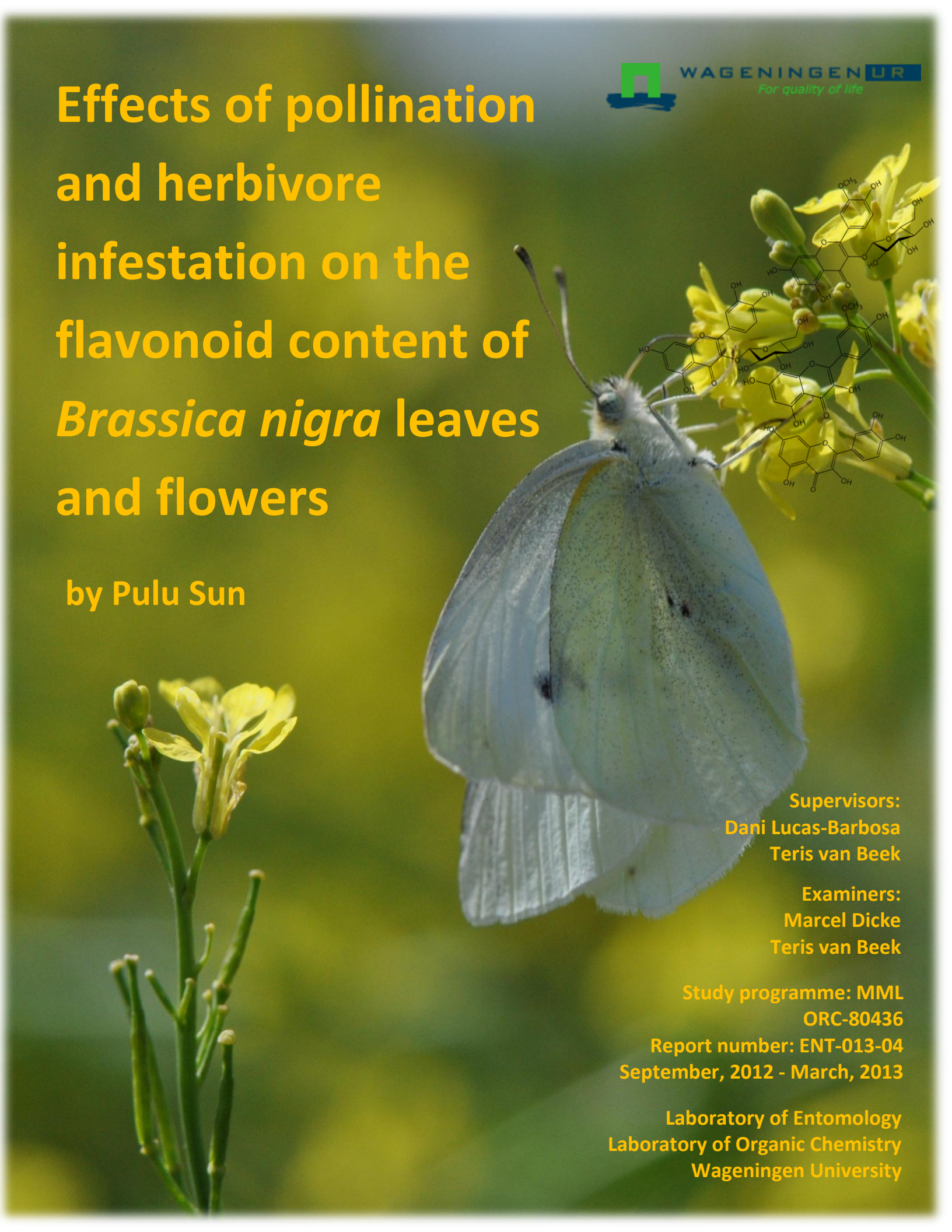
Effects of pollination and herbivore infestation on the flavonoid content of *Brassica nigra* leaves and flowers

by Pulu Sun



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Study programme: MML
ORC-80436
Report number: ENT-013-04
September, 2012 - March, 2013

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Abstract

Flavonoids are important pigments colouring most flowers in nature. They are characterized by C_6-C_3-C_6 carbon framework and phenylbenzopyran functionality. In nature, they play a role in host recognition and host acceptance by herbivores. Flavonoids may stimulate to or deter herbivore larvae from feeding on plants. Furthermore, flavonoids may also be involved in interactions between plants and insect pollinators. Changes in colours that might occur upon pollination may influence how pollinators perceive flowers. The objective of this study was to investigate whether herbivore infestation by *Pieris brassicae* and pollination influence the flavonoid content of *Brassica nigra* leaves and flowers. In this study, reverse-phase ultra-high pressure liquid chromatography (RP-UHPLC) and liquid chromatography–mass spectrometry (LC-MS) were used for separation and quantification. Flavonoids compounds were characterized by their absorbance profile in the UV-Vis and some of the compounds were identified by LC-MS. Only compounds that had similar UV patterns as that of flavonoids were quantified and regarded as flavonoid compounds. In this study, 55 flavonoid compounds were quantified in leaf-extracts and 92 were quantified in flower-extracts. Mainly flavonols were identified in leaves and flowers of *B. nigra.* Isorhamnetin hexoside and quercetin dihexosides, for instance, were characteristic of the flower profile. Leaf profile and flower profile were different, regarding their flavonoid content. *Brassica nigra* plants responded to herbivore infestation as well as pollination with changes in flavonoid content of leaves and flowers. Herbivore infestation and pollination influenced the flavonoid content of leaves and flowers of *B. nigra* plants differently, and the change in flavonoid content influenced the UV-Vis absorbance of flower extracts, which may in turn influence the behaviour of insect pollinators. The total flavonoid content of pollinated flowers of infested plants was greater than that of pollinated flowers of non-infested plants. Additionally, when the plants were subject to both pollination and infestation, an increase on total flavonoid content was also observed in leaves, when compared to plants that were only subjected to pollination. It seems, therefore, that there is a synergistic effect between herbivore infestation and pollination. These results bring new insights into the increasing number of studies addressing a potential trade-off between plant defence and plant reproduction.

Table of Contents

Introduction

Flavonoids are important pigments colouring most flowers in nature (Harborne and Grayer, 1993). They are characterized by a phenylbenzopyran functionality and a C_6-C_3-C_6 carbon framework (Marais et al., 2006; Stobiecki and Kachlicki, 2006). As pigments for flowers, flavonoids colour petals in pink, purple, yellow, white and so on (Harborne and Grayer, 1993). For example, yellow flavonoids, such as chalcones, aurones, 6- and 8-hydroxy flavonols, are important pigments of many yellow-flowered plants such as *Primula* and *Coreopsis* (Harborne and Grayer, 1993). White-flowered species also have flavonoid pigments, but in the form of flavone or flavonol glycosides present in the epidermal cells of the petals (Harborne and Grayer, 1993). Flavonoids do not only colour flowers but are also content of different organs of the plants, such as seeds, roots, leaves, flowers, and fruits (Stobiecki and Kachlicki, 2006). As plant second metabolites, flavonoids are involved in different biochemical and physiological plant metabolic processes (Stobiecki and Kachlicki, 2006). Flavonoids can protect the internal tissues of plants from abiotic stresses (Treutter, 2006) as well as biotic stresses, such as microbes (Mathpal et al., 2011) and play an important role in interactions with insects (Iwashina, 2003). Regarding plant-insect interactions, flavonoids can be deterrents or stimulants of feeding and oviposition and are involved in the attraction of pollinators (Simmonds, 2001; Tabashnik, 1987).

In plants, flavonoids play an important role in host recognition and host acceptance by herbivores (Simmonds, 2001). In nature, it is vital for adult herbivores such as butterflies to select a suitable host plant for their less mobile offspring (Tabashnik, 1987). Adult herbivores can detect flavonoids visually due to changes in colour of flowers (Eisikowitch and Lazar, 1987) or via contact, sensing through their forelegs and antennae (Simmonds, 2001). For instance, the female butterfly *Danaus plexippus* uses flavonoids, such as glucosylgalactose and rutinoside, which are constitutively present in *Asclepias curassavica* as contact cues to choose a suitable plant for oviposition (Haribal and Renwick, 1996). Herbivore larvae might be stimulated or deterred from feeding on the plants and this behaviour has been associated with the flavonoid content of the plants. Flavonoids may deter generalist herbivores from eating from the plants. For instance, third instar larvae of *Dione juno* was deterred from feeding on the leaves of *Passiflora mollisima* and *P. quadrangularis* when 40 ppm of a flavonol, ermanin, were applied on the leaves (Echeverri et al., 1991). However, flavonoids might also render plants attractive to specialist insects. Indeed, some specialist insects can sequester flavonoids and use them in intraspecific/interspecific visual communication and to attract mates (Burghardt et al., 1997; Ferreres et al., 2009; Harborne and Grayer, 1993). For example, flavonoids sequestered by the herbivore larvae of *Polyommatus icaus* will be stored in wings of their adults butterflies that generate UV patterns, which is used in species discrimination and mate selection (Burghardt et al., 1997). Apart from that, flavonoids might be sequestered and used by insects as defence against their enemies (Ferreres et al., 2009). Besides, whether herbivore larvae are stimulated to or deterred from feeding on a plant can also depend on the concentration of constitutively present flavonoids (Simmonds, 2001). For example, final instar larvae of *Heliothis zea* and *Heliocoverpa armigera* were deterred from feeding the plants with higher concentration of rutin spray (more than 10^{-3} M), but feeding activity was stimulated when rutin concentration was lower (less than 10^{-4} M) (Simmonds, 2001). If a higher concentration of flavonoid deters an insect from eating the plant, it will be beneficial for the plant to increase flavonoid concentration upon herbivore attack to suppress further damage. It is known that plants can indeed

activate defences in response to herbivory, both locally and systemically, and in leaves and flowers (Dicke and Baldwin, 2010; Lucas-Barbosa et al., 2011). However, whether plants respond to herbivore damage by changing the flavonoid content is still unknown.

Flavonoids can also play a role in interactions between plants and pollinating insects. Insect pollinators may associate both visual and odour cues with the quality of nectar and pollen offered by flowers as reward for pollination (Harborne and Grayer, 1993; Milet-Pinheiro et al., 2012; Weiss, 1991). For example, open-pollinated blueberry flowers emitted 32% less volatiles (mainly cynammil alcohol) compared to unpollinated flowers (Rodriguez-Saona et al., 2011). Thus, pollinators could reach the unpollinated flowers more precisely based on their olfactory sense. Besides, plants can also guide pollinators to the non-pollinated flowers by changing the colour of their flowers in order to increase the efficiency of pollination (Eisikowitch and Lazar, 1987; Niesenbaum et al., 1999; Weiss, 1991). For example, butterflies *Agraulis vanilla* and *Junonia coenia* mostly visit the yellow flowers of *Lantana* instead of red flowers, which have been pollinated and have little or no nectar (Weiss, 1991). Colour change in the flowers is probably caused by the significant decrease in the concentration of one or several pigments (Eisikowitch and Lazar, 1987), but the exact mechanism has not been investigated to date.

In this research project, I focused on how herbivore infestation by *Pieris brassicae* and pollination affect the flavonoid content of leaves and flowers of *Brassica nigra* plants. Plant responses to herbivore infestation and pollination might result in changes of colours in leaves and flowers, and as a consequence, influence how insects perceive the plant. Separation and quantification of the flavonoid compounds from *B. nigra* leaves and flowers were done via a reverse phase ultra-high pressure liquid chromatography with a diode array detector (RP-UHPLC-DAD). Flavonoid compounds present in plants were identified using UHPLC system tandem mass spectrometer (UHPLC-MS). Specifically, the objectives of my research can be articulated in four questions, as follows:

1) Is the flavonoid content of *B. nigra* leaves different from the content of flowers?
2) Does herbivore infestation affect the flavonoid content of *B. nigra* leaves and flowers?
3) Does the flavonoid content of *B. nigra* leaves and flowers change upon pollination?
4) What is the net effect on the flavonoid content of *B. nigra* leaves and flowers when plants have been exposed to both herbivore infestation and pollination?

Materials and methods

Study system

Brassica nigra (L.) Koch (Brassicaceae) is an annual herb that is considered to be an obligately out-crossing plant and produces only hermaphroditic flowers (Conner and Neumeier, 1995; Lucas-Barbosa et al., 2013). *Brassica nigra* produces hundreds of yellow flowers during its growing season, and new flowers open every day. In the Netherlands, *B. nigra* is often colonized by the Large Cabbage White butterfly, *Pieris brassicae*. *Pieris brassicae* L. (Lepidoptera: Pieridae) is a specialist herbivore that feeds on plants from the Brassicaceous family. Females *P. brassicae* butterflies lay clutches of on average 30–100 eggs (D. Lucas-Barbosa, personal communication), on the underside of the host plant leaves. After caterpillars had hatched from eggs, they mainly feed on the leaves during their first-instar, but start from second-instar they migrate to the buds and flowers and start feeding on the flowers (Lucas-Barbosa et al., 2013; Smallegange et al., 2007). Figure 1 shows the study system.

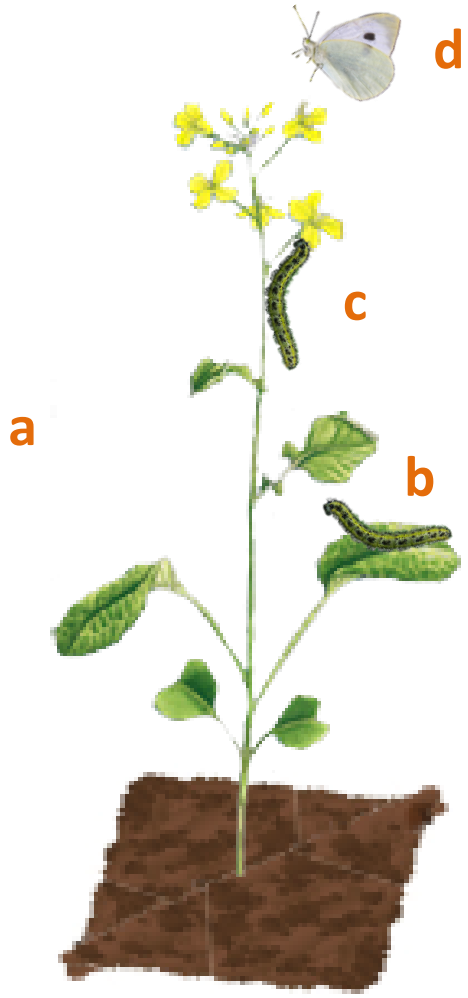

Figure 1. Schematic representation of the study system: (a) *Brassica nigra* plant (b) folivorous *Pieris brassicae* caterpillar (c) florivorous *P. brassicae* caterpillar and (d) *P. brassicae* butterfly.

Caterpillars of *P. brassicae* were reared on *Brassica oleracea* L. var. *gemmifera* (Brussels sprout) plants in a climate room (22 ± 1 °C, 50–70% r.h., L16:D8). A solution with 10% sucrose was supplied as feed for the adults. Original seeds of *B. nigra* plant were obtained from the Centre of Genetic Resources (CGR, Wageningen, The Netherlands) from an early-flowering accession (CGN06619, feral population gathered in 1975 from the Peloponnesus, Greece), and reproduced in Wageningen by exposing plants to open pollination. Seeds for this experiment were collected randomly from 25 individuals and combined in order to obtain a representative sample of the population. The individuals of *B. nigra* were in pots grown inside a greenhouse compartment (23 ± 2 °C, 70% r.h., L16:D8). Plants with at least one open flower (i.e. growth stage 4.2 according to Harper and Berkenkamp, 1975) were used for the experiments.

Plant treatments

Herbivore infestation

To investigate whether herbivore infestation by *P. brassicae* caterpillars affects the flavonoid content of *B. nigra* leaves and flowers, I infested plants with butterfly eggs at two time points: 24 h and 120 h after the caterpillars had hatched from the eggs. Plants were infested by being exposed to adult individuals of *P. brassicae* in an oviposition cage (100 cm × 70 cm × 82 cm). Each plant was infested with one egg clutch of 30 eggs. Surplus of eggs were gently removed with forceps. The flowers of each plant were covered with a mesh bag during infestation to prevent butterflies from visiting and pollinating them. For the 120 h herbivore treatment, 50% of the caterpillars were removed from the plants 48 h after hatching to simulate predation and dispersion. At each time point, for every plant subjected to treatment, there was another plant in the same growth stage used as control. In total, 11 pairs of plants were used for 24 h herbivore treatment and 10 pairs for 120 h treatment (Table 1).

Control and infested plants were kept in the same greenhouse compartment (23 ± 2 °C, 70% r.h., L16:D8) until leaves and flowers could be harvested and frozen. One and five days (24 h and 120 h) after the caterpillars had hatched from the eggs, leaves and flowers of each plant were harvested, frozen in liquid nitrogen and kept at −80 °C, until they could be freeze dried (model 2040, Snijders Scientific b.v., Tilburg, The Netherlands) for four consecutive days. All herbivores were removed from the plants before freezing the samples. Dried samples were ground and weighed and stored at −20 °C.

Pollination

To investigate whether the flavonoid content of *B. nigra* leaves and flowers change upon pollination, flowers were hand-pollinated five days after the first flower had open, with pollen from a different individual plant (cross-pollination). Plants from the same stage as treated plants that, therefore, were not exposed to pollination, were kept as control plants. Leaves and flowers of control and treated plants were harvested 24 h and 120 h after they had been pollinated. For the 24 h treatment, all open flowers of the plants used for the pollination treatment were marked with a small black thread after being hand-pollinated with a painting brush, so that pollinated flowers could be separated from unpollinated flowers before harvesting. All samples were harvested, frozen, dried and ground as described above. In total, eight pairs of plants of each time point were used for hand-pollination (Table 1).

As a control for the hand-pollination experiment, plants have been also exposed to pollination by male butterflies. To ensure cross-pollination by butterflies, groups of two plants were put into each of the two insect rearing tents (75 cm × 75 cm × 115 cm) inside a greenhouse compartment (23 ± 2 °C, 70% r.h., L16:D8), being one tent for control plants and the other to apply the treatment (Figure 2). Two male butterflies were released into the treatment tent for 6 h (from 9:00 a.m. until 3:00 p.m.). In order to increase frequency of flower visitation and consequently efficiency of pollination, butterflies were starved for 16 h before the experiment. Male butterflies were used for the experiments at 3 to 7 days after eclosion. One and five days after flowers had been exposed to the butterflies, plants were harvested, frozen, dried and ground as described above. In total, 16 plants were used in this experiment (Table 1)

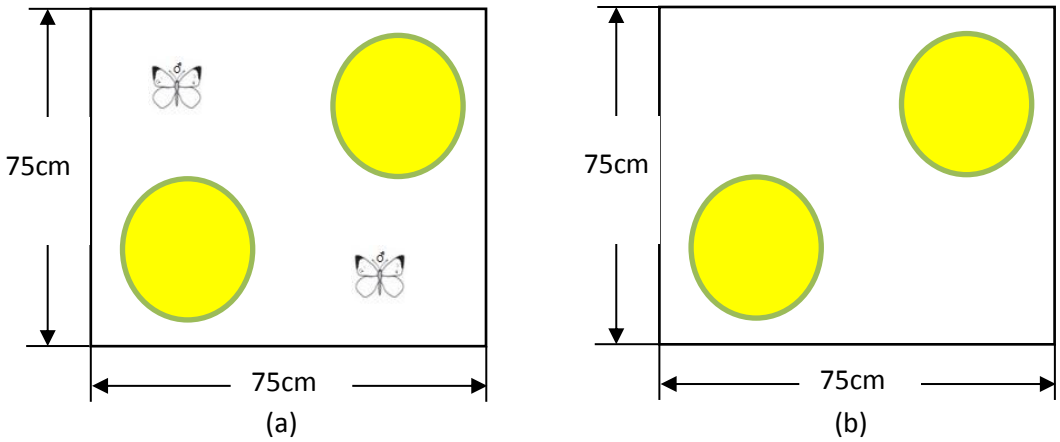

Figure 2. Schematic representation of a tent (a) with two flowering plants and two male *Pieris brassicae* butterflies and (b) with only two flowering plants.

Pollination and herbivore infestation

To investigate what is the net effect of pollination and herbivore infestation on the flavonoid content of *B. nigra* leaves and flowers, plants were infested with one clutch of 30 eggs as described above. On the day of caterpillars hatched, all open flowers of infested plants were hand-pollinated with pollen from a different plant. Plants from the same stage that were exposed to neither infestation nor pollination were used as control plants. For the 24 h treatment, all pollinated flowers were marked with black thread as describe above, and harvested separately from the upollinated flowers. Flowers and leaves of treated and control of plants were harvested frozen, dried and ground one and five days later after pollination, as described above. In total, eight pairs of plants of each time point were used for both herbivore infestation and pollination (Table 1).

Table 1. Overview of plant treatments and number of replicates

Treatments	Time point	Plant part	Number of replicates
Control	24 h	flowers	27
		leaves	27
	120 h	flowers	27
		leaves	27
Herbivore infestation	24 h	flowers	11
		leaves	11
	120 h	flowers	10
		leaves	10
Hand-pollination	24 h	pollinated flowers	7*
		unpollinated flowers	8
		leaves	8
	120 h	flowers	8
		leaves	8
Herbivore infestation and pollination	24 h	pollinated flowers	8
		unpollinated flowers	8
		leaves	8
	120 h	flowers	8
		leaves	8
Control for insect-pollination	24 h	flowers	4
		leaves	4
	120 h	flowers	4
		leaves	4
Insect-pollination	24 h	pollinated flowers	3**
		unpollinated flowers	4
		leaves	4
	120 h	flowers	4
		leaves	4

* One sample of the pollinated flower treatment was excluded because ground material was not enough for extraction.

** Plant materials of two samples of pollinated flower treatment were combined so that enough material could be obtained for extraction.

Flavonoids extraction

The flavonoid content of ground leaves and flowers of *B. nigra* subjected to different treatments were extracted with MeOH:H_2O 8:2. 200 mg of each sample was extracted with 20 mL of methanol (HPLC grade, J.T.Baker®, Avantor Performance Materials, USA) -ultrapure water (Milli-Q, Merck Millipore, Billerica, USA) 8:2 (v/v) by sonication at room temperature for 10 min (Gaspar et al., 2009). The solid-liquid mixture stood for 25 min and the supernatant was filtered into a 20 mL glass vial using a disposable polypropylene syringe (10 mL and 20 mL) through a PTFE syringe filter (13 and 17 mm ϕ 0.45 µm, Grace, Deerfield, USA). Extracts were stored at −20 °C until they could be analysed. An aliquot (1 mL) of filtered solution was used for the UHPLC analysis.

Sample analysis

Separation and quantification

Resulting extracts were analysed using reverse-phase UHPLC Agilent Eclipse XDB-C18 2.1 mm × 150 mm 1.8-micron column equipped with a binary pump, a diode array detector (DAD), an autosampler and a thermostatted column compartment (all Agilent 1290 Infinity LC, Agilent Technologies, Santa Clara, USA). Two solvents were used as mobile phase: (A) pH=3 buffer and (B) acetonitrile (HPLC grade, Sigma-Aldrich, St. Louis, USA). The pH=3 buffer used as solvent A was consisted of formic acid (160 mM, 99% pure, Thermo Fisher Scientific, Geel, Belgium), ammonium formate (40 mM, HPLC grade, Sigma-Aldrich, St. Louis, USA) and EDTA (98%, Sigma-Aldrich, St. Louis, USA) in ultrapure water (0.04 mM) (Villela et al., 2011). To separate the compounds present in the samples, a gradient starting with 7% B, was performed to reach 18% at 10 min, 65% at 13.6 min, be back to 7% at 14 min and maintained isocratic for 6 min. The flow rate was 0.45 mL min^{-1} and the injection volume was 2 µL. The column was kept at a temperature of 50 ˚C. Individual compounds were scanned from 200 to 800 nm, and for quantification of individual flavonoid compounds, chromatograms were recorded at 330 nm.

Identification

In this study, flavonoids were characterized by their absorbance profile in the UV-Vis and some of the compounds were identified by mass spectrometry. Only compounds that had similar UV patterns as that of flavonoids were quantified in this study and regarded as flavonoid compounds, throughout this report. Typically the UV-Vis profile of flavonoids is characterized by two absorptions bands (Figure 3). Band A of flavones (Figure 4 a) lies between 310 and 350 and band B between 250 and 290. For flavonols (Figure 4b), the band A of lies between 350 and 385 and band B between 250 and 290, and band A of flavanones (Figure 4 c) and dihydroflavonols (Figure 4 e) lies between 300–330 nm and band B between 277–295 nm (Iwashina, 2003; Tsimogiannis et al., 2007). Flavanones cannot be discriminated from dihydroflavonols based on the UV pattern; flavanols (Figure 4d) and phenolic absorb in the same region (270–290 nm) and therefore cannot be selective characterized based on UV pattern (Tsimogiannis et al., 2007).

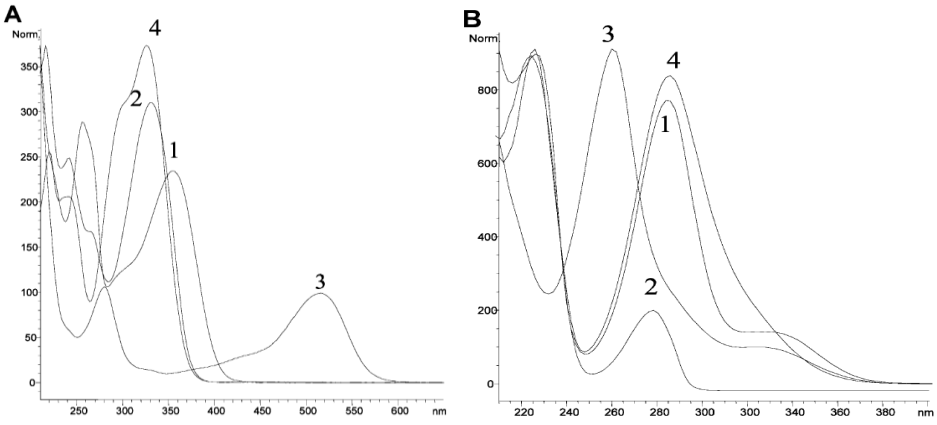

Figure 3. Typical UV-Vis absorbance profiles of: (A) 1, flavonol (quercetin 3-O-galactoside); 2, flavone (sinengetin); 3, anthocyanin (cyaniding-3-O-galactoside); 4, chlorogenic acid. (B) 1, flavanone (hesperidin); 2, flavanol (Epicatechin); 3, isoflavone (genistin); 4, dihydrochalcone (phloridzin) (Lin and Harnly, 2007).

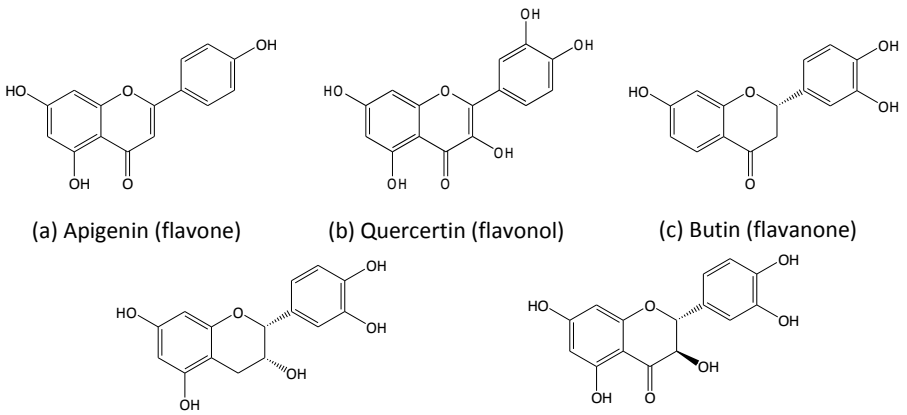

Figure 4. Examples of flavonoid compounds of different subgroups.

Liquid chromatography-mass spectrometer (LC-MS) was used for identification of some of unknown compounds obtained from the sample separation by UHPLC. One sample of each treatment was analysed, and 25 µL of chrysin (0.505 mg/mL) was added to the samples. Separated flavonoid individual was injected to an Exactive mass spectrometer (Exactive MS) (Thermo Fisher Scientific, Waltham, USA), which was equipped with electrospray ionisation (ESI) interface. Mass spectra were recorded in the negative and positive ionisation modes. To obtain optimal conditions for ionisation, for negative mode, the sheath gas flow rates, auxiliary gas flow rate and spray voltage were adjusted to 30 (arbitrary unit), 30 (arbitrary unit) and 5 kV, respectively. Capillary temperature, capillary voltage and tube lens voltage were adjusted to 257 °C, −27.5 V and −130 V, respectively. For positive mode, the sheath gas flow rates, auxiliary gas flow rate and spray voltage were adjusted to 32 (arbitrary unit), 30 (arbitrary unit) and 6 kV, respectively. Capillary temperature, capillary voltage and tube lens voltage were adjusted to 257 °C, 95 V and 120 V, respectively. The full scan mass range was set from m/z 100 to 1000, and at ultra-high resolution (100 k at 1 Hz).

UV-Vis absorbance measurements of flavonoid extracts

Absorbance spectra of the extracts of leaves and flowers of *B. nigra* subjected to different treatments were recorded to correlate changes in absorbance among treatments with changes in colour. Six samples of each treatment were scanned from 200 to 800 nm in 1 minute, using Cary® 100 UV-Vis spectrophotometer (Varian, Agilent Technologies, Santa Clara, USA) and quartz cuvettes of 3 mL. Extracts were sonicated for 2–5 min and diluted with MeOH:H_2O 8:2, in a proportion of 1:15 before measurement.

Statistical analysis

In order to compare if the total flavonoid content present in leaves and flowers of *B. nigra* plants subjected to different treatments differ, Friedman's Two-Way Analysis of Variance by Ranks and Wilcoxon signed rank tests were used. A projection to latent structures discriminant analysis (PLS-DA) was used to determine qualitative and quantitative differences between flavonoid profiles of samples subjected to the different treatments. Non-parametric Mann-Whitney U test and Kruskal-Wallis tests were used to determine quantitative differences in the concentration of individual flavonoid compounds.

Results

Flavonoid profile of *B. nigra* leaves and flowers

The flavonoid profile of the extracts of leaves of *B. nigra* plants is qualitatively and quantitatively different from that of flowers (Figure 5). In this study, 55 compounds in leaf-extracts and 92 compounds in flower-extracts were quantified (see appendix Table A 1 and Table A 2). The maximum absorbance for the leaf-extracts was 0.37 measured at 327 nm, on average, and for the flower-extracts was 0.80 measured at 330 nm (Figure 5).

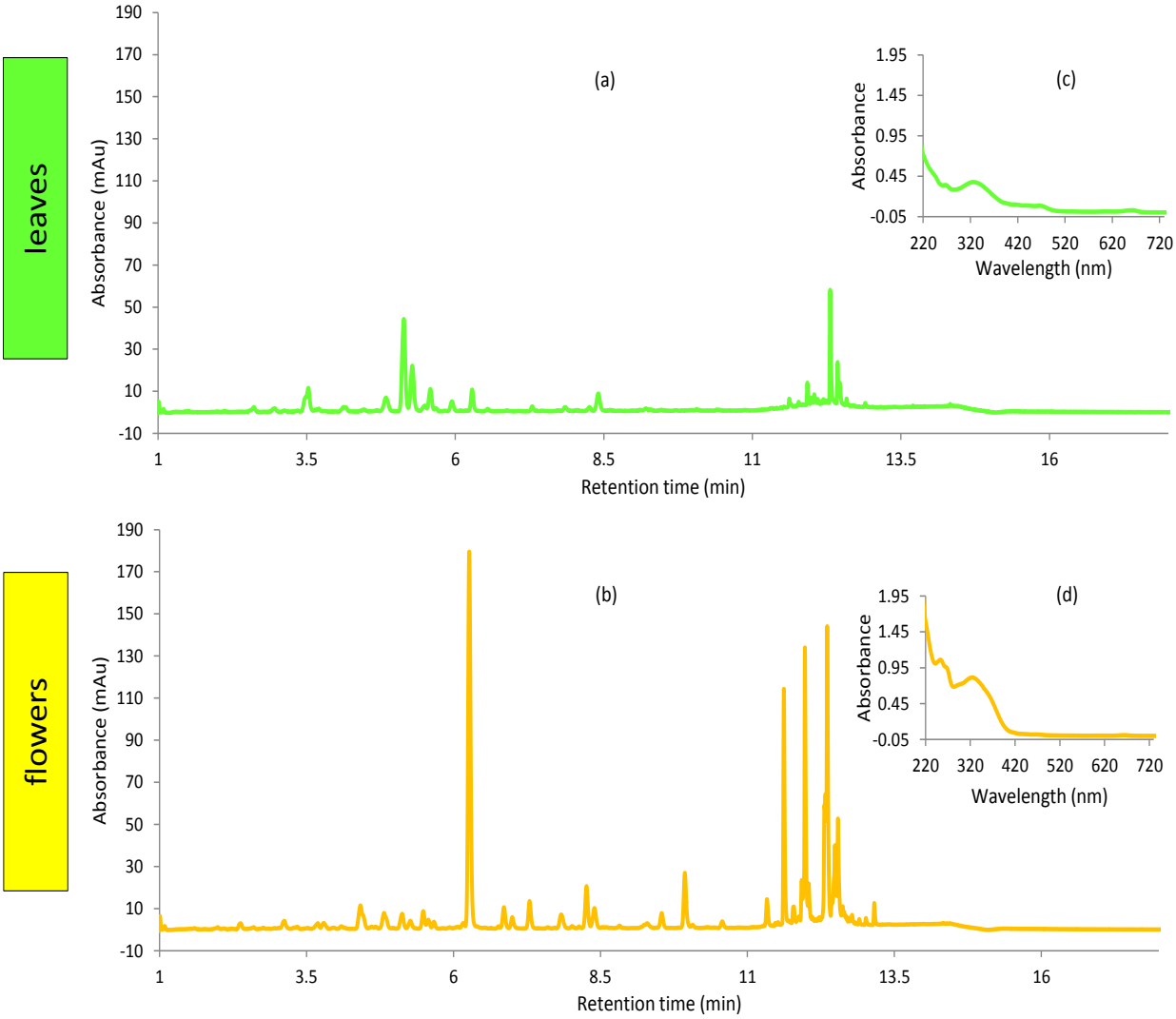

Figure 5. Typical UHPLC flavonoid profiles of the extracts of *Brassica nigra* leaves (a) and flowers (b) recorded at 330 nm. UV-Vis spectra of the extracts of *B. nigra* flowers and leaves are shown from 220 nm to 720 nm. UV-Vis spectra show average profile for 6 samples, in each case.

Effects of herbivore infestation on the flavonoid content of *B. nigra* leaves and flowers

Herbivore infestation changed the flavonoid profiles of leaves and flowers quantitatively and qualitatively. A Projection to Latent Structures Discriminant Analysis (PLS-DA) (SIMCA P+ 12.0, Umetrics AB, Umeå, Sweden) was used to analyse the flavonoid profiles of *B. nigra* leaves and flowers of 24 h and 120 h herbivore infested plants and control plants at the same growth stages (Figure 6). Flavonoid profile of leaves was different from that of flowers, regardless of the herbivore treatment or time point investigated, as could be also observed in their UHPLC profiles (Figure 5). The second principal component separated samples of flowers of infested plants from samples of control flowers, at both time points (Figure 6), and the leaves profile of infested plants also differ from profile of the control leaves, but only after 120 h of exposition to herbivory (Figure 6). The total flavonoid content measured in flowers was greater than in leaves (Figure 7); these differences were quantified at both time points, 24 h (Wilcoxon signed rank text, $P = 0.005$) and 120 h (Wilcoxon signed rank text, $P < 0.001$). Regarding the effects of herbivore infestation, the total flavonoid content was larger in control leaves than in infested leaves after plants were exposed to herbivores for 120 h (Wilcoxon signed rank text, $P = 0.034$).

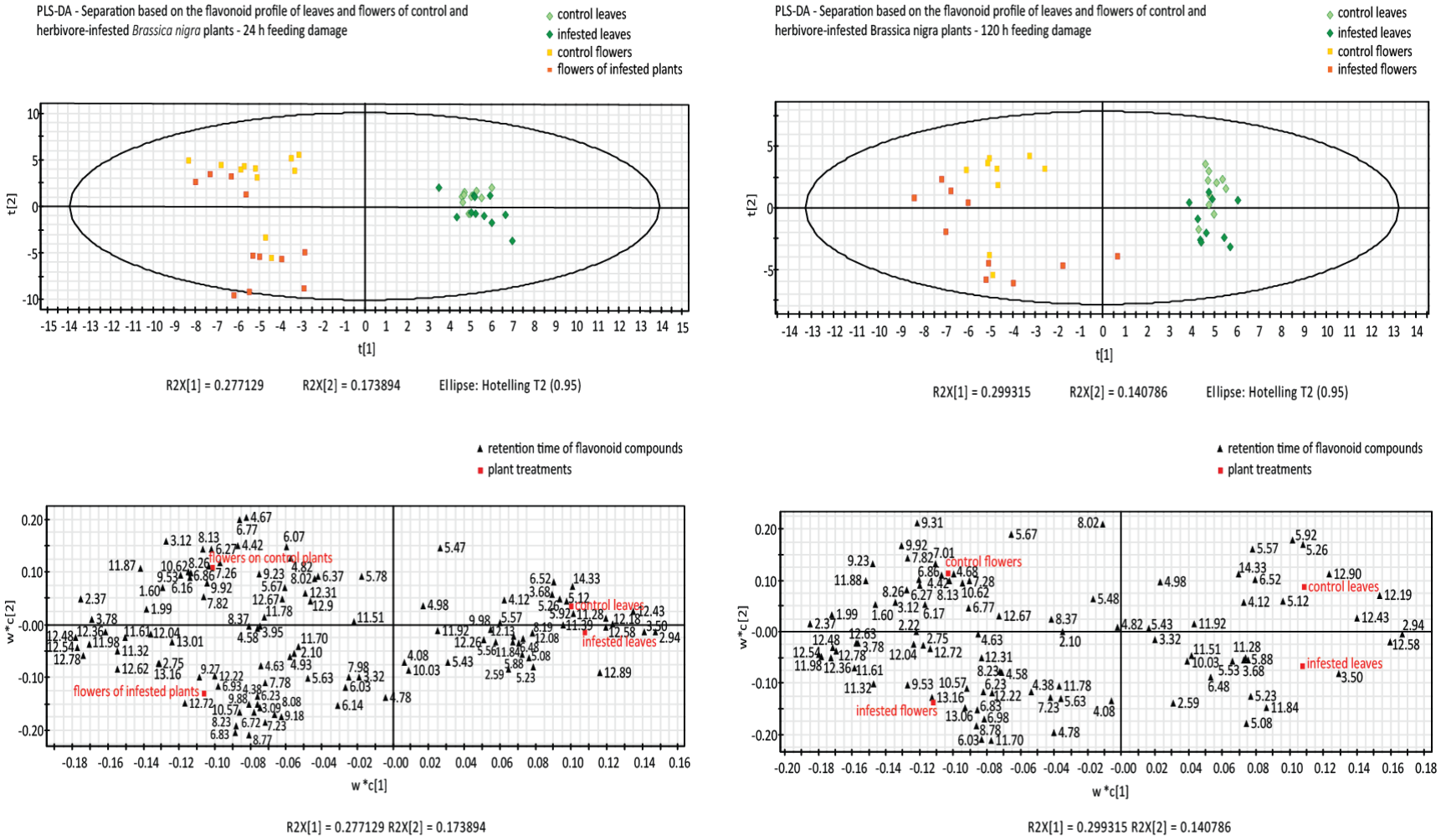

Figure 6. PLS-DA on flavonoid compounds extracted from leaves and flowers of *Pieris brassicae*-infested plants and control *Brassica nigra* plants of the same stage, at two time points: 24 h and 120 h after plant had been treated. Projection to Latent Structure Discriminant Analysis (PLS-DA) on the peak area of flavonoids compounds from leaves and flowers of *B. nigra* plants. (a) Grouping pattern of samples based on the first two principal components, and the Hotelling's T2 ellipse confining the confidence region (95%) of the score plot; (b) Loading plot of the PLS-DA components shows the contribution of individual flavonoid compounds to the first two principal components.

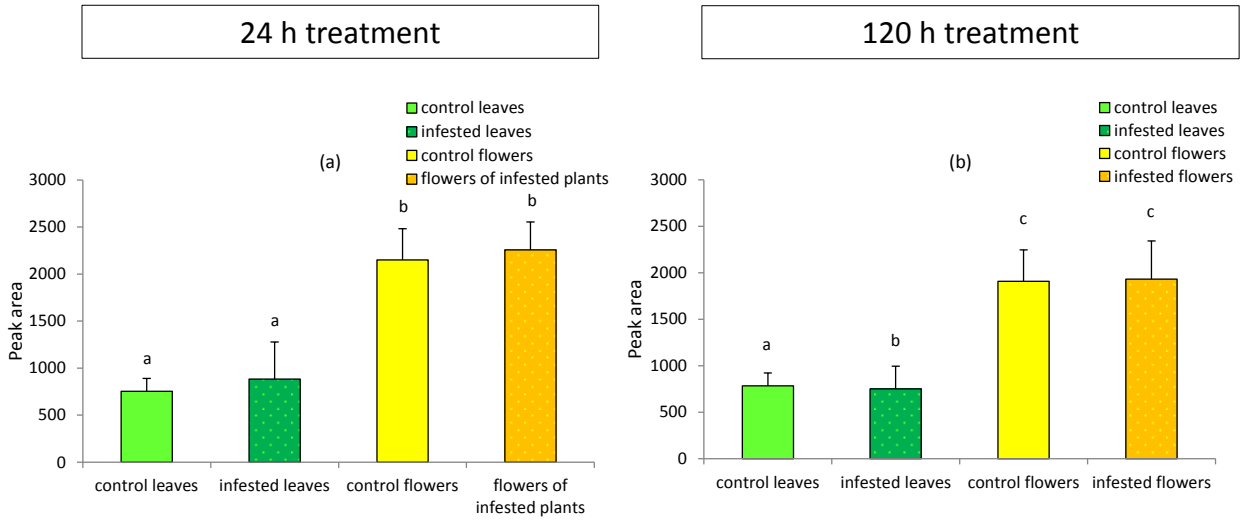

Figure 7. Total content of flavonoid compounds (mean ± SD) in leaves and flowers of *Brassica nigra* infested with *Pieris brassicae* caterpillars and in leaves and flowers of control plants at two time points: (a) 24 h and (b) 120 h after plants had been treated. Peak area measured in UHPLC-DAD at 330 nm. Different letters (a,b,c) indicate significant difference between medians at the 0.05 level (Wilcoxon signed rank text).

Effects of pollination and pollination plus herbivore infestation on flavonoid content of *B. nigra* leaves and flowers

Flavonoid profiles of leaves and flowers of *B. nigra* plants were different upon pollination and also when plants were subjected to pollination and infestation with *P. brassicae* caterpillars (Figure 8). PLS-DA was used to analyse the flavonoid profiles of flowers of plants that have been only pollinated and of flowers that have been pollinated and infested with *P. brassicae* (Figure 9). The first principal component separated pollinated flowers from unpollinated flowers, whereas the second principal component separated the flowers of plants that were infested from the flowers of the plants without infestation.

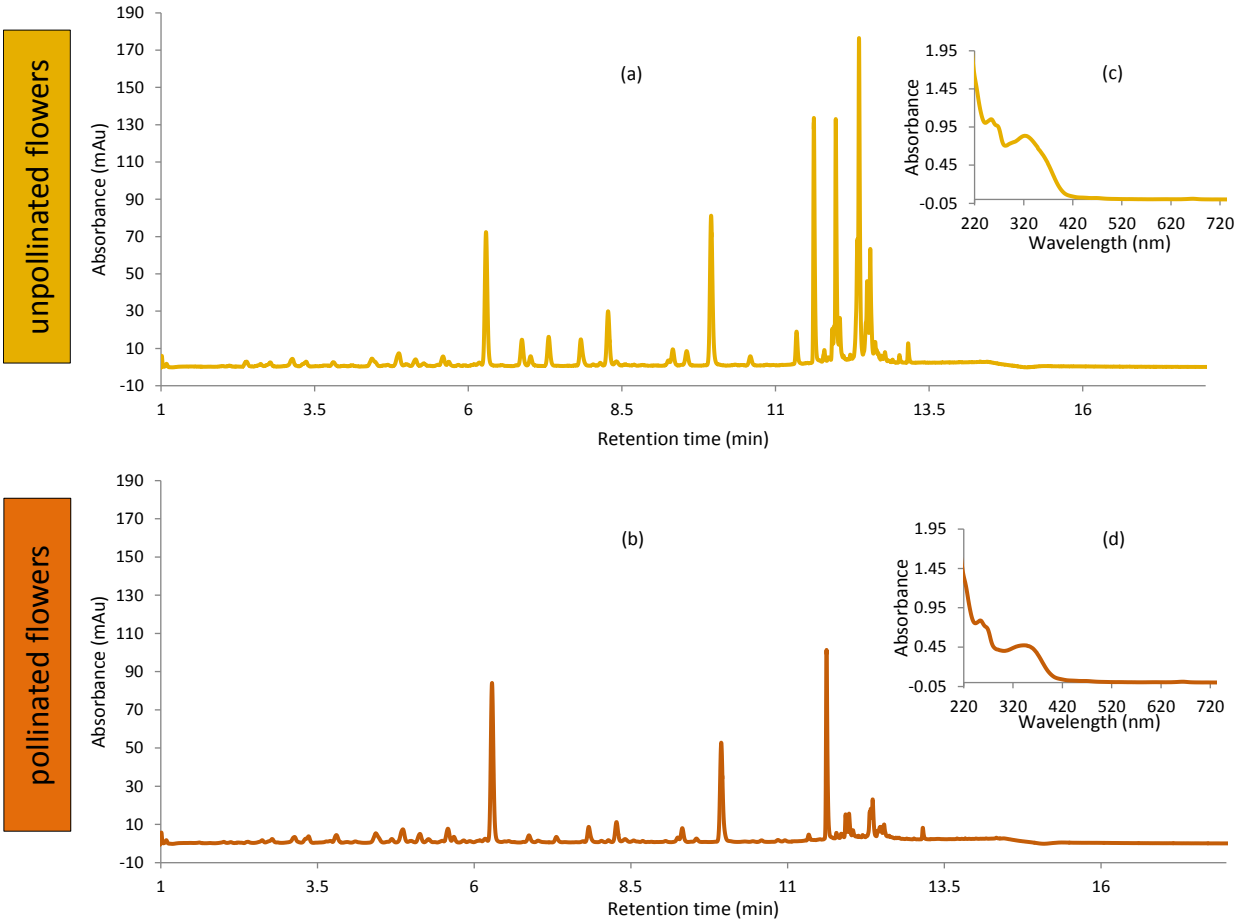

Figure 8. Typical UHPLC flavonoid profiles of the extracts of *Brassica nigra* unpollinated flowers (a) and pollinated flowers (b) recorded at 330 nm. UV-Vis spectra of the extracts of *B. nigra* flowers and leaves are shown from 220 nm to 720 nm. UV-Vis spectra show average profile for 6 samples, in each case.

In terms of total flavonoid contents, the quantification results of flowers extracts of the plants that were subjected to different treatments, at both time points, are shown in Figure 11. When comparing different treatments at 24 h time point, total flavonoid content of unpollinated flowers was larger than pollination flowers, regardless of herbivore infestation (Wilcoxon signed rank test, for non-infested plants $P < 0.001$, for infested plants $P = 0.001$). Herbivore infestation influenced the total flavonoid content of pollinated flowers of non-infested plants when compared with the pollinated flowers of infested plants (Wilcoxon signed rank test, $P = 0.005$). For 120 h treatment, the total flavonoid content of flowers of infested plants was only different from pollinated plants (Wilcoxon signed rank test, $P =$ 0018).

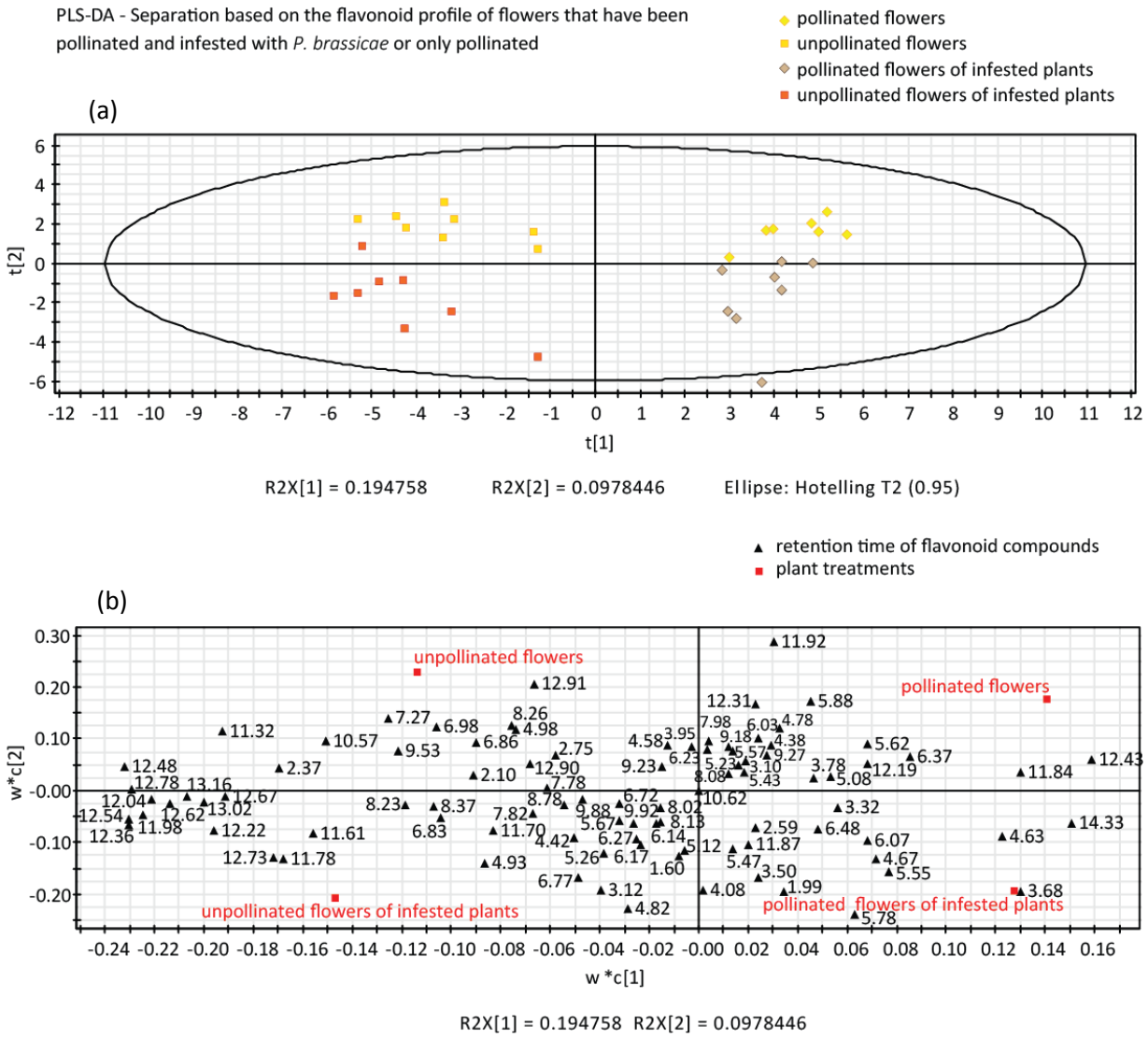

Figure 9. PLS-DA on flavonoids compounds extracted from pollinated flowers and unpollinated flowers of pollinated *Brassica nigra* plants and of plants that were pollinated and infested with *Pieris. brassicae* caterpillars. Projection to Latent Structure Discriminant Analysis (PLS-DA) on the peak area of flavonoids compounds from flowers of *B. nigra* plants. (a) Grouping pattern of samples based on the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot; (b) Loading plot of the PLS-DA components shows the contribution of individual flavonoids compounds to the first two principal components.

Flavonoid compounds quantified in infested leaves, leaves of pollinated plants and leaves of pollinated and infested *B. nigra* plants, from 24 h treatments, were subjected to PLS-DA (Figure 10). The first principal component separated the leaves of pollinated plants from the leaves of non-pollinated plants, while the second principal component separated the leaves of infested plants from the leaves of non-infested plants.

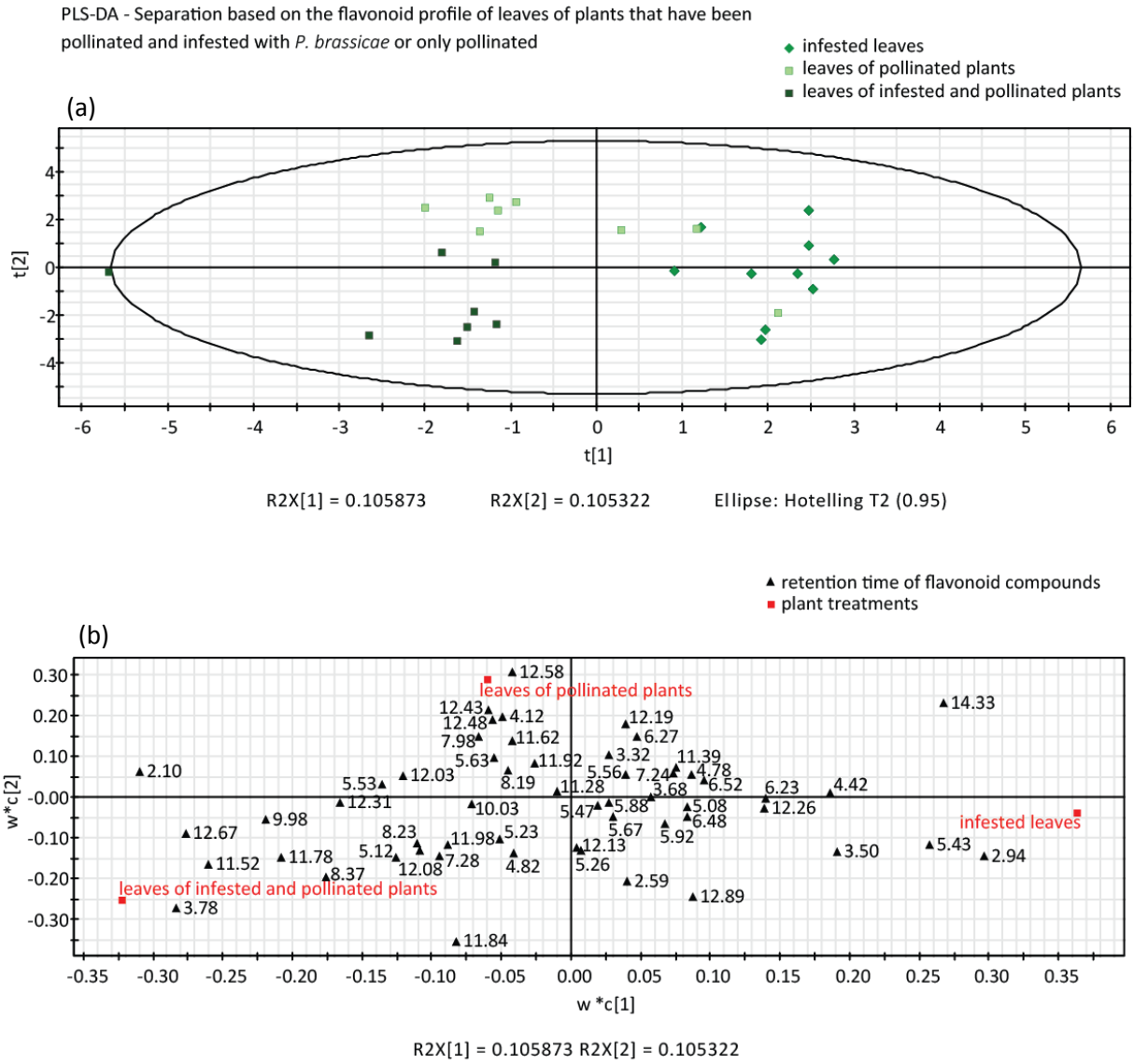

Figure 10. PLS-DA on flavonoid compounds extracted from leaves of 24 h *Pieris brassicae*-infested plants, pollinated plants and of *Brassica nigra* plants that were pollinated and infested with *P. brassicae* caterpillars. Projection to Latent Structure Discriminant Analysis (PLS-DA) on the peak area of flavonoid compounds from leaves of *B. nigra* plants. (a) Grouping pattern of samples based on the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot; (b) Loading plot of the PLS-DA components shows the contribution of individual flavonoids compounds to the first two principal components.

Quantification results, in terms of total flavonoid content of flowers extracts of the plants that were subjected to different treatments, at both time points, are shown in Figure 11. The total flavonoid content of leaves of the plants that had been pollinated was different from leaves of the plants