160.4	no	11.45	-0.16	0.01	26.9
GC-2054	Unknown	0.25294	<0.001	P06	162.4	no	11.47	-0.18	0.03	27.0
				P07	66.2	no	3.09	0.19	-0.16	8.1
GC-1910	Unknown	0.24696	<0.001	P06	163.4	no	10.74	-0.16	0.03	25.5
				P07	66.2	no	3.14	0.19	-0.2	8.3
LC-5046	Unknown	-0.27578	<0.001	P06	94.9	yes	4.11	0.14	-0.07	10.7
LC-1245	Unknown	0.25022	<0.01	P05a	31.0	no	3.29	0.05	0.01	8.6
				P05b	51.8	no	5.31	0.09	-0.01	13.6
LC-4145	Quercetin x-O-rhamnoside y-O-rhamnoside II	0.23814	<0.01	n.d[h]	n.d[h]					
LC-3925	Unknown	0.23455	<0.01	P05b	22.0	no	27.11	0.24	-0.18	52.4
LC-3072	Unknown	-0.22615	<0.01	P06	129.1	no	4.31	0.10	0.03	11.1
LC-2514	Phloretin-C-diglycoside	0.22400	<0.01	P06	77.7	no	8.64	-0.09	0.00	21.1
LC-6462	N544	0.22352	<0.01	P11	17.5	no	3.55	0.05	0.05	9.3
LC-5738	Unknown	0.22420	<0.01	P11	39.1	no	4.51	0.11	0.02	11.6
LC-1980	Unknown	0.22363	<0.01	P03a	34.2	no	7.14	0.12	-0.08	17.8
LC-6540	Unknown	-0.21982	<0.01	n.d	n.d					
LC-3601	Unknown	0.22198	<0.01	P05b	36.2	no	9.46	0.09	-0.10	22.8
LC-6636	Naringenin chalcone-hexose	0.22056	<0.01	P06	19.8	no	5.48	-0.10	-0.13	13.9
LC-2703	Unknown	-0.21484	<0.01	P06	148.4	no	3.22	0.02	0.17	8.5
LC-5964	Unknown	-0.21561	<0.01	P05a	22.7	no	3.16	-0.07	-0.02	8.3
LC-1558	p-Hydroxybenzoic acid	-0.20338	<0.01	P01	85.3	no	3.31	0.12	-0.02	8.7
LC-5703	Capsianoside III-2	-0.20580	<0.01	P06	77.7	yes	3.71	0.06	-0.01	9.7
LC-4860	Unknown	0.20175	<0.01	P05b	41.2	no	6.16	0.09	-0.07	15.5

[a] GC and LC indicate metabolites detected by GC-MS and LC-MS, respectively, [b] Based on Pearson correlation, [c] Position of the QTL, in cM from the top of linkage group
[d] mQTLs were considered as co-localized with resistance QTLs when there was an overlap between the 2-LOD intervals regions compared to the resistance QTLs (Chapter 4). 2-LOD interval were determined using MapChart 2.2 (Voorrips, 2002), [e] Logarithm of the odds (LOD). QTLs were deemed significant when the LOD exceeded 3.6 (threshold corresponding to a genome-wide confidence level of 0.95, estimated from permutation tests with 1,000 iterations), [f] Negative values indicate that *C. annuum* alleles have lower phenotypic values than *C. chinense* alleles , [g] Percentage of phenotypic variance explained by each QTL
[h] not detected

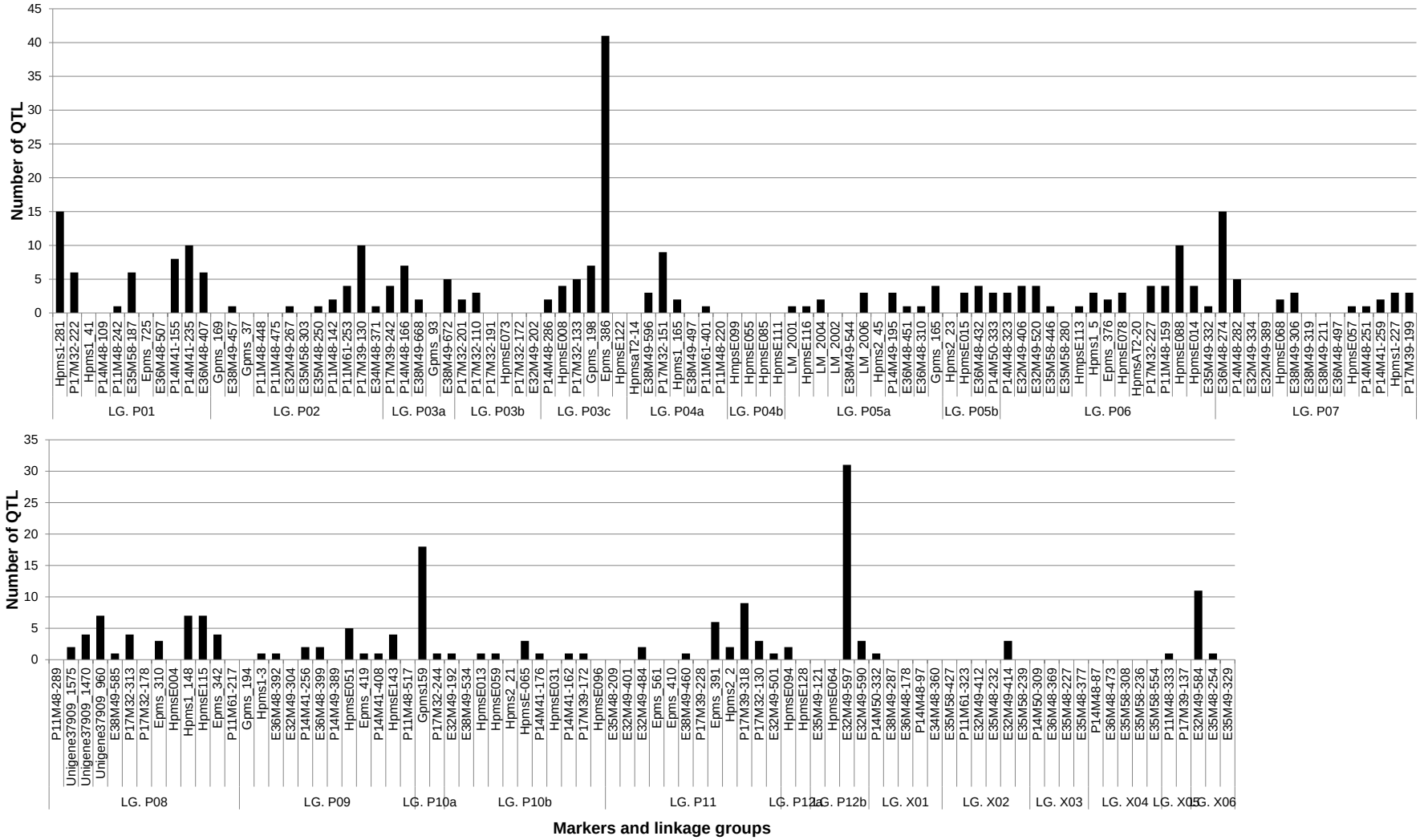

Figure 1. Frequency distribution of metabolite QTLs on a linkage map in pepper developed from an F2 populationof a *Capsicum annuum* X *C. chinense* cross. Each mQTL is assigned to the marker closest to the maximum LOD score.

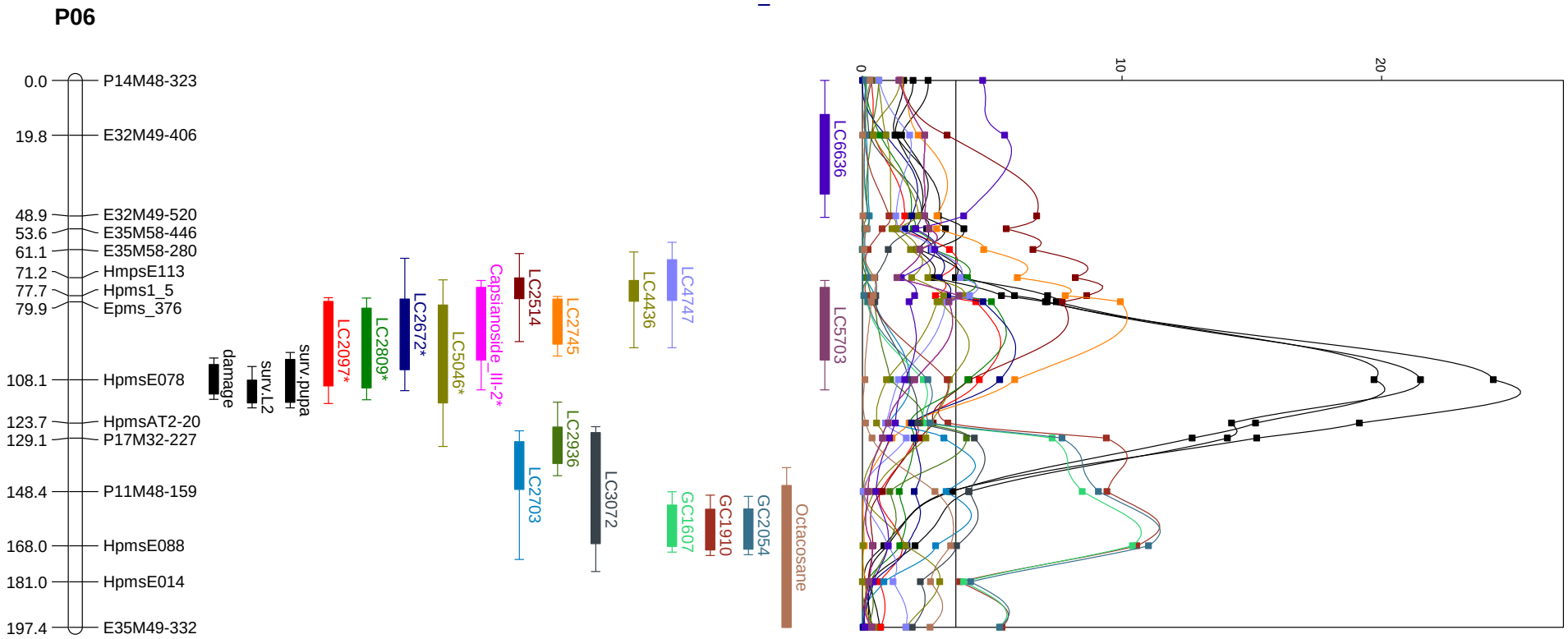

Figure 2. LOD profiles of several metabolites QTLs (mQTLs) detected on linkage groups P06 (chromosome 6). mQTLs significantly co-localized to the QTL for resistance (black solid bars) are indicated by *. In the graph on the right, the Y-axis indicates the LOD values; the line at LOD 3.6 indicates the LOD threshold.

Discussion

Metabolites correlated to resistance

In this study we detected twenty one metabolites which are significantly correlated to one resistance parameter, i.e., second instar larval stage (L2) survival that was observed before (Chapter 4). However, the correlation of these metabolites with resistance is relatively low ($0.14<R<0.27$). Survival of L2 larvae was chosen since it was the parameter that produced the most clear separation among resistant, intermediate and susceptible accessions (Chapter 3). Seven metabolites have a negative correlation and thirteen a have positive correlation with larval survival. Unfortunately, most of the metabolites detected by GC-MS (3 out of 4) and LC-MS (12 out of 16) that correlated to thrips resistance could not be identified. This is still a major drawback in the field of metabolomics (Allwood et al., 2008; Scalbert et al., 2009; Lei et al., 2011; Okazaki & Saito, 2012).

In our previous study (Chapter 3) we reported 13 metabolites that were significantly correlated with resistance to thrips. In the current study, only four of those metabolites were detected again: tocopherol, heptacosane, octacosane and nonacosane; of those four only octacosane was correlated with resistance in the current study. These differences between the two studies could be caused by differences in plant growth conditions, sample collection and extraction, despite our efforts to keep these as constant as possible. For instance in the previous study we harvested the leaf material in the winter (of 2008) , while in the current study it was harvested in the summer (of 2011). Such differences are known to affect reproducibility and sensitivity of the analysis (Scalbert et al., 2009). Another, probably more important difference between the studies is that the current study used an F2 population, while the previous study compared nine unrelated accessions belonging to four different species; since Capsicum species differ with respect to metabolite profiles, at least in fruits (Wahyuni et al., 2012), it is probable that more different metabolites occurred in the material of that study. This may perhaps also explain some of the associations detected in Chapter 3.

Of the thirteen metabolites that were significantly correlated with resistance in our previous study (Chapter 3) only octacosane was also correlated to resistance in the current F2 analysis. This supports our previous result about the negative correlation of octacosane with resistance to thrips.

It is interesting that some of the identified metabolites in the current study have been associated with insect resistance in other crops. For example, quercetin derivatives have been frequently reported to be involved in plant-insect interactions (Iwashina, 2003; Simmonds, 2003; Pereira et al., 2009) and *p*-hydroxybenzoic acid is a phenolic acid that has been reported for its relation to pest and disease resistance in plants (Bennett & Wallsgrove, 1994). However, other compounds that could not be annotated may be important as well since they are also significantly correlated with thrips resistance in pepper.

Mapping metabolite QTLs in pepper

We detected mQTLs for 88% of the metabolites segregating in the F2 population, which were spread unequally resulting in 'hotspots' and 'coldspots' of mQTLs. Hotspots and coldspots are common phenomenona in mQTL studies. Several recent studies have reported the presence of mQTL hotspots and coldspots such as in *Arabidopsis thaliana* (Lisec et al., 2008), apple (Khan et al., 2012), and potato (Carreno-Quintero et al., 2012). Hotspots for mQTLs suggest the presence of a regulator gene controlling the expression of a large group of metabolites at that map position. Many of the metabolites detected may be biochemically related and therefore have similar genetic control (Keurentjes et al., 2006).

Co-localization of metabolite QTLs and thrips resistance QTLs in pepper

Co-localization of QTLs for thrips resistance and metabolites may indicate a causal relationship between the two. However, the number of correlated metabolites closely linked with the resistance QTLs is very low. Our study shows that only two out of the twenty one metabolites that significantly correlated with resistance to thrips co-localize with resistance QTLs (Figure 2). A possible explanation for the low number of co-localization metabolites is that the correlations of those metabolites with resistance, although highly significant, are weak (Table 1). Conversely, three metabolites co-localized with the resistance QTL although they were not correlated with resistance. This lack of correlation may be due to the relatively low percentage of variation explained by these mQTLs, as well as to the fact that two of the three metabolites had additional mQTLs elsewhere on the genome.

The mQTLs for three metabolites that were previously found to be correlated with resistance to thrips in pepper (Chapter 3) do not co-localize with the resistance QTL. The mQTL closest to the resistance QTL among these metabolites is the one for octacosane. However, based on the 2-LOD intervals, the mQTL for octacosane is well separated from the resistance QTL (Figure 2).

Thus our results here may also indicate the possibility that the resistance factor(s) is not a metabolite. Although several studies have reported that some metabolites correlated with resistance to thrips, such as jasmonic acid in *Arabidopsis thaliana* (L.) Heynh. (Abe et al., 2008), chlorogenic acid in chrysanthemum (Leiss et al., 2009b) and pyrrolizzidine alkaloids, jacobine, jaconine, and kaempferol glucosides in *Senecio* spp. (Leiss et al., 2009a), none of these correlations have been confirmed in a segregating population. Therefore, based on the results presented in the current study, it is imperative to confirm these relationships of metabolites with thrips resistance in a segregating population.

In conclusion, the co-localization of two mQTLs and the resistance QTL may indicate that the two metabolites LC-5046 and capsianoside III are involved in resistance against thrips. However, the correlations of these two metabolites with larval survival of thrips are weak. Further work is still required to annotate LC-5046 and to confirm the role of these two metabolites in thrips resistance in

pepper. In addition, it is possible that the resistance of *C. annuum* AC1979 to thrips is not or only partially determined by the presence or absence of specific metabolites.

Acknowledgements

The research was financially supported by the Royal Netherlands Academy of Arts and Sciences in the framework of the Scientific Programme Indonesia – the Netherlands (SPIN).

Chapter 6

General Discussion

The need for thrips resistance in pepper

Direct and indirect damage caused by thrips significantly decrease yield both in greenhouse and field cultivation of pepper (Siemonsma & Piluek, 1994). Thrips control practices include chemical treatments, biological control, crop management, and Integrated Pest Management (IPM). However, the effectiveness of chemical treatments is limited due to the cryptic habit of thrips (Herron & James, 2005) and their ability to rapidly develop resistance to pesticides (Jensen, 2000a). Also there is a rising of awareness of the risk of pesticides to the environment (Delbeke et al., 1997; Bielza, 2008). Biological control of thrips is difficult because the natural enemies generally have a lower reproduction and different environmental requirements for optimal growth than thrips (Cloutier et al., 1995). Crop management practices, e.g. the use of silver plastic mulches, soil sterilization to kill pupae, mass trapping with sticky traps or ribbon to trap adult and larvae (Castane et al., 1999; Weintraub, 2007) and the use of organic mineral fertilizers (Almeida et al., 2009), are most of the times too costly to be successful, thus unwanted by farmer. Even Integrated Pest Management (IPM), the combination of biological control, crop management practices and chemical applications to control thrips with consideration of ecological requirements, is not really effective because of its complexity, high cost, and consequently low adoption by farmers especially in developing countries (Vos et al., 1995a; Reitz et al., 2003; Weintraub, 2007). An increase in the effectiveness and decrease of the disadvantages of current thrips control practices outlined above can be achieved by introducing genetic resistance to thrips in pepper varieties. Therefore, breeding for resistant varieties, to be used preferably in combination with other measures in an IPM strategy, is considered as the best approach against insect pests (Broekgaarden et al., 2011).

Resistance is a term that is often used imprecisely, referring to antixenosis, antibiosis and/or tolerance. Antixenosis is the presence of morphological or chemical factors resulting in low preference of the insect for the crop (Kogan & Ortman, 1978). Antibiosis is defined as a condition where resistance factors in a plant can negatively affect the survival and reproduction of insects (Smith, 1989). Tolerance is the ability of plants to produce offspring and/or marketable yield in spite of insect attack (Fery & Schalk, 1991). In our study we did not measure tolerance, as we did not study the yield or quality of harvested fruits. Most of our tests focused on antibiosis: we studied larval and adult survival and reproduction in non-choice situations, mostly in an *in vitro* setup (Chapter 2). Some of our tests were conducted in greenhouses or screenhouses in a choice situation; in these tests both antibiosis and antixenosis could have influenced the results (Chapter 2). The reason to concentrate on antibiosis was that in practical cultivation a variety is often grown in a monoculture; thrips in the crop do not have an option to select more attractive plants and therefore need to survive and multiply on the available variety. Antixenosis will not reduce the problem in this situation, but antibiosis will limit the proliferation of thrips. Tolerant varieties may initially alleviate the problems, but since tolerance will not limit the development of thrips at some point even tolerant varieties will suffer when the thrips population density becomes too high.

Sources of resistance to thrips

Breeding for resistance requires one or more sources of resistance and reliable and effective selection methods. Our study revealed that sources of antibiosis based resistance to thrips can be found within *Capsicum annuum* and closely related species. In a panel of 32 pepper accessions from five *Capsicum* species the level of resistance to thrips has been assessed by a variety of test methods (Chapter 2) followed by a confirmation based on a study of thrips life-cycle parameters in a smaller set (Chapter 3). This resulted in the identification of a few accessions with high levels of resistance to two different thrips species. Among the accessions tested in our study, we identified several with higher levels of resistance than the most resistant accession reported before, which is Keystone Resistant Giant (Fery & Schalk, 1991). Since *C. annuum* is the major cultivated pepper species (Bosland & Votava, 2000) and the resistant accessions found in our study belong mostly to *C. annuum* (Chapter 2) it should be relatively easy to transfer the resistance from these accessions into commercial varieties of pepper through conventional crossing and selection.

Apart from the screening of germplasm, sources of resistance may be obtained by introducing resistance through genetic modification (GM), RNAi, or even by mutations. Possibilities to obtain resistance to thrips through GM have been shown by Outchkourov et al (2004) in potato. RNAi approaches have been used recently in insect management (Burand & Hunter, 2013; Gu & Knipple, 2013) and it might also be applicable to thrips control in pepper. Mutation breeding is also an option to obtain resistance to insects in various plants (Van Harten, 1998). However, using GM, RNAi, and mutation induction have some drawbacks compared to working with natural variation. The main advantages of using natural variation are that it is technically simple, relatively cheap, it avoids regulatory issues associated with GM, and it avoids the public resistance to GM crops which is present in many countries especially in Europe. The use of RNAi approaches in insects is still in its infancy and many road blocks need to be overcome before its potential for use as viable insect pest control strategy is realized (Burand & Hunter, 2013), while mutation breeding is also limited mostly because of its random, imprecise and/or uncertain way of operating. We found resistance in closely related, crossable accessions; therefore we did not pursue a GM strategy, RNAi approach or mutation induction approach.

Apart from sources of resistance also good evaluation methods are needed. A good evaluation method has at least the following properties: (1) The results obtained correspond to the practical cultivation situation in the sense that the genotypes that perform best in practice also have a very high chance of being selected using the test method. (2) It is reproducible, preferably unaffected by season so that tests can be performed all year round. (3) It can be applied on a relatively large scale and is not too expensive. (4) Especially in the case of insects, there should be no risk to contaminate other experiments. While the first point might be expected to be achieved best by testing whole plants in a greenhouse or screenhouse situation, this offers only little control over environmental factors, is quite expensive and poses risks in terms of contamination. Therefore we developed *in vitro* tests. The results of the *in vitro* detached leaf and leaf disk tests corresponded well with the greenhouse and

screenhouse tests (Chapter 2). These tests are performed under well-defined and reproducible conditions, are quite cheap in terms of equipment and labor. Also the thrips are confined to the lab, minimizing the risk of contaminating other experiments.

The nature of thrips resistance in pepper

Resistance factors in leaves

Thrips can attack leaves, flowers and fruits. However the presence of resistance in leaves is more desirable. Although adults are naturally attracted to flowers and also feed on pollen, they mostly return to leaves to deposit eggs (Hake et al., 1996; Lewis, 1997). Therefore the early larval stages need to be able to feed on leaves at the beginning of the life cycle in order to reach maturity, and resistance factors in the leaves affecting larval stages will therefore be very effective in controlling thrips. Secondly, in the presence of pollen thrips reproduce faster and their life cycle is shortened (Murai & Loomans, 2001). So, it is important that the thrips population should be eliminated or at least suppressed before the plants start to flower, otherwise the population may increase too rapidly. This can be done if the plants possess resistance factors in leaves, particularly during the vegetative stages. A third reason why it is important to study resistance in leaves is that thrips feeding damages the leaves, reducing the photosynthetic capacity, resulting in reduced fruit production (Shipp et al., 1998). Our study revealed suppression of larval survival and of reproduction caused by resistance factors in leaves (Chapter 3).

Antibiosis as resistance mechanism

Our study showed that antibiosis, not antixenosis, is the main resistance mechanism to thrips in pepper. We found high and very significant correlations between damage scores in a non-choice (leaf assay) versus a choice situation (greenhouse test) (Chapter 2). Also we found no significant differences in the number of female adults of *F. occidentalis* on leaves of resistant and susceptible accessions in a choice setup (*data not shown*). The antibiosis we observed affected larval survival and oviposition by adults as shown by clear and significant differences in survival of larvae reared on leaves of different pepper accessions (Chapter 3), a clear segregation of larval survival measured on leaves of an F2 population developed from a cross between resistant and susceptible accessions (Chapter 4), and negative effects on oviposition by female adult thrips reared on resistant accessions (Chapter 3).

The antibiosis identified in our study is more likely to reduce virus transmission by thrips than tolerance and (incomplete) antixenosis would do. Since the viruses are acquired during the first and early second larval stages and are reintroduced into the plant by adults (Moritz et al., 2004; Jones, 2005), the inhibition of the larval survival and the negative effects on adults on resistant accessions will restrict the multiplication and transmission of viruses. This was indeed found by Maris (2003): impeded thrips population development restricted and delayed the spreading of *Tomato Spotted Wilt Virus* (TSWV). Resistance to thrips may therefore provide a significant protection to TSWV infection, even

when the crop is fully susceptible to the virus. Tolerance would allow thrips to survive and reproduce in the plant and to move between them and thereby to spread viruses, even if the plant would not show direct symptoms or damage due to thrips feeding. Strong antixenotic factors may be able to reduce direct damage, virus acquisition and transmission (Mutschler et al., 2006). However, incomplete antixenosis might increase thrips probing and movements which can enhance the spread of viruses (Joost & Riley, 2005).

QTL mapping

We analyzed damage and larval survival to the L2 and pre-pupa stages in a mapping population, an F2 population derived from a cross between resistant and susceptible accessions. This resulted in the identification of one single highly significant resistance QTL affecting all three resistance parameters located on chromosome 6, explaining about 50% of the genetic variation (Chapter 4). The other 50% unexplained genetic variation could not be explained by another major QTL, as the multiple-QTL mapping (MQM) approach did not detect another QTL while our map does cover almost the entire genome. Our results conflict with those of Linders et al. (2010) who described a major QTL on chromosome 5 that was not detected in our study, in spite of the fact that we included several markers linked to it (Chapter 4). As we used the same resistant parent (*C. annuum* AC1979), but a different susceptible parent, this difference may be caused by the presence of at least two major resistance factors in the shared resistance parent, with only one of those segregating in each mapping population. If it is true, then both factors would be necessary for resistance.

Plant traits associated with resistance

Any leaf character that interferes with the thrips life-cycle is a potential resistance factor which may contribute to the mechanism of defense against thrips. It is known that both morphological and chemical characters of leaves can play a role in defense against insects (Rosenthal & Kotanen, 1994). Pepper leaves possess morphological characters which may be related to insect resistance, such as trichomes (Yadwad et al., 2008; Firdaus et al., 2011), wax layer, color, toughness, and thick cuticles (Firdaus et al., 2011). Some morphological characters have been reported in relation to thrips resistance in other crops such as color in *Gerbera jamesonii* and chrysanthemum (Blumthal et al., 2005), wax layer in gladiolus (Zeier & Wright, 1995) and cabbage (Voorrips et al., 2008; Žnidarčič et al., 2008). However, no significant correlation was found for those morphological characters with the resistance level to thrips in the 32 pepper accessions and in the F2 mapping population. Additionally we showed convincingly that the major QTL for trichome density was on a different chromosome than the resistance QTL, again suggesting that there is no relation between trichomes density with resistance to thrips in pepper.

Metabolites in pepper leaves might also play a role in defense against insects. Extracts of *C. annuum* leaves were shown to have negative effects on oviposition of the leafminer *Liriomyza trifolii* (Dekebo et al., 2007) and on larval growth and development of the cotton bollworm *Helicoperva armigera* (Tamhane et al., 2005). The presence or absence of several metabolites had also been reported

to be related to the reproduction and development of thrips (De Jager et al., 1996; Leiss et al., 2009a; Leiss et al., 2009b; Yang et al., 2012) as well as damage caused by thrips (Mirnezhad et al., 2010; Cheng et al., 2011). Our first investigation in Chapter 3 indicated that metabolites in pepper could also play role in resistance against thrips. By application of LC-MS in combination with GC-MS in the same F2 population used for QTL analysis (Chapter 4), we achieved a greater coverage of the metabolome (Chapter 5). Correlation analysis resulted in the identification of several metabolites with a small but significant positive or negative correlation with resistance parameters. The QTL analysis of metabolites detected using GC-MS and LC-MS in this mapping population resulted in the detection of two mQTLs co-located with the resistance QTL which might indicate a causal relationship between those metabolites and resistance. However, the correlations of these two metabolites with resistance were weak and the co-localization could be accidental. The mQTLs for metabolites that correlated with resistance in Chapter 3 did not co-localize with the resistance QTL. Since the resistant (*C. annuum* and *C. baccatum*) and susceptible (*C. chinense*) accessions in that study belonged to different species it is more likely that the correlations in Chapter 3 were due to the differences between species rather than difference in resistance. Wahyuni et al., 2013 reported that pepper species can be grouped based on the metabolite composition of the fruits. A Random Forest analysis of our leaf metabolite data (unpublished) showed the same. All this suggests that the resistance is not or only partially determined by the presence or absence of specific metabolites in the leaves.

Other factors beyond the scope of our investigations here might also be the key factors of resistance to thrips. Since we found that resistance is clearly expressed in detached leaves, we can exclude some factors such as metabolites in fruit, pollen and architecture of the plant. Besides metabolites and leaf anatomical and morphological characters, leaf proteins may play a role; this has been reported in the thrips resistance mechanism in several plant species such as pepper, cucumber, lettuce and tomato (Mollema & Cole, 1996). The possible role of proteinase inhibitors has also been reported in *C. annuum* against *Helicoverpa armigera* (Tamhane et al., 2005). The lack of evidence of any relation of leaf morphological characters to thrips resistance might be caused by the fact that we limited our observation to the most important traits previously reported for their relation to thrips and/or insect resistance: color, trichome density, toughness and cuticle thickness. Conceivably other morphological traits may be involved in resistance e.g. cell wall modification (Passardi et al., 2004). Also we should not completely exclude the possible relationship of metabolites with thrips resistance. It is possible that other metabolite classes, that were not detected by our methods or procedures, act as key factors in resistance against thrips as well. For example, alkaloids, that are strongly linked with insect resistance including thrips (Leiss et al., 2009a; Cheng et al., 2011), could not be detected in our study. Therefore other techniques beside GC-MS and LC-MS such as NMR or HPLC, and other extraction methods could be implemented to extend this investigation.

Implications for breeding and implementation into Integrated Pest Management

Thrips resistant varieties can be developed using the resistance source AC 1979 which belongs to *C. annuum*. As this is the dominant pepper crop species, it can be assumed that the introgression of this region to other *C. annuum* will be straightforward. To develop resistant pepper varieties based on the resistance QTL identified in *C. annuum* AC 1979, introgression of the QTL region on chromosome 6 is needed.

We showed that resistance in pepper can be scored based on larval mortality. However, scoring damage is much easier than scoring larval survival, and these parameters are highly correlated. Therefore for practical applications scoring based on damage is recommended. Further, *in vitro* (detached leaf or leaf punch) laboratory tests correlate well with greenhouse tests. To minimize the risk of contamination and uncontrolled environment factors, *in vitro* laboratory tests are recommended to support breeding programs. The developmental period and adult survival were not correlated with larval mortality in this study. Thus, there may be a possibility to combine these resistance parameters in breeding to obtain even more effective resistance against thrips.

Future direction

In this thesis we have described the development and validation of test methods for thrips resistance in pepper. We have identified accessions with high levels of antibiosis resistance and shown that this resistance, which is effective in the leaves, primarily affects the development of larvae. We have developed a mapping population and found one major QTL for resistance, located on chromosome 6. We have studied leaf morphological characters and leaf metabolites, but not found convincing evidence that these play a role in the resistance.

Several questions related to thrips resistance in pepper remain unanswered by this thesis. The resistance factors in pepper leaves that affect the resistance to thrips have not been identified clearly. Since our metabolomics results indicate that the resistance factors may not be metabolites, future research to investigate other possible factors such as other leaf morphological characters, proteins and more specifically proteinase inhibitors. However, the possible role of the few metabolites with mQTL co-localizing with the resistance QTL should be investigated further as well.

The major resistance QTL detected in this thesis should be confirmed in at least one other population, e.g. a population of F3 lines. The major resistance QTL described in this thesis is located on chromosome 6, whereas Linders et al. (2010) detected a different major QTL on chromosome 5, using a mapping population with the same resistant parent as ours. These contrasting results need to be resolved. Further studies can be directed towards the mapping of additional resistance parameters (e.g. affecting oviposition), to the effects of environmental and culture conditions, to the interaction of the resistance QTL with different genetic backgrounds and with different thrips species.

Finally, applied research is needed on how to use this resistance in an Integrated Pest Management system such that its potential is fully exploited to obtain the best effect, allowing us to achieve a more healthy, profitable and sustainable pepper cultivation.

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Summary

Pepper (*Capsicum*) production is constrained by heavy infestations of thrips, causing direct and indirect (by transmitting viruses) damage. Thrips control using chemical insecticides, biological agents, culture practices and integrated pest management has limited success. The availability of thrips-resistant varieties would increase the effectiveness of thrips control and may also delay and reduce the transmission of viruses. This thesis is aimed at obtaining more knowledge regarding thrips resistance in pepper, including the identification of new sources of resistance, the elucidation of resistance mechanisms, identification of factors contributing to resistance and a QTL analysis.

We developed several test methods to evaluate plant resistance to thrips and showed that in vitro tests correlate well with greenhouse tests. We used these methods to test a collection of Capsicum accessions of widely different origin and crop types. This resulted in the identification of a few accessions (mostly *C. annuum*) with high levels of resistance to two thrips species: *Frankliniella occidentalis* and *Thrips parvispinus*. Since *C. annuum* is the most widely cultivated species, the finding of resistance in *C. annuum* is means that the resistance can be easily introgressed through conventional crossing and selection.

The effect of resistance in pepper on thrips reproduction and development was studied using three highly resistant, three medium resistant and three susceptible accessions selected based on damage ratings. Adult and pre-adult survival, developmental time and reproduction rate were assessed in a detached leaf system. Resistance factors in leaves of resistant pepper accessions were shown to have significant effects on oviposition rate, larval mortality and life-cycle period, indicating that this resistance is based on antibiosis.

In order to map QTL for resistance we developed an F2 population from the cross between a susceptible C. chinense accession and the resistant C. annuum AC 1979. A genetic linkage map for this population was based on AFLP and SSR markers, where the SSR markers served to assign and orient most linkage groups to pepper chromosomes. As larval stages were highly affected by resistance in pepper leaves, damage caused by larvae and larval survival were used as parameters to detect QTLs conferring resistance to thrips. Interval mapping detected one QTL for each of these parameters, all co-localizing near the same marker on chromosome 6. This QTL explained about 50% of the genetic variation, and the resistance allele of this QTL was inherited from the resistant parent. No other resistance QTLs were detected in this population.

Since resistance to thrips was clearly expressed in pepper leaves we proceeded to study leaf traits that may contribute to resistance. Morphological leaf characters and metabolites have frequently been linked with resistance to thrips in other plant species. However, we found no convincing evidence that any of these traits played a role in thrips resistance in pepper. In the F2 mapping population we found no correlation and no QTL co-localization of resistance with leaf morphological characters previously linked to resistance in pepper against insect pest and in other plant species against thrips e.g. color, toughness, trichome density, and cuticula thickness. GC-MS (Gass Chromatography – Mass Spectrometry) analysis of the three resistant, three intermediate and three

susceptible accessions mentioned above showed that seven metabolites were correlated with resistance to thrips and six compounds with susceptibility. However, when we applied GC-MS and LC-MS (Liquid Chromatography – Mass Spectrometry) to leaves of the F2 mapping population, we found no strong correlation between resistance and any detected metabolites. Two metabolite QTLs co-localized with the resistance QTL. However, these QTLs explained only a small proportion of the variance and the co-localization was not supported by strong correlations of the metabolites with resistance. This suggests that the major resistance factor(s) in pepper against thrips may not or only partially be determined by the presence or absence of specific metabolites.

This thesis provides a strong basis for the development of thrips resistant pepper varieties through introgression of the resistance QTL region on chromosome 6 originating from resistant C. annuum accessions. However, the effect of resistance QTL on chromosome 6 should be confirmed in another population such as a population of F3 lines. In vitro leaf assay can be used as evaluation methods in pepper breeding program. This has the advantages of minimizing the risk of contamination and of controlled environmental conditions. Elucidation of factors contributing to resistance should be continued by giving attention to other possibilities such as proteins, specifically proteinase inhibitors, or other leaf anatomical and morphological traits. Also other extraction and detection methods may be used to discover other metabolites that might be related to resistance. Finally, for practical applications it is necessary study how to use the antibiosis based mechanism against thrips found in this thesis in thrips control and/or management practices.

Samenvatting

In de teelt van pepers (*Capsicum*) veroorzaakt trips vaak grote problemen, zowel door directe schade als indirect door het overbrengen van virussen. De bestrijding van trips via chemische insecticiden, biologische bestrijding, teeltmaatregelen en geïntegreerde bestrijding heeft slechts een beperkt effect. Trips-resistente rassen zouden de effectiviteit van bestrijding kunnen verhogen en ook de verspreiding van virussen kunnen vertragen en verminderen. Het promotieonderzoek was gericht op het vergroten van onze kennis over resistentie tegen trips in peper, in het bijzonder over nieuwe resistentiebronnen, resistentiemechanismen, factoren die bijdragen aan resistentie en een genetische (QTL) analyse van de resistentie.

We hebben enkele toetsmethoden ontwikkeld voor het bepalen van het resistentieniveau tegen trips, en aangetoond dat resultaten van *in vitro* toetsen goed correleren met die van kasproeven. We hebben deze toetsmethoden gebruikt voor het evalueren van een collectie *Capsicum* accessies van uiteenlopende herkomst en verschillende gewastypen. Hiermee konden we enkele accessies (voornamelijk *C. annuum*) identificeren met een hoog niveau van resistentie tegen twee tripssoorten: *Frankliniella occidentalis* and *Thrips parvispinus*. Aangezien de meeste geteelde pepers tot *C. annuum* behoren en de resistentie in deze zelfde soort is gevonden zal deze eenvoudig ingekruist kunnen worden.

Het effect van de resistentie op de reproductie en ontwikkeling van trips werd bestudeerd in drie resistente, drie intermediaire en drie vatbare accessies, die geselecteerd waren op basis van de hoeveelheid schade die ze ondervonden door trips. In een proefopzet met afgeknipte bladeren werd de overleving van volwassen trips en larven, de ontwikkelingsduur en de reproductie gemeten. De resistentie bleek een significant effect te hebben op eileg, larvale mortaliteit en duur van de levenscyclus, wat aangaf dat deze resistentie op antibiose is gebaseerd.

Voor een QTL analyse van de resistentie ontwikkelden we een F_2 populatie uit een kruising van een vatbare *C. chinense* accessie met de resistente *C. annuum* AC 1979. Een genetische merkerkaart van deze populatie was gebaseerd op AFLP en SSR merkers, waarbij de SSR merkers zorgden voor de toekenning en oriëntatie van de koppelingsgroepen aan de peperchromosomen. Aangezien de larvale stadia het meest door de resistentie beïnvloed werden is de QTL analyse uitgevoerd voor de parameters larvale overleving en schade veroorzaakt door larven. Via interval mapping werd één QTL voor elk van de parameters gedetecteerd, alle bij dezelfde merker op chromosoom 6. Dit QTL verklaarde ongeveer 50% van de genetische variatie en het resistentie-allel was afkomstig van de resistente ouder. Naast dit QTL werden geen andere resistentie-QTLs gevonden.

Aangezien de tripsresistentie duidelijk tot expressie kwam in de bladeren onderzochten we vervolgens verschillende bladkenmerken die zouden kunnen bijdragen aan de resistentie. Morfologische bladkenmerken en metabolieten zijn in andere plantensoorten vaak in verband gebracht met tripsresistentie. We vonden echter geen overtuigende aanwijzingen dat deze eigenschappen een rol

spelen in tripsresistentie in pepers. In de F_2 populatie vonden we geen correlatie en ook geen colocalizatie van QTLs tussen resistentie en morfologische bladkenmerken zoals kleur, taaiheid, trichoomdichtheid en cuticula-dikte, die in ander onderzoek wel betrokken waren bij resistentie van peper tegen andere insecten, of bij resistentie van diverse andere gewassen tegen trips. Een GC-MS (gaschromatografie – massaspectometrie) analyse van de drie resistente, intermediaire en vatbare accessies die hierboven genoemd werden resulteerde in de identificatie van zeven metabolieten die met resistentie, en zes die met vatbaarheid gecorreleerd waren. In de bladeren van de F_2 populatie vonden we met GC-MS en LC-MS (vloeistofchromatografie – massaspectrometrie) echter geen sterke correlaties tussen resistentie en metabolieten. QTLs voor twee metabolieten vielen samen met het resistentie-QTL. Deze metaboliet-QTLs verklaarden echter slechts een klein deel van de variatie, en deze metabolieten waren slechts zwak gecorreleerd met resistentie. Deze resultaten suggereren dat de tripsresistentie in peper niet of slechts in beperkte mate op de aan- of afwezigheid van bepaalde metabolieten berust.

Dit proefschrift kan als basis gebruikt worden voor de ontwikkeling van trips-resistente peperrassen via introgressie van het resistentie-QTL op chromosoom 6, afkomstig van een resistente *C. annuum* accessie. Het effect van dit QTL moet echter nog bevestigd worden in een andere populatie, bijvoorbeeld in een populatie van F_3-lijnen. *In vitro* bladtoetsen kunnen gebruikt worden als resistentietoets in een veredelingsprogramma. Dit heeft als voordeel dat de kans op ontsnapping van trips wordt geminimaliseerd, en de toetsen kunnen onder goed gereguleerde condities worden uitgevoerd. Verder onderzoek naar resistentiebepalende factoren is nodig en zou zich moeten richten op eiwitten zoals proteinase inhibitors en mogelijk ook op andere anatomische en/of morfologische bladkenmerken. Ook zouden met andere extractie- en detectiemethoden mogelijk alsnog metabolieten kunnen worden gevonden die een rol spelen in de resistentie. Als laatste is van belang om te onderzoeken hoe de in dit proefschrift beschreven, op antibiose gebaseerde resistentie het best kan worden toegepast bij het reduceren of voorkomen van tripsproblemen in de praktijk.

Ringkasan

Produksi cabai terkendala oleh tingginya serangan thrips yang dapat menyebabkan kerusakan langsung maupun tidak langung (dengan menularkan virus) pada tanaman. Efektivitas pengendalian thrips menggunakan pestisida, agen hayati, kultur teknis, dan program pengendalian hama terpadu memiliki keterbatasan. Keberadaan varietas cabai tahan thrips akan meningkatkan keefektifan pengendalian thrips serta dapat menunda dan menurunkan penularan virus. Thesis ini bertujuan untuk menggali informasi terkait dengan ketahanan thrips pada cabai, termasuk identifikasi sumber ketahanan baru, identifikasi mekanisme ketahanan, identifikasi faktor-faktor yang berkontribusi terhadap ketahanan terhadap thrips, serta analisis QTL.

Dalam thesis ini, peneliti mengembangkan beberapa metode evalusi ketahanan tanaman cabai terhadap thrips yang mana menunjukkan bahwa uji secara *in vitro* berkorelasi nyata dengan uji ketahanan yang dilakukan di rumah kaca. Kami menggunakan metode pengujian ini untuk melakukan pengujian tingkat ketahanan koleksi aksesi *Capsicum* yang terdiri dari atas aksesi dari berbagai daerah asal dan tipe tanaman. Pengujian tersebut berhasil mengidentifikasi beberapa aksesi (sebagian besar *C. annuum*) dengan tingkat ketahanan yang tinggi terhadap dua spesies thrips: *Fraknliniella occidentalis* dan *Thrips parvispinus*. Dikarenakan *C. annuum* merupakan spesies cabai yang paling banyak dibudidayakan, identifikasi sumber ketahanan pada *C. annuum* dapat diartikan faktor ketahanan terhadap thrips dapat secara mudah ditransfer melalui persilangan dan seleksi secara konvensional.

Pengaruh faktor ketahanan terhadap pertumbuhan dan perkembangan thrips diteliti menggunakan tiga aksesi cabai yang sangat tahan, tiga aksesi agak tahan dan tiga aksesi rentan yang diseleksi menggunakan tingkat kerusakan akibat serangan thrips. Tingkat keberhasilan hidup stadia dewasa (*adult*) dan pra-dewasa (*pre-adult*), masa perkembangan, dan tingkat reproduksi diamati dalam percobaan secara *in vitro* pada daun. Faktor ketahanan pada daun dari kelompok tanaman tahan memiliki pengaruh yang sangat signifikan terhadap jumlah telur, tingkat kematian larva dan siklus hidup, yang mengindikasikan bahwa ketahanan terhadap thrips pada cabai adalah antibiosis.

Untuk memetakan QTL ketahanan terhadap thrips, peneliti membentuk populasi F2 hasil dari persilangan antara aksesi yang tahan yaitu *C. annuum* AC 1979 sebagai sebagai tetua betina dan aksesi rentan yaitu *C. chinense* sebagai tetua jantan. Peta pautan genetik pada populasi ini dibentuk berdasarkan marka AFLP dan SSR, dimana marka SSR digunakan untuk menduga posisi dan orientasi kromosom. Dikarenakan stadia larva sangat dipengaruhi oleh faktor ketahanan pada tanaman cabai, kerusakan akibat larva dan tingkat keberhasilan hidup larva digunakan sebagai parameter untuk mendeteksi QTL ketahanan thrips. Analisis *interval mapping* mendeteksi satu QTL untuk setiap parameter ketahanan, yang mana terko-lokalisasi dekat dengan marka yang sama pada kromosom 6. QTL tersebut menjelaskan sekitar 50% variasi genetik, dan alel ketahanan pada QTL tersebut diturunkan dari tetua tahan. Tidak terdapat QTL ketahanan lain yang dideteksi pada populasi ini.

Dikarenakan ketahanan terhadap thrips terekspresi secara jelas di daun, peneliti melanjutkan penelitian terkait dengan karakter daun yang mungkin memiliki peran dalam ketahanan. Karakter morfologi daun dan karakter metabolit seringkali dikaitkan dengan ketahanan terhadap serangga termasuk thrips. Namun demikian, dalam penelitian ini tidak didapatkan bukti yang meyakinkan bahwa karakter-karakter tersebut memiliki peran yang nyata dalam mekanisme ketahanan terhadap thrips pada cabai. Pada populasi F2 yang digunakan untuk peta genetik, tidak ditemukan juga korelasi dan juga ko-lokalisasi antara QTL ketahanan dengan QTL karakter morfologi yang telah banyak dilaporkan sebelumnya mengenai kaitannya dengan ketahanan terhadap serangga dan thrips seperti warna, kekekaran, kepadatan trikoma, dan ketebalan kutikula. Analisis GC-MS (*Gas Chromatography – Mass Spectrometry*) terhadap tiga aksesi tahan, tiga aksesi agak tahan dan tiga aksesi rentan yang disebutkan di atas menunjukkan terdapat tujuh metabolit yang berkorelasi dengan ketahanan dan enam metabolite yang berkorelasi dengan kerentanan terhadap thrips. Namun demikian, ketika peneliti mengaplikasikan GC-MS ditambah dengan LC-MS (*Liquid Chromatography – Mass Spectrometry*) pada daun dari populasi F2 di atas, tidak diketemukan korelasi yang tinggi antara ketahanan dan metabolit yang terdeteksi. Dua QTL metabolit berko-lokalisasi dengan QTL ketahanan. Namun QTL tersebut hanya sedikit menjelaskan variasi genetik dan ko-lokalisasi tersebut tidak didukung dengan korelasi yang tinggi. Hal ini menandakan bahwa faktor ketahanan mayor pada cabai terhadap thrips kemungkinan tidak atau hanya sedikit sekali dipengaruhi oleh ada atau ketidakadaan metabolit tertentu.

Tesis ini merupakan dasar informasi yang bermanfaat untuk mengembangkan varietas cabai tahan thrips melalui intogresi bagian QTL pada kromosom 6 dari aksesi *C. annuum*. Namun demikian, pengaruh QTL ketahanan di kromosom 6 tersebut perlu dikonfirmasi lebih lanjut pada populasi yang berbeda seperti populasi F3. Uji daun *in vitro* dapat digunakan sebagai metode evaluasi dalam program pemuliaan cabai. Metode tersebut memiliki keunggulan diantaranya mampu meminimalisir resiko kontaminasi dan mendapatkan lingkungan yang terkendali. Identifikasi lebih lanjut terkait faktor yang berkontribusi terhadap ketahanan perlu dilanjutkan dengan turut mempertimbangkan kemungkinan lain disamping yang telah diteliti dalam tesis ini seperti protein, secara spesifik protein inhibitor, atau karakter anatomi dan morfologi daun yang lain. Selain itu metode ekstrasi dan deteksi metabolit yang lain juga perlu digunakan untuk mendeteksi golongan metabolit lain yang kemungkinan terkait pula dengan ketahanan. Pada akhirnya, untuk aplikasi praktis, diperlukan penelitian lanjutan mengenai bagaimana memanfaatkan mekanisme antibiosis yang dijelaskan pada tesis ini dalam usaha pengendalian dan/atau manajemen thrips.

Acknowledgements

This PhD thesis could not have been realized without invaluable contributions of many people. Therefore, I would like to express my grateful thanks to everyone, who helped me a lot before and during my PhD period. I don't know if I can find the right words to thank you properly for everything you have given to me.

I want to thank Prof. Richard Visser, my promotor, who gave me this wonderful opportunity to join the PhD program at Wageningen UR Plant Breeding. The last five years have been one of the best periods in my life, in which I have grown both as a man and, with immense joy, as a researcher. I would like to mention my supervisor, Dr. Roeland Voorrips, especially. Dear Roeland, thank you to guide and help me during my PhD period. I have been happy and extremely honoured to be a PhD candidate under your supervision. Through our discussions and interactions I have got to known you a bit. What I know about you is that you are a true professional who always pays attention to every little detail, who always responds to my curiousity and hesitation very well with courage. Thank for your dedicated time and patience to supervise me. I also want to thank my co-supervisor, Dr. Ben Vosman, who always encouraged and motivated me during my PhD period, and who also provided me with some important clues. You know exactly which buttons to press. I want to thank Dr. Agus Purwito, my Indonesian supervisor, who gave me high motivation and the possibility to apply to this program, and also gave support and guidance during my PhD period. I also want to thank my external supervisor, Prof. C. (Titti) Mariani. Thank you for your motivative words to finish my PhD.

I need to extend my thanks to other INDOSOL team members: Dr. Sjaak van Heusden, Dr. Arnaud Bovy, Prof. Suharsono, Dr. Enny Sudarmonowaty, Dr. Asadi, Dr. Ence, Dr. Ana Rosa Ballister, Dr. Gerard van der Weerden, Dr. Ronald van den Berg, and also other Indosol PhD candidates who gave valuable suggestions and contructive criticism for the improvement of my research, especially during the regular Indosol workshops and meetings. I would particularly mention Syarifin Firdaus and Wahyuni, thank you for your significant support and sharing of ideas during our PhD period.

My thesis could also not be finished without financial support by the Royal Netherlands Academiy of Arts and Sciences (KNAW) through the SPIN program. For me, SPIN is a source of immense motivation and energy for young researchers. I also want to thank Dr. Douwe Zuidema from the Graduate School 'Experimental Plant Sciences' for his help during my PhD period. My deepest appreciation and thanks are addressed to the Ministry of National Education of Indonesia, the Rector, the Dean of Faculty of Agriculture, and the Head of Agronomy and Horticulture Department of Bogor Agricultural University who granted me the permission to take the PhD program. I also want to thank Center for Tropical Horticulture Studies and Indonesian Ministry of Research and Technology for their given support to complete my PhD program.

During my time in Wageningen, I've worked alongside some wonderful researchers and above all some wonderful people. It's hard to find the words to thank them all. In particular, I want to thank those who helped me a lot with my thesis work: Greet Steenhuis-Broers, Asep Harpenas, Koen Pelgrom, Francel

Verstappen, Roland Mumm, Ric C.H. de Vos, Bert Schippers, Gerrie Wiegers, Paul Dijkhuis, Fien Meijer-Dekens, Andre Maassen, Maarten Peters, Teus van den Brink, Aernoudt Aardse, Sri Sunarti, and Aatika Akhirtana. All members of Non-host and insect resistance group, thanks for encouraging discussions which very benefitted me to improve my research.

Several parts of my thesis were conducted in Purwakarta. During that time at PT East West Seed Indonesia (EWINDO, Purwakarta), I also worked with some highly motivated researchers who also contributed to my thesis. Therefore I want to thank Rien Rodenburg, Ramadhani Safitri, Buari, Muryanto, Rochim, and Adi.

I want to thank the Secretariat of Wageningen UR Plant Breeding: Mariame Gada, Anie Marchal, Letty Dijker, Janneke van Deursen and Nicole Trefflich, who helped me a lot during my working period at Plant Breeding. My appreciation and thanks are also addressed to Indonesian Embassy in DenHaag, especially to Dr. Ramon Mohandas and Bu Rina Bergsma who helped me a lot during my stay in Wageningen. I want to thank Greet Steenhuis-Broers and Frans, Dr. Hadiyanto familiy, Marco familiy, Peter family, Raymond von Schukkmann, for helping me to learn more about Dutch culture and helping me settle to live in Wageningen. Demie Moore, thank you for improving my English.

For all my international friends and all my Indonesian friends in Wageningen, thank you for the friendship and memories. I really enjoyed our togetherness in Wageningen. All of you have made Wageningen a second home to me. To be honest, I wanted to spend a longer time with you in Wageningen. Unfortunately, in life there comes a time when you have to move on and look forward. My destiny is indeed to go back to Indonesia. Those of you who really know me know that I'll do so with great pride and determination, as I've always tried to do both as a man and as professional.

There are still so many people I have enjoyed being with during my PhD in the Netherlands, therefore I'd like to say thank you to each and every one of you. I want you to know that you will always remain in my heart, even though I can't name you all.

To all my seniors and colleagues in Indonesia, especially at the Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agriculture University. Especially to Prof. Chozin, Prof. Sri Setyati Hariyadi, Prof. Sriani Sujipriharti (alm), Prof. Nurhayati Ansori Mattjik, Prof. Sudarsono, Prof. Wattimena, Prof. Bambang S. Purwoko, all members of plant biotech and plant genetic and breeding section who inspired me and supported me a lot. Dr. Sudradjat, Dr. Muhamad Syukur, Prof. Sobir, Prof. Syafrida Wanuwoto, Dr. Rimbawan, Dr. Rahmat Suhartanto, Dr. Syarifah Iis Aisyah, who helped me to adapt during my early comeback period in Indonesia. Dr. Dwi Guntoro and Dr. Willy Bayuardi who kept me updated about information related to Indonesia, including reseach project opportunities and political situation.

Finally, I would like to thank all my family members for their constant support. Especially to my beloved wife, Siska Megasari, thank you for your affection, your sacrifices, your trust, your support and your love.

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Awang Maharijaya was born on 8 September 1980 in Blitar, East Java, Indonesia. He got his undergraduate degree from Bogor Agricultural University majoring in Horticulture in 2003. Before his graduation, he was selected as the Best Student for Leadership and Service in Horticulture by Bogor Agricultural University and was awarded the Best National Student Creative Innovation by Ministry of National Education. In 2005 he joined Bogor Agricultural University as permanent faculty member. After that, he was granted by Directorate Generale of Higher Education, Ministry of Education, Indonesia to take master degree in Agronomy and Horticulture from 2005 – 2007 at Bogor Agricultural University. He was awarded two young scientist research grants about biotic stress in potato at this period. In 2008 he was selected as one of five PhD candidates of INDOSOL (Indonesia Netherlands Solanaceae) funded by KNAW through SPIN program, which allowed him to join the PhD programme at Plant Breeding Department, Wageningen University and Research Centre. In 2011, he won best innovation paper among Indonesian scholars overseas in Paris, France. After defending his PhD thesis in 2013, he will continue working on insect-resistance related topics at Bogor Agricultural University and Center for Tropical Horticulture Studies, Indonesia. He can be reached by email: awangmaharijaya@ipb.ac.id; awang.maharijaya@gmail.com

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Supplementary Table 1. QTL effects of metabolites detected by GC-MS and LC-MS on F2 population of pepper from a cross between Capsicum annuum AC. 1979

Metabolite code[1]	Linkage group	QTL position[2]	LOD[3]	Additive[4]	Dominance	% Expl.[5]	Metabolite putative identification
GC-0105	P08	68,2	3,61	0,10	-0,04	9,4	Unknown
GC-0105	P06	168,0	3,40	-0,08	0,08	8,9	Unknown
GC-0523	P01	20,2	3,31	0,06	0,06	8,7	Unknown
GC-1428	P10b	16,5	6,84	-0,15	-0,06	17,1	Octacosane
GC-1428	P06	148,4	4,41	-0,16	0,17	11,4	Octacosane
GC-1428	P08	68,2	3,84	0,12	0,00	10,0	Octacosane
GC-1469	P01	92,4	3,50	-0,08	-0,02	9,2	Nonacosane
GC-1469	P06	148,4	3,16	-0,09	0,11	8,3	Nonacosane
GC-1469	P08	68,2	3,16	0,07	-0,01	8,3	Nonacosane
GC-1607	P06	168,0	10,57	-0,13	0,01	25,2	Unknown
GC-1685	P08	68,2	3,08	0,07	0,01	8,1	Unknown
GC-1835	P11	17,5	3,79	0,09	0,00	9,9	α-tochoperol
GC-1892	P11	17,5	3,72	0,09	0,00	9,7	Unknown
GC-1910	P06	168,0	10,40	-0,15	0,02	24,8	Unknown
GC-1910	P07	66,2	3,14	0,19	-0,16	8,3	Unknown
GC-2054	P06	168,0	11,02	-0,15	0,02	26,1	Unknown
GC-2054	P07	66,2	3,09	0,19	-0,16	8,1	Unknown
GC-2334	P11	74,8	3,99	-0,06	-0,08	10,4	Unknown
GC-2334	P01	92,4	3,85	-0,10	-0,03	10,0	Unknown
GC-2334	P10a	16,5	3,07	-0,10	0,03	8,1	Unknown
GC-2364	P11	17,5	3,81	0,08	0,01	9,9	Unknown
GC-2380	P08	68,2	3,74	0,09	-0,01	9,7	Unknown
GC-2540	P08	68,2	3,84	0,09	-0,01	10,0	Unknown
GC-2840	P08	68,2	3,97	0,08	-0,01	10,3	Unknown
LC-0071	P01	61,1	7,81	0,11	-0,05	19,3	Unknown
LC-0081	P01	0,0	6,84	-0,02	-0,12	17,1	Unknown
LC-0117	P01	61,1	8,60	0,13	-0,06	21,0	Unknown
LC-0133	P01	0,0	8,87	-0,02	-0,17	21,6	Unknown
LC-0188	P01	61,1	9,10	0,14	-0,06	22,1	Unknown
LC-0258	P01	0,0	3,47	-0,03	-0,12	9,1	Unknown
LC-0302	P01	61,1	6,54	0,09	-0,04	16,4	Unknown
LC-0326	P12b	33,1	4,75	0,07	-0,02	12,2	Benzyl alcohol-hexose-pentose + FA
LC-0326	P09	25,9	3,36	0,03	-0,04	8,8	Benzyl alcohol-hexose-pentose + FA
LC-0392	P01	61,1	4,45	0,04	-0,03	11,5	Unknown
LC-0445	P05a	53,4	3,22	0,05	0,01	8,4	Unknown
LC-0457	P03c	40,4	3,10	0,09	-0,05	8,1	Unknown
LC-0490	P12b	33,1	3,37	0,01	0,11	8,8	Unknown
LC-0490	P01	20,2	3,12	-0,02	-0,10	8,2	Unknown
LC-0532	P03c	40,4	3,45	0,11	-0,07	9,0	Unknown
LC-0568	P04a	23,7	16,57	0,11	-0,06	36,5	sinapoyl (206 Da) + Chlorogenic acid methyl ester
LC-0583	P01	61,1	6,25	0,08	-0,03	15,7	Unknown
LC-0671	P02	168,6	3,27	-0,08	-0,10	8,6	Unknown
LC-0696	P02	102,7	3,54	-0,06	-0,06	9,2	Unknown
LC-0772	P02	102,7	3,87	-0,05	-0,04	10,1	Unknown
LC-0779	P09	25,9	6,22	0,03	0,07	15,7	Unknown
LC-0822	P09	32,8	3,48	0,07	-0,06	9,1	Unknown
LC-0850	P08	68,2	4,72	-0,06	0,04	12,1	Unknown
LC-0863	P07	55,0	6,60	-0,11	0,01	16,6	Unknown
LC-0863	P06	79,9	3,19	0,05	-0,04	8,4	Unknown
LC-0940	P06	148,4	11,98	0,22	-0,17	28,0	Unknown
LC-0963	P08	7,6	3,83	-0,13	-0,04	10,0	Caffeic acid 3-glucoside
LC-0963	P09	15,1	3,45	-0,10	-0,10	9,0	Caffeic acid 3-glucoside
LC-0968	P02	82,9	7,90	-0,15	0,08	19,5	Unknown
LC-0968	P01	105,7	3,04	-0,10	0,01	8,0	Unknown
LC-0975	P05a	28,1	4,03	-0,02	0,08	10,5	Naringenin O-Pentose-diglucose
LC-0979	P12b	6,4	3,35	0,14	-0,09	8,8	dihydroxybenzoic acid xyloside III
LC-0990	P08	32,9	5,40	0,09	-0,08	13,8	Unknown
LC-0998	P08	32,9	4,73	0,12	-0,07	12,2	Unknown
LC-1007	P01	92,4	4,22	-0,09	-0,05	10,9	Unknown
LC-1009	P07	30,8	3,18	-0,06	0,00	8,4	N152
LC-1015	P03c	40,4	13,62	-0,13	0,02	31,2	Unknown
LC-1019	P09	75,6	3,05	0,10	-0,03	8,0	Unknown
LC-1023	P08	32,9	4,32	0,08	-0,07	11,2	Benzyl alcohol-dihexose
LC-1034	P08	32,9	5,63	0,12	-0,09	14,3	Unknown
LC-1047	P09	15,1	4,24	-0,08	-0,04	11,0	Benzyl alcohol-dihexose + FA
LC-1047	P01	92,4	3,50	-0,09	-0,06	9,2	Benzyl alcohol-dihexose + FA
LC-1056	P03c	40,4	3,03	0,08	-0,04	8,0	Caffeic acid
LC-1067	X02	73,9	3,05	0,04	0,21	8,0	Unknown
LC-1147	P05b	51,8	10,51	0,20	-0,01	25,0	Unknown
LC-1162	P03c	40,4	7,29	0,15	-0,04	18,1	Unknown

Supplementary Table 1. (continued)

Metabolite code[1]	Linkage group	QTL position[2]	LOD[3]	Additive[4]	Dominance	% Expl.[5]	Metabolite putative identification
LC-1162	P06	168,0	3,06	-0,09	0,07	8,0	Unknown
LC-1170	P05a	53,4	3,62	0,07	0,01	9,5	Unknown
LC-1176	P07	21,2	6,09	-0,07	-0,04	15,4	Unknown
LC-1191	P03a	20,9	6,32	0,12	0,05	15,9	Unknown
LC-1196	P03a	34,2	3,39	0,09	0,01	8,9	Unknown
LC-1199	P07	102,9	4,29	0,15	-0,22	11,1	Unknown
LC-1199	P04a	23,7	3,11	0,07	-0,01	8,2	Unknown
LC-1211	P01	92,4	3,29	-0,06	-0,03	8,6	Unknown
LC-1245	P05b	51,8	5,31	0,09	-0,01	13,6	Unknown
LC-1245	P05a	30,9	3,29	0,05	0,01	8,6	Unknown
LC-1253	X06	18,5	3,36	0,08	-0,04	8,8	Unknown
LC-1253	P01	74,3	3,19	0,07	-0,04	8,4	Unknown
LC-1259	P10b	23,0	11,88	0,09	-0,09	27,8	Unknown
LC-1266	P03c	7,5	5,70	-0,07	0,01	14,5	Unknown
LC-1272	P05a	53,4	3,52	0,08	0,02	9,2	Unknown
LC-1278	P06	53,6	3,18	0,00	0,11	8,4	Quercetin x-O-glucoside y-O-rhamnoside
LC-1289	P03c	40,4	4,91	0,12	-0,04	12,6	Unknown
LC-1304	P03c	40,4	3,74	0,10	-0,01	9,7	Unknown
LC-1312	P03c	40,4	3,75	0,08	-0,01	9,8	Unknown
LC-1316	P02	136,7	4,15	0,00	0,09	10,7	Unknown
LC-1316	P05b	36,2	3,72	0,06	0,02	9,7	Unknown
LC-1317	P08	32,9	5,38	0,11	-0,10	13,7	Unknown
LC-1323	P01	92,4	3,71	-0,11	-0,02	9,7	trans-p-Sinapoyl beta-D-glucopyranoside
LC-1323	P08	7,6	3,50	-0,10	-0,03	9,2	trans-p-Sinapoyl beta-D-glucopyranoside
LC-1337	P11	45,9	5,39	-0,07	-0,04	13,7	Unknown
LC-1337	P10b	36,1	3,39	0,05	-0,06	8,9	Unknown
LC-1337	P02	24,1	3,26	0,00	0,45	8,6	Unknown
LC-1337	P07	30,8	3,18	-0,05	-0,03	8,3	Unknown
LC-1340	P08	41,2	3,03	0,05	-0,04	8,0	Unknown
LC-1368	P07	32,3	6,42	-0,08	0,00	16,1	Unknown
LC-1377	P03c	25,2	4,39	-0,08	-0,01	11,3	Unknown
LC-1384	P01	0,0	8,92	-0,01	-0,16	21,7	Unknown
LC-1402	P11	39,1	4,11	-0,08	-0,05	10,7	Luteolin 6,8-di-C-hexoside
LC-1402	P02	136,7	3,76	0,05	0,07	9,8	Luteolin 6,8-di-C-hexoside
LC-1402	P07	21,2	3,34	-0,04	-0,07	8,7	Luteolin 6,8-di-C-hexoside
LC-1402	X02	62,9	3,18	0,01	0,10	8,3	Luteolin 6,8-di-C-hexoside
LC-1513	P06	168,0	5,23	-0,09	0,09	13,4	Kaempferol 3-O-rutinoside
LC-1513	P03c	40,4	4,99	0,10	-0,05	12,8	Kaempferol 3-O-rutinoside
LC-1553	P07	21,2	3,15	-0,04	-0,06	8,3	Unknown
LC-1553	P01	92,4	3,05	-0,07	0,00	8,0	Unknown
LC-1558	P01	85,3	3,31	0,12	-0,02	8,7	p-Hydroxybenzoic acid
LC-1566	P03c	40,4	6,25	0,10	-0,04	15,7	Dihydrokaempferol-hexose or Eriodictyol chalcone-hexose III
LC-1591	P08	8,2	3,85	0,09	-0,08	10,0	Unknown
LC-1591	P04a	34,0	3,75	0,10	-0,02	9,8	Unknown
LC-1599	P03c	25,2	15,04	0,12	0,01	33,8	Unknown
LC-1599	P03a	52,1	3,99	-0,06	0,03	10,4	Unknown
LC-1621	P05b	36,2	5,11	-0,06	0,00	13,1	Unknown
LC-1633	P07	32,3	4,20	-0,06	-0,03	10,9	Quercetin-Methyl-O-hexose-O-rhamnose
LC-1644	P01	0,0	4,52	0,00	-0,12	11,7	Unknown
LC-1665	X02	73,9	3,48	0,02	0,14	9,1	Quercetin-3-O-deoxyhexose-C5H8O4
LC-1668	P07	30,8	4,42	-0,07	-0,02	11,4	Unknown
LC-1680	P03c	25,2	3,90	-0,05	0,00	10,1	Unknown
LC-1680	P07	32,3	3,34	0,01	-0,06	8,7	Unknown
LC-1689	P01	0,0	9,21	-0,03	-0,24	22,3	Benzyl glucopyranoside; ?-D-form, 2-O-Sulfate
LC-1689	P06	181,0	3,27	0,01	0,15	8,6	Benzyl glucopyranoside; ?-D-form, 2-O-Sulfate
LC-1694	P11	56,3	14,11	-0,12	-0,06	32,1	Unknown
LC-1694	P08	76,2	3,84	0,06	0,04	10,0	Unknown
LC-1718	X02	73,9	3,59	0,00	0,12	9,4	Kaempferol-deoxyhexose-C5H8O4 (II)
LC-1718	P06	168,0	3,08	-0,05	0,06	8,1	Kaempferol-deoxyhexose-C5H8O4 (II)
LC-1738	P01	92,4	4,17	-0,07	-0,01	10,8	Flavonoid-C-hexose-pentose
LC-1739	P11	91,6	4,48	-0,10	0,02	11,6	Delphinidin 3-(cis-coumaroyl)-rutinoside-5-glucoside + H2O
LC-1741	P03c	40,4	8,22	0,08	-0,05	20,2	Quercetin 3-O-rutinoside-7-O-glucoside
LC-1751	P08	7,6	4,49	-0,06	-0,02	11,6	Unknown
LC-1751	P01	92,4	3,23	-0,06	-0,01	8,5	Unknown
LC-1751	P06	181,0	3,01	0,03	0,06	7,9	Unknown

Supplementary Table 1. (continued)

Metabolite code[1]	Linkage group	QTL position[2]	LOD[3]	Additive[4]	Dominance	% Expl.[5]	Metabolite putative identification
LC-1774	P03c	40,4	3,22	-0,08	-0,01	8,5	Unknown
LC-1776	P04a	34,0	3,65	0,07	-0,02	9,5	Unknown
LC-1792	P01	105,7	7,93	-0,13	0,10	19,5	Unknown
LC-1792	P03c	40,4	4,33	-0,13	0,04	11,2	Unknown
LC-1792	P08	9,0	3,35	-0,11	0,00	8,8	Unknown
LC-1792	P09	99,5	3,27	-0,15	0,15	8,6	Unknown
LC-1811	P01	105,7	8,29	-0,12	0,09	20,3	Unknown
LC-1811	P03c	40,4	4,09	-0,11	0,05	10,6	Unknown
LC-1811	P08	8,2	3,10	-0,10	0,00	8,1	Unknown
LC-1811	P09	99,5	3,02	-0,13	0,15	8,0	Unknown
LC-1819	P03c	40,4	4,32	0,09	-0,04	11,2	Apigenin 6-C-pentoside-8-C-hexoside
LC-1819	P06	168,0	3,11	-0,06	0,08	8,2	Apigenin 6-C-pentoside-8-C-hexoside
LC-1848	P01	105,7	7,83	-0,10	0,07	19,3	Unknown
LC-1848	P03c	40,4	4,28	-0,10	0,03	11,1	Unknown
LC-1890	P06	168,0	4,03	0,05	0,06	10,5	Unknown
LC-1916	P03a	43,2	3,90	-0,06	0,02	10,1	Unknown
LC-1932	P06	197,4	3,10	0,04	0,03	8,1	Unknown
LC-1947	P01	115,4	3,74	0,09	-0,09	9,7	Unknown
LC-1947	P02	136,7	3,14	0,10	-0,07	8,3	Unknown
LC-1980	P03a	34,2	7,14	0,12	-0,08	17,8	Unknown
LC-1984	P08	9,0	3,78	0,06	-0,08	9,8	Unknown
LC-1984	P04a	50,6	3,28	0,07	-0,04	8,6	Unknown
LC-2011	P10a	16,5	4,54	0,08	0,04	11,7	Unknown
LC-2035	P03c	40,4	4,03	0,08	0,02	10,4	Unknown
LC-2035	P06	168,0	3,70	0,04	0,08	9,6	Methyl salicylate malonyl dihexose-pentose
LC-2035	P11	39,1	3,10	-0,06	-0,05	8,1	Methyl salicylate malonyl dihexose-pentose
LC-2069	X02	73,9	3,65	0,01	0,14	9,5	Kaempferol-deoxyhexose-C5H8O4 (II)
LC-2070	P12b	44,0	4,26	0,10	-0,13	11,0	Unknown
LC-2097	P06	108,1	4,50	0,06	0,03	11,6	Unknown
LC-2097	P07	30,8	4,27	-0,07	0,00	11,0	Unknown
LC-2115	P07	102,9	3,60	0,12	-0,17	9,4	Unknown
LC-2167	P08	76,2	3,05	0,05	-0,02	8,0	Icariside E5
LC-2186	P01	0,0	7,30	0,00	0,14	18,1	Ferulic acid-hexose II
LC-2187	P12b	33,1	3,56	-0,04	-0,05	9,3	Unknown
LC-2196	P07	92,4	3,82	0,16	-0,20	9,9	Unknown
LC-2222	P05a	8,1	4,20	0,06	-0,01	10,9	Unknown
LC-2237	P11	45,9	4,06	-0,05	-0,04	10,5	Unknown
LC-2237	P06	197,4	3,31	0,02	0,07	8,7	Unknown
LC-2240	P03c	7,5	5,42	0,11	0,09	13,8	C14H18O9
LC-2240	P01	0,0	4,43	0,01	-0,17	11,4	C14H18O9
LC-2240	P06	181,0	4,19	0,06	0,13	10,8	C14H18O9
LC-2244	P06	181,0	3,50	0,04	0,06	9,1	Unknown
LC-2244	P02	168,6	3,29	0,03	0,16	8,6	Unknown
LC-2274	P06	168,0	3,68	0,04	0,05	9,6	Unknown
LC-2277	P05b	22,0	3,67	-0,05	0,04	9,6	Unknown
LC-2282	P04a	50,6	4,46	0,08	-0,04	11,5	Unknown
LC-2282	X06	18,5	3,73	0,08	-0,04	9,7	Unknown
LC-2282	P08	8,2	3,51	0,05	-0,08	9,2	Unknown
LC-2317	P03c	40,4	5,98	0,09	-0,05	15,1	Kaempferol rhamnoside I
LC-2317	P06	168,0	3,48	-0,06	0,07	9,1	Kaempferol rhamnoside I
LC-2333	P01	105,7	3,64	0,05	-0,01	9,5	Unknown
LC-2333	P02	49,0	3,40	0,06	-0,04	8,9	Unknown
LC-2333	P08	41,2	3,31	0,05	-0,02	8,7	Unknown
LC-2355	P06	129,1	3,42	0,12	-0,08	8,9	Dihydrokaempferol-hexose or Eriodictyol chalcone-hexose III
LC-2358	P10b	23,0	8,37	0,08	-0,09	20,5	Unknown
LC-2359	P06	129,1	3,39	0,05	0,03	8,9	Unknown
LC-2384	P05a	8,1	3,09	0,02	0,08	8,1	Unknown
LC-2412	P03b	11,5	4,04	0,07	-0,01	10,5	Kaempferol 3-O-rhamnosyl-glucoside 7-O-rhamnoside
LC-2422	P07	21,2	3,70	-0,06	-0,03	9,7	Unknown
LC-2433	P06	168,0	5,19	0,06	0,04	13,3	Unknown
LC-2433	P02	136,7	3,75	0,00	0,09	9,8	Unknown
LC-2433	P09	67,9	3,73	0,06	0,01	9,7	Unknown
LC-2433	P07	21,2	3,33	-0,04	-0,05	8,7	Unknown
LC-2433	P01	20,2	3,30	-0,02	-0,11	8,6	Unknown
LC-2454	P08	48,2	8,78	-0,07	-0,01	21,4	Kaempferol 7-O-rhamnoside 3-O-glucosylglucoside
LC-2470	P03c	40,4	15,10	0,12	-0,10	33,9	Flavonoid glycosides
LC-2472	P04a	34,0	4,82	0,06	-0,04	12,4	Unknown
LC-2480	P10b	9,3	3,89	0,06	0,00	10,1	Quercetin 3-O-glucoside
LC-2497	P07	92,4	5,72	0,20	-0,23	14,5	Unknown

Supplementary Table 1. (continued)

Metabolite code[1]	Linkage group	QTL position[2]	LOD[3]	Additive[4]	Dominance	% Expl.[5]	Metabolite putative identification
LC-2503	P08	76,2	3,30	-0,04	0,00	8,7	Unknown
LC-2514	P06	77,7	8,64	-0,09	0,00	21,1	N344 Phloretin-C-diglycoside
LC-2514	P09	25,9	4,64	0,07	0,01	12,0	N344 Phloretin-C-diglycoside
LC-2530	P01	0,0	11,61	-0,05	-0,20	27,3	C14H18O9
LC-2545	P11	45,9	7,13	-0,09	-0,02	17,7	Unknown
LC-2545	P07	32,3	3,33	-0,05	-0,03	8,7	Unknown
LC-2545	P10b	18,6	3,22	0,05	-0,04	8,4	Unknown
LC-2552	P05a	28,1	3,04	-0,05	-0,11	8,0	Kaempferol 3-O-glucoside
LC-2552	P04a	62,9	3,02	-0,09	-0,10	8,0	Kaempferol 3-O-glucoside
LC-2571	P03b	11,5	3,10	0,04	-0,04	8,2	Unknown
LC-2571	P05a	8,1	3,10	0,02	0,06	8,1	Unknown
LC-2574	P03c	25,2	3,38	-0,07	0,05	8,9	Unknown
LC-2576	P10b	40,5	3,23	0,05	-0,03	8,5	Unknown
LC-2589	P04a	34,0	4,04	0,06	-0,05	10,5	Unknown
LC-2589	P07	66,2	3,99	0,16	-0,15	10,4	Unknown
LC-2606	P07	21,2	3,05	-0,06	-0,02	8,0	Unknown
LC-2643	P07	21,2	3,44	-0,03	-0,02	9,0	Unknown
LC-2643	P06	197,4	3,34	0,03	0,02	8,7	Unknown
LC-2672	P06	79,9	4,97	0,06	0,02	12,7	Unknown
LC-2672	P10b	43,1	3,54	-0,05	0,02	9,2	Unknown
LC-2703	P06	148,4	3,22	0,02	0,17	8,5	Unknown
LC-2725	P03c	40,4	4,70	0,06	0,03	12,1	Glc-Glc + C20H22O6(dihydroconiferyl alcohol)
LC-2725	P02	136,7	3,15	0,02	0,06	8,3	Glc-Glc + C20H22O6(dihydroconiferyl alcohol)
LC-2745	P06	79,9	9,94	-0,10	-0,05	23,9	Unknown
LC-2767	P03c	25,2	3,06	-0,08	0,00	8,0	Unknown
LC-2784	P08	9,0	4,51	0,07	-0,06	11,6	Unknown
LC-2784	P04a	62,9	3,56	0,05	-0,05	9,3	Unknown
LC-2784	P01	105,7	3,19	0,05	-0,05	8,4	Unknown
LC-2784	X06	18,5	3,09	0,06	-0,03	8,1	Unknown
LC-2792	P08	68,2	7,10	0,07	-0,03	17,7	Unknown
LC-2792	P11	39,1	4,07	-0,06	-0,02	10,6	Unknown
LC-2797	P08	48,2	7,78	0,11	-0,04	19,2	Unknown
LC-2797	P11	56,3	4,57	-0,09	-0,01	11,8	Unknown
LC-2809	P06	108,1	5,29	0,06	0,05	13,5	Unknown
LC-2828	P06	181,0	3,40	0,03	0,05	8,9	Icariside E5
LC-2842	P06	168,0	7,11	0,08	-0,06	17,7	Unknown
LC-2864	P05a	30,9	3,65	-0,01	0,11	9,5	Unknown
LC-2879	P06	181,0	5,30	0,08	-0,10	13,5	p-Coumaric acid
LC-2913	P07	92,4	3,49	0,09	-0,15	9,1	Unknown
LC-2918	P01	0,0	4,11	0,00	-0,07	10,6	Unknown
LC-2936	P06	129,1	4,01	-0,08	-0,12	10,4	Unknown
LC-2936	P01	0,0	3,59	-0,10	-0,11	9,4	Unknown
LC-2936	P11	74,8	3,04	-0,06	-0,12	8,0	Unknown
LC-2941	P11	56,3	21,36	0,17	0,16	44,3	4,5-Dicaffeoylquinic acid
LC-2950	P09	60,4	5,26	0,06	0,00	13,4	Unknown
LC-2950	P11	56,3	4,84	0,05	0,04	12,4	Unknown
LC-2995	P01	105,7	4,63	-0,10	-0,08	11,9	Unknown
LC-3018	P11	39,1	4,36	-0,06	-0,03	11,3	Luteolin (apiosyl-acetyl)-glucoside
LC-3055	P12b	33,1	5,51	-0,05	-0,09	14,0	Unknown
LC-3072	P06	129,1	4,31	0,10	0,03	11,1	Unknown
LC-3079	P03c	40,4	12,78	0,13	-0,05	29,6	Unknown
LC-3079	P08	68,2	3,03	0,02	0,09	8,0	Unknown
LC-3096	P03c	25,2	4,27	-0,08	0,00	11,0	Unknown
LC-3113	P03c	40,4	16,84	0,19	-0,09	37,0	Apigenin 6-C-pentoside-8-C-hexoside
LC-3113	P08	68,2	3,13	0,03	0,13	8,2	Apigenin 6-C-pentoside-8-C-hexoside
LC-3157	P10b	14,9	10,65	-0,11	0,08	25,3	Luteolin 6-C-hexoside
LC-3157	P02	136,7	6,50	-0,07	0,12	16,3	Luteolin 6-C-hexoside
LC-3165	P01	0,0	5,35	0,03	-0,21	13,6	Unknown
LC-3169	P05a	8,1	3,44	0,03	0,07	9,0	Unknown
LC-3179	P01	0,0	11,06	0,01	-0,23	26,2	Caffeic acid-hexose I
LC-3223	P01	0,0	3,73	0,01	0,11	9,7	Dehydrodiconiferyl alcohol glucoside
LC-3250	P06	148,4	11,60	0,13	-0,12	27,2	Isorhamnetin 3-O-glucoside 7-O-rhamnoside
LC-3250	P11	74,8	3,09	-0,07	0,14	8,1	Isorhamnetin 3-O-glucoside 7-O-rhamnoside
LC-3273	P03a	20,9	4,26	0,06	-0,02	11,0	Unknown
LC-3285	P10b	23,0	13,98	0,12	-0,10	31,8	Unknown
LC-3285	P02	136,7	3,08	0,06	-0,05	8,1	Unknown
LC-3293	P11	39,1	3,83	-0,06	-0,02	10,0	Unknown
LC-3293	P07	32,3	3,50	-0,05	-0,02	9,1	Unknown
LC-3296	P03c	40,4	14,91	0,21	-0,15	33,6	Kaempferol rhamnoside II
LC-3301	P05a	28,1	3,16	0,00	0,07	8,3	Unknown
LC-3306	P01	92,4	5,00	-0,05	0,08	12,8	Unknown

Supplementary Table 1. (continued)

Metabolite code[1]	Linkage group	QTL position[2]	LOD[3]	Additive[4]	Dominance	% Expl.[5]	Metabolite putative identification
LC-3306	P03b	6,6	4,14	0,07	-0,05	10,7	Unknown
LC-3306	P03c	40,4	4,10	-0,05	0,08	10,6	Unknown
LC-3344	P12b	6,4	3,07	0,08	-0,05	8,1	ferulic acid+coniferyl alcohol+glucose I
LC-3348	P08	48,2	3,54	-0,07	0,02	9,3	Unknown
LC-3365	P09	60,4	3,61	0,03	0,03	9,4	Unknown
LC-3398	P03c	16,2	5,07	-0,11	-0,03	13,0	Unknown
LC-3422	P08	76,2	3,05	-0,03	0,00	8,0	Unknown
LC-3448	P03a	20,9	12,04	-0,11	0,03	28,1	Unknown
LC-3506	P12b	33,1	4,42	-0,10	-0,05	11,4	Unknown
LC-3537	P03a	20,9	9,41	-0,07	0,06	22,7	Unknown
LC-3537	P07	21,2	3,36	-0,03	-0,05	8,8	Unknown
LC-3554	P04a	34,0	3,56	0,10	-0,01	9,3	Unknown
LC-3554	P07	92,4	3,23	0,20	-0,26	8,5	Unknown
LC-3554	P10b	47,5	3,19	-0,07	-0,13	8,4	Unknown
LC-3558	P06	197,4	3,05	0,02	0,05	8,0	Unknown
LC-3571	P08	8,2	3,78	0,09	-0,04	9,8	Unknown
LC-3596	P03b	8,5	3,91	0,06	-0,01	10,2	Unknown
LC-3601	P05b	36,2	9,46	0,09	-0,10	22,8	Unknown
LC-3615	P01	92,4	3,29	-0,06	-0,01	8,6	Unknown
LC-3632	X06	18,5	3,56	0,10	-0,04	9,3	Unknown
LC-3632	P08	8,2	3,20	0,08	-0,09	8,4	Unknown
LC-3632	P04a	34,0	3,12	0,08	-0,08	8,2	Unknown
LC-3639	P08	76,2	4,09	-0,05	-0,06	10,6	Luteolin 6-C-hexoside
LC-3639	P05a	53,4	3,07	0,07	-0,02	8,1	Luteolin 6-C-hexoside
LC-3654	P05a	30,9	3,95	-0,02	0,08	10,3	Unknown
LC-3654	P07	21,2	3,14	-0,03	-0,04	8,3	Unknown
LC-3659	P08	9,0	4,28	0,11	-0,06	11,1	Unknown
LC-3659	P01	105,7	4,04	0,09	-0,06	10,5	Unknown
LC-3659	P04a	34,0	3,64	0,09	-0,06	9,5	Unknown
LC-3659	X06	18,5	3,47	0,10	-0,05	9,1	Unknown
LC-3661	P03c	16,2	7,35	-0,12	-0,04	18,2	Unknown
LC-3671	P01	92,4	3,16	-0,07	0,00	8,3	Unknown
LC-3676	P03c	40,4	15,53	0,16	-0,09	34,7	Unknown
LC-3690	P05b	51,8	3,31	0,08	-0,13	8,7	Unknown
LC-3713	P03a	20,9	24,46	-0,17	0,07	48,9	Unknown
LC-3751	P05b	36,2	3,45	-0,06	0,10	9,0	Unknown
LC-3751	P07	25,5	3,10	-0,05	-0,01	8,1	Unknown
LC-3777	P03c	16,2	5,59	-0,10	-0,01	14,2	Unknown
LC-3788	X03	31,8	3,08	-0,04	0,10	8,1	Unknown
LC-3797	P10a	16,5	3,63	0,06	-0,07	9,5	Unknown
LC-3809	P05a	22,7	4,41	-0,06	-0,03	11,4	Unknown
LC-3815	P04a	23,7	4,27	0,08	-0,01	11,0	Unknown
LC-3815	P02	66,1	3,27	0,06	-0,08	8,6	Unknown
LC-3815	X06	18,5	3,15	0,06	-0,05	8,3	Unknown
LC-3829	P03c	40,4	16,90	0,15	-0,07	37,1	Apigenin 6-C-pentoside-8-C-hexoside
LC-3840	P07	30,8	4,90	-0,06	-0,01	12,6	Unknown
LC-3856	P08	41,2	3,93	0,10	-0,16	10,2	Unknown
LC-3856	P12b	33,1	3,38	-0,15	-0,01	8,8	Unknown
LC-3888	P02	136,7	3,40	0,05	-0,05	8,9	Unknown
LC-3888	P03b	37,3	3,40	0,06	-0,03	8,9	Unknown
LC-3913	P03c	40,4	6,59	-0,06	0,08	16,5	Unknown
LC-3919	P03c	40,4	23,37	0,24	-0,14	47,3	Unknown
LC-3925	P05b	22,0	27,11	0,24	-0,18	52,4	Unknown
LC-3937	P01	92,4	6,88	-0,10	-0,07	17,2	Unknown
LC-3937	P08	8,2	3,75	-0,06	-0,02	9,8	Unknown
LC-3937	P04a	55,0	3,40	-0,05	-0,05	8,9	Unknown
LC-3937	P03a	20,9	3,12	0,05	-0,02	8,2	Unknown
LC-3938	P01	85,3	3,07	0,08	-0,01	8,1	Unknown
LC-3945	P03c	40,4	19,80	0,17	-0,08	41,9	Apigenin 6-C-pentoside-8-C-hexoside
LC-3958	P02	136,7	6,77	0,12	-0,13	16,9	Unknown
LC-3958	P01	92,4	3,25	0,12	0,02	8,5	Unknown
LC-3958	P10b	23,0	3,02	0,11	-0,03	8,0	Unknown
LC-3969	P11	56,3	9,86	-0,08	-0,02	23,7	Unknown
LC-3969	P08	76,2	5,40	0,06	-0,01	13,8	Unknown
LC-3984	P08	41,2	3,59	0,07	-0,10	9,4	Unknown
LC-3984	P12b	33,1	3,06	-0,08	-0,04	8,0	Unknown
LC-4004	P03c	40,4	5,19	-0,07	0,04	13,3	Unknown
LC-4036	P03c	40,4	17,64	0,17	-0,06	38,3	Unknown
LC-4036	P08	41,2	3,52	0,07	0,05	9,2	Unknown
LC-4074	P03c	25,2	3,66	-0,09	-0,02	9,5	Unknown
LC-4074	P04a	34,0	3,33	0,09	0,03	8,7	Unknown
LC-4099	P07	21,2	3,86	-0,05	0,00	10,0	Unknown
LC-4099	P10b	9,3	3,32	0,05	-0,02	8,7	Unknown
LC-4111	P01	105,7	3,04	-0,06	-0,06	8,0	Unknown

Supplementary Table 1. (continued)

Metabolite code[1]	Linkage group	QTL position[2]	LOD[3]	Additive[4]	Dominance	% Expl.[5]	Metabolite putative identification
LC-4118	P08	76,2	5,89	0,08	-0,03	14,9	Unknown
LC-4118	P02	136,7	3,58	0,06	0,04	9,4	Unknown
LC-4150	P05a	22,7	7,24	-0,12	-0,05	18,0	4,5-Dicaffeoylquinic acid
LC-4162	P09	60,4	3,05	0,02	0,04	8,0	Unknown
LC-4180	P08	48,2	3,28	-0,05	0,00	8,6	Unknown
LC-4183	P08	48,2	6,57	-0,09	-0,01	16,5	Luteolin-Methyl-Acetyl-apiofuranosyl-hexose
LC-4183	P04a	34,0	3,92	0,07	-0,05	10,2	Luteolin-Methyl-Acetyl-apiofuranosyl-hexose
LC-4183	X06	18,5	3,23	0,07	-0,04	8,5	Luteolin-Methyl-Acetyl-apiofuranosyl-hexose
LC-4202	P03a	34,2	3,44	-0,06	-0,04	9,0	Unknown
LC-4222	P03c	16,2	4,21	-0,09	-0,03	10,9	Unknown
LC-4222	P09	32,8	3,36	-0,06	0,07	8,8	Unknown
LC-4245	P01	92,4	3,23	-0,07	0,00	8,5	Unknown
LC-4249	P03c	40,4	13,21	0,15	-0,14	30,4	Unknown
LC-4259	P03c	16,2	4,49	-0,09	-0,04	11,6	Unknown
LC-4259	P01	20,2	3,54	-0,01	0,16	9,2	Unknown
LC-4266	P03a	52,1	11,73	-0,17	-0,12	27,5	Unknown
LC-4266	P09	60,4	4,65	0,11	-0,04	12,0	Unknown
LC-4266	P02	24,1	3,07	0,00	0,81	8,1	Unknown
LC-4277	P08	8,2	5,12	0,17	-0,08	13,1	Unknown
LC-4277	P09	32,8	3,39	-0,13	-0,05	8,9	Unknown
LC-4284	P12b	33,1	5,76	-0,12	0,00	14,6	double charge chinense
LC-4284	P10a	16,5	3,81	-0,09	0,00	9,9	double charge chinense
LC-4300	P07	21,2	3,04	-0,04	-0,03	8,0	Unknown
LC-4315	P03c	40,4	4,36	0,06	-0,05	11,3	Unknown
LC-4324	X02	73,9	3,29	0,07	-0,05	8,6	Unknown
LC-4348	P05a	22,7	6,85	-0,10	-0,01	17,1	Quercetin Hexose-Deoxy-Feruloyl
LC-4376	P12b	33,1	3,16	-0,09	-0,02	8,3	Unknown
LC-4376	P08	42,2	3,15	0,07	-0,08	8,3	Unknown
LC-4404	P08	7,6	3,47	-0,08	-0,03	9,1	Unknown
LC-4428	P05a	28,1	4,17	0,07	-0,02	10,8	Unknown
LC-4436	P06	77,7	3,96	-0,05	-0,01	10,3	glucose
LC-4455	P03a	52,1	7,80	-0,11	-0,07	19,3	Unknown
LC-4455	P02	24,1	3,62	0,00	0,66	9,5	Unknown
LC-4461	P03c	40,4	3,11	-0,07	0,04	8,2	Unknown
LC-4466	P01	105,7	5,83	-0,13	-0,05	14,8	Unknown
LC-4466	P08	9,0	3,73	-0,09	-0,02	9,7	Unknown
LC-4489	P04a	34,0	3,67	0,10	-0,04	9,6	Unknown
LC-4489	P10b	47,5	3,09	-0,07	-0,14	8,1	Unknown
LC-4502	P03c	40,4	20,22	0,21	-0,11	42,6	Unknown
LC-4526	P05a	38,8	5,14	-0,10	-0,04	13,1	Unknown
LC-4533	P08	8,2	5,28	0,08	-0,07	13,5	Unknown
LC-4533	P04a	34,0	4,03	0,07	-0,06	10,5	Unknown
LC-4533	P01	105,7	3,97	0,06	-0,06	10,3	Unknown
LC-4542	P02	136,7	3,43	0,07	-0,06	9,0	Unknown
LC-4559	P02	136,7	5,17	0,07	-0,07	13,2	Unknown
LC-4594	P08	41,2	3,47	0,06	-0,10	9,1	Unknown
LC-4598	P01	56,1	5,16	0,09	-0,05	13,2	Unknown
LC-4598	P08	9,0	3,49	0,06	-0,09	9,1	Unknown
LC-4601	P01	0,0	6,67	-0,02	-0,15	16,7	Homovanillic acid-O-hexoside
LC-4613	P03c	40,4	15,59	0,14	-0,09	34,8	Unknown
LC-4619	P12b	33,1	3,89	-0,15	0,06	10,1	Unknown
LC-4619	P10a	16,5	3,74	-0,10	-0,02	9,7	Unknown
LC-4645	P09	12,7	12,54	-0,08	-0,05	29,1	Unknown
LC-4660	P08	76,2	4,74	0,06	-0,01	12,2	Unknown
LC-4660	P02	168,6	3,19	0,07	0,06	8,4	Unknown
LC-4688	P07	97,0	5,33	0,04	0,10	13,6	Unknown
LC-4688	P11	56,3	4,27	0,09	0,02	11,1	Unknown
LC-4694	P03c	40,4	18,63	0,17	-0,11	40,0	Unknown
LC-4720	P12b	33,1	3,90	-0,13	0,01	10,1	double charge chinense
LC-4720	P10a	16,5	3,42	-0,10	-0,01	9,0	double charge chinense
LC-4720	P01	20,2	3,28	0,02	0,16	8,6	double charge chinense
LC-4747	P06	77,7	4,13	-0,05	-0,02	10,7	Unknown
LC-4747	P09	64,1	3,54	0,04	0,04	9,3	Unknown
LC-4755	P01	74,3	3,07	0,07	-0,04	8,1	Unknown
LC-4780	P03c	40,4	5,88	0,13	-0,08	14,9	Flavonoid glycosides
LC-4797	P03c	40,4	4,40	0,05	-0,07	11,4	Isorhamnetin Hexose-Deoxy-Coum I
LC-4797	P05a	17,2	3,92	-0,06	-0,03	10,2	Isorhamnetin Hexose-Deoxy-Coum I
LC-4820	P05a	22,7	3,07	-0,05	-0,02	8,1	Unknown
LC-4826	P08	41,2	3,16	0,06	-0,09	8,3	Unknown
LC-4860	P05b	51,8	6,13	0,11	-0,12	15,5	Unknown
LC-4870	P07	32,3	3,85	0,09	-0,04	10,0	Unknown
LC-4875	P03c	40,4	6,52	0,08	-0,04	16,4	Unknown
LC-4911	P12b	33,1	5,76	-0,05	-0,08	14,6	Unknown
LC-4911	P01	0,0	3,46	0,03	0,09	9,1	Unknown

Supplementary Table 1. (continued)

Metabolite code[1]	Linkage group	QTL position[2]	LOD[3]	Additive[4]	Dominance	% Expl.[5]	Metabolite putative identification
LC-4911	P10a	16,5	3,13	-0,08	0,04	8,2	Unknown
LC-4950	P01	115,4	4,12	0,07	-0,07	10,7	Unknown
LC-4992	P12b	33,1	5,20	-0,02	-0,10	13,3	Unknown
LC-4992	P03c	25,2	3,69	0,06	0,02	9,6	Unknown
LC-4992	P07	102,9	3,36	0,01	0,08	8,8	Unknown
LC-5013	P05a	22,7	7,39	-0,10	-0,04	18,3	Kaempferol-hexose-dehydrohexose, -C12H12O5 (236)
LC-5023	P03c	40,4	7,52	0,16	-0,10	18,6	Unknown
LC-5026	P12b	33,1	5,57	-0,09	-0,06	14,2	Unknown
LC-5027	P12b	33,1	4,49	-0,04	-0,04	11,6	Unknown
LC-5037	P01	115,4	7,43	0,05	-0,06	18,4	Unknown
LC-5037	P04a	23,7	3,58	-0,02	-0,11	9,3	Unknown
LC-5046	P06	108,1	3,35	0,11	-0,02	8,8	Unknown
LC-5070	P12b	33,1	4,00	-0,05	-0,09	10,4	Capsianoside XVII
LC-5083	P05b	51,8	4,07	0,10	-0,05	10,6	Unknown
LC-5089	P05b	51,8	3,28	0,07	-0,05	8,6	Unknown
LC-5096	P02	136,7	7,19	-0,12	-0,13	17,9	Unknown
LC-5096	P08	48,2	3,60	0,09	-0,09	9,4	Unknown
LC-5101	P12b	33,1	4,42	-0,07	-0,04	11,4	Unknown
LC-5101	P10a	16,5	3,19	-0,08	0,02	8,4	Unknown
LC-5115	P03a	20,9	6,31	0,10	-0,05	15,9	Unknown
LC-5116	P12b	33,1	4,35	-0,12	0,04	11,2	Capsianoside XVII
LC-5116	P09	32,8	3,56	-0,07	0,04	9,3	Capsianoside XVII
LC-5151	P03c	40,4	3,33	0,09	-0,05	8,7	Unknown
LC-5162	P05b	51,8	6,78	0,14	-0,14	17,0	Unknown
LC-5169	P06	148,4	3,37	0,16	-0,04	8,8	Unknown
LC-5177	P03c	40,4	4,49	0,10	-0,06	11,6	Unknown
LC-5177	P04a	23,7	3,26	0,05	-0,12	8,5	Unknown
LC-5199	P12b	44,0	3,14	0,07	0,00	8,2	Unknown
LC-5249	P01	105,7	5,29	-0,12	0,01	13,5	Unknown
LC-5249	P08	8,2	3,41	-0,10	-0,01	8,9	Unknown
LC-5260	P03c	40,4	3,13	0,08	-0,02	8,2	Unknown
LC-5326	P05a	53,4	3,43	0,08	0,01	9,0	Unknown
LC-5350	P12b	33,1	4,61	-0,04	-0,08	11,9	Capsianoside XVII
LC-5350	P01	20,2	4,16	0,00	0,14	10,8	Capsianoside XVII
LC-5362	P11	56,3	8,50	0,11	0,04	20,8	Unknown
LC-5362	P07	97,0	7,79	0,05	0,10	19,2	Unknown
LC-5374	P11	56,3	12,33	0,19	0,03	28,7	Unknown
LC-5397	X02	73,9	3,30	-0,04	0,10	8,6	Capsianoside III
LC-5426	P12b	33,1	3,07	-0,01	0,12	8,1	Unknown
LC-5442	P11	56,3	3,50	0,08	0,02	9,2	Unknown
LC-5442	P07	21,2	3,17	0,07	-0,03	8,3	Unknown
LC-5451	P03c	25,2	3,33	-0,06	-0,02	8,7	Unknown
LC-5460	P03c	40,4	10,05	0,17	-0,09	24,1	Unknown
LC-5496	P07	32,3	3,43	0,07	-0,03	9,0	Unknown
LC-5567	P07	97,0	5,71	-0,01	0,14	14,5	Unknown
LC-5584	P06	19,8	6,07	0,07	-0,03	15,3	Unknown
LC-5595	P10b	40,5	3,67	0,04	-0,13	9,6	Unknown
LC-5616	P06	48,9	3,52	0,08	0,04	9,2	Unknown
LC-5631	X02	73,9	3,13	-0,04	0,11	8,2	Unknown
LC-5653	P01	20,2	4,55	0,02	0,14	11,7	Unknown
LC-5666	P12b	33,1	6,27	-0,06	-0,09	15,8	Capsianoside XVII
LC-5672	P03c	40,4	3,08	0,06	-0,06	8,1	Unknown
LC-5679	P06	19,8	4,33	0,09	0,02	11,2	Unknown
LC-5693	P12b	33,1	6,19	-0,07	-0,07	15,6	Unknown
LC-5703	P06	77,7	3,71	0,06	-0,01	9,7	Capsianoside III-2 (Phytatetraene-diol-diglucose-rhamnose diglucose)
LC-5703	P09	75,6	3,52	-0,06	0,03	9,2	Capsianoside III-2 (Phytatetraene-diol-diglucose-rhamnose diglucose)
LC-5735	P12b	44,0	3,33	0,07	0,09	8,7	Unknown
LC-5738	P11	39,1	4,51	0,11	0,02	11,6	Unknown
LC-5770	P06	53,6	3,72	0,02	0,11	9,7	Capsianoside IV (Hydroxy-phytatetraen-oic acid-O-diglucose)
LC-5812	P03c	40,4	4,72	0,08	-0,07	12,1	Unknown
LC-5821	P08	36,8	3,44	0,07	-0,01	9,0	Unknown
LC-5859	P06	48,9	3,31	0,12	0,09	8,7	Unknown
LC-5877	P07	97,0	7,63	-0,01	0,18	18,9	Unknown
LC-5915	P06	19,8	3,69	0,06	-0,03	9,6	Unknown
LC-5915	P01	85,3	3,19	0,06	-0,03	8,4	Unknown
LC-5922	P01	115,4	9,46	0,10	-0,08	22,8	Unknown
LC-5923	P08	41,2	3,16	0,10	-0,07	8,3	Unknown
LC-5923	P06	148,4	3,12	-0,10	-0,03	8,2	Unknown
LC-5931	P03c	7,5	3,16	-0,03	0,08	8,3	Unknown
LC-5946	P05a	22,7	3,16	-0,07	-0,02	8,3	Unknown
LC-5964	P09	75,6	4,65	-0,10	0,05	12,0	Unknown
LC-6000	P06	48,9	3,71	0,09	0,09	9,7	Unknown
LC-6011	P03c	7,5	4,13	0,05	-0,05	10,7	Unknown

Supplementary Table 1. (continued)

Metabolite code[1]	Linkage group	QTL position[2]	LOD[3]	Additive[4]	Dominance	% Expl.[5]	Metabolite putative identification
LC-6011	P03b	6,6	3,59	0,04	-0,06	9,4	Unknown
LC-6072	P12b	33,1	3,93	-0,05	-0,10	10,2	Unknown
LC-6173	P03c	7,5	3,13	-0,04	0,08	8,2	Unknown
LC-6176	P01	115,4	9,75	0,08	-0,07	23,5	Unknown
LC-6209	P01	115,4	5,54	0,07	-0,08	14,1	trans-Dihydrodehydrodiconiferyl alcohol-9-O-beta-D-glucoside
LC-6212	P03c	7,5	6,75	0,08	-0,09	16,9	C28H42O11
LC-6212	P03b	6,6	4,22	0,06	-0,08	10,9	C28H42O10
LC-6237	P06	48,9	3,56	0,09	0,05	9,3	Capsianoside IX
LC-6278	P09	75,6	7,39	-0,13	-0,05	18,3	Capsianoside VIII
LC-6294	P08	48,2	3,24	-0,06	-0,08	8,5	Unknown
LC-6295	P12b	33,1	3,61	-0,04	-0,05	9,4	Unknown
LC-6296	X03	31,8	3,05	-0,02	0,10	8,0	Unknown
LC-6317	P10b	36,1	3,76	-0,02	0,14	9,8	Unknown
LC-6317	P11	23,7	3,13	0,08	-0,01	8,2	Unknown
LC-6319	P05a	53,4	4,39	0,11	0,01	11,3	Unknown
LC-6319	P10b	16,5	3,14	0,00	0,11	8,2	Unknown
LC-6348	P12b	33,1	3,06	-0,02	-0,07	8,1	Unknown
LC-6350	P03a	52,1	4,81	-0,10	-0,07	12,4	Unknown
LC-6350	P09	60,4	3,55	0,09	-0,03	9,3	Unknown
LC-6357	P12b	44,0	4,30	0,05	0,06	11,1	Unknown
LC-6387	P06	48,9	3,02	0,07	0,00	8,0	Unknown
LC-6400	P12b	33,1	4,28	-0,06	-0,03	11,1	Unknown
LC-6423	P12b	33,1	3,40	-0,08	0,05	8,9	Unknown
LC-6429	P06	53,6	3,05	0,09	0,03	8,0	Capsianoside IX
LC-6462	P11	17,5	3,55	0,05	0,05	9,3	Unknown
LC-6486	P12b	33,1	3,85	-0,08	-0,10	10,0	Unknown
LC-6516	P03a	52,1	7,45	-0,12	-0,10	18,5	Unknown
LC-6516	P09	60,4	5,02	0,10	-0,04	12,8	Unknown
LC-6542	P09	75,6	8,19	-0,15	0,00	20,1	Unknown
LC-6563	P12b	33,1	4,45	0,01	0,15	11,5	Unknown
LC-6570	P11	27,6	4,39	0,10	0,00	11,3	Unknown
LC-6577	P12b	44,0	5,80	0,10	-0,05	14,7	Unknown
LC-6617	P06	48,9	3,99	0,12	0,09	10,4	Capsianoside IX
LC-6625	X02	73,9	3,25	-0,03	0,12	8,5	Unknown
LC-6625	P06	48,9	3,02	0,08	0,09	7,9	Unknown
LC-6636	P10a	16,5	8,25	0,14	-0,08	20,2	Unknown
LC-6636	P06	19,8	5,48	-0,10	-0,13	13,9	Unknown
LC-6636	P12b	33,1	4,37	0,14	-0,11	11,3	Unknown
LC-6688	P12b	33,1	3,50	0,13	-0,05	9,1	Unknown
LC-6688	P01	0,0	3,27	-0,03	-0,12	8,6	Unknown
LC-6701	P05a	53,4	3,93	0,06	0,03	10,2	Unknown
LC-6721	P12b	33,1	3,49	0,08	0,06	9,1	Unknown
LC-6735	P03c	25,2	3,47	-0,06	-0,03	9,1	Unknown
LC-6838	P10a	16,5	3,52	-0,09	0,01	9,2	Unknown
LC-6848	P01	105,7	4,23	-0,08	0,07	10,9	Unknown
LC-6896	P12b	33,1	6,44	-0,18	0,04	16,2	Unknown
LC-6896	P02	168,6	3,21	-0,12	-0,28	8,4	Unknown
LC-6902	P10a	16,5	3,19	-0,08	0,03	8,4	Unknown
LC-6904	P12b	33,1	13,99	0,11	0,05	31,9	Unknown
LC-6904	P10a	16,5	4,46	0,09	-0,05	11,5	Unknown
LC-6929	P03c	40,4	6,71	0,08	-0,09	16,8	Unknown
LC-6942	P12b	33,1	8,79	-0,13	-0,07	21,4	Unknown
LC-6963	P12b	33,1	7,69	-0,08	-0,18	19,0	Unknown
LC-6985	P06	19,8	7,31	0,13	-0,15	18,2	Unknown
LC-6985	P12b	33,1	3,48	0,09	0,10	9,1	Unknown
LC-6985	P10a	16,5	3,30	0,16	-0,07	8,7	Unknown

[1] GC and LC indicate metabolites detected by GC-MS and LC-MS, respectively

[2] Position of the QTL, in cM from the top of linkage group

[3] Logarithm of the odds (LOD). QTLs were deemed significant when the LOD exceeded 3.6 (threshold corresponding to a genome-wide confidence level of 0.95, estimated from permutation tests with 1,000 iterations)

[4] Negative values indicate that *C. annuum* alleles have lower phenotypic values than *C. chinense* alleles

[5] Percentage of phenotypic variance explained by each QTL

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Awang Maharijaya
Date: 18 November 2013
Group: Plant Breeding, Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Thrips Resistance in Pepper	Feb 25, 2008
► **Writing or rewriting a project proposal**	
Candidate Genes for Thrips Resistance in Pepper	2008
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Student Day, Leiden University	Feb 26, 2009
EPS PhD Student Day, Wageningen University	May 20, 2011
► **EPS theme symposia**	
EPS theme symposia 2 'Interaction between plants and biotic agents', Utrecht University	Nov 16, 2009
EPS theme symposia 5 'Plant-Insect Interaction workshop', Wageningen University	Nov 11, 2010
EPS theme symposia 2'Interaction between plant and biotic agents', University of Amsterdam	Feb 03, 2011
EPS theme symposia 5 'Plant-Insect Interaction workshop, University of Amsterdam	Nov 23, 2011
EPS theme symposia 2'Interaction between plant and biotic agents', Wageningen University	Feb 10, 2012
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
NERN Conference	Feb 09-10, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2012
► **Seminars (series), workshops and symposia**	
Plant Breeding Research Day	Jun 17, 2008
First annual INDOSOL programme symposium, Bogor-Indonesia	Nov 06, 2008
Application of Molecular Marker in Breeder Right Issues and Phylogenetic Studies	Nov 10-13, 2008
Plant Breeding Research Day	Mar 03, 2009
KNAW seminar: Performing Research	Nov 16, 2009
KNAW seminar: Valorisation of Knowledge	Nov 17, 2009
Agriculture Beyond Food	Nov 18-20, 2009
Second annual INDOSOL Symposium, Wageningen, the Netherlands	May 31-Jun 01, 2010
Plant Breeding Research Day	Mar 09, 2011
Mini-symposium "How to write a world-class paper", Wageningen University	Oct 26, 2011
Plant Breeding Research Day	Feb 28, 2012
SPICY Workshop: Improving yield prediction	Mar 07, 2012
Bioinformatics and statistical genetic and genomic	Mar 08, 2012
Understanding the impact of crop characteristics on yield through crop growth modelling	Mar 09, 2012
► **Seminar plus**	
► **International symposia and congresses**	
XVIth Eucarpia Symposium of the Tomato Working Group, Wageningen University	May 12-15, 2008
4th International Indonesian Biotechnology Conference, Bogor-Indonesia	Aug 05-07, 2008
XIVth Eucarpia Capsicum and Eggplant Symposium, Valencia-Spain	Aug 30-01, 2010
Eusol-Latsol-Indosol Joint Conference, Natal-Brazil	Nov 13-16, 2010
5th International Indonesian Biotechnology Conference, Bogor-Indonesia	Jul 04-07, 2012
International Seminar on Future Adaptation in the Tropics	Sep 05-06, 2012
XVth Eucarpia Capsicum and Eggplant Symposium, Turin-Italy	Sep 02-04, 2013
► **Presentations**	
4th International Indonesian Biotechnology Conference (oral)	Aug 05-07, 2008
First annual INDOSOL programme symposium (oral)	Nov 06, 2008
EPS PhD Student Day (poster)	Feb 26, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren (poster)	Apr 06-07, 2009
NERN Conference (oral)	Feb 09-10, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren (poster)	Apr 19-20, 2010
XIVth Eucarpia Capsicum and Eggplant Symposium, Valencia-Spain (oral)	Aug 30-01, 2010
XVth Eucarpia Capsicum and Eggplant Symposium, Turin-Italy (poster)	Sep 02-04, 2013
EPS theme symposia 5 'Plant-Insect Interaction workshop', Wageningen University (oral)	Nov 11, 2010
Eusol-Latsol-Indosol Joint Conference, Natal-Brazil (poster)	Nov 13-16, 2010
Plant Breeding Day (poster)	2010
ALW meeting 'Experimental Plant Sciences', Lunteren (oral)	Apr 04-05, 2011
International Seminar on Future Adaptation in the Tropics	Sep 05-06, 2012
► **IAB interview**	Dec 02, 2009
► **Excursions**	
Scientific Excursion to PT East West Seed Indonesia and Indonesian Vegetables Research Institut	Nov 07, 2008
Scientific Excursion to Rijkwaan (pepper maintainance)	Mar 09, 2009
Subtotal Scientific Exposure	*30.8 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Plant Metabolomics	Apr 26-28, 2011
Bioinformatics: a user approach	Aug 29-Sep 02,
► **Journal club**	
Participating in journal and literature study group	2008-2012
► **Individual research training**	
Subtotal In-Depth Studies	*5.4 credits**

4) Personal development	*date*
► **Skill training courses**	
PhD course Techniques for Writing and Presenting Scientific Papers	Dec 14-17, 2010
Writing a grant proposal course, RUG	Oct 21-Nov 18,
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**47,4**

Herewith the Graduate School declares that the PhD candidate has complied with the educational

** A credit represents a normative study load of 28 hours of study.*

Thesis lay-out: By the author
Cover design: Unknown resistance factors in leaves of pepper defend the pepper plant from thrips attacks, drawn by the author

Printing: Ipskamp Drukkers BV Postbus 333, 7500 AH Enschede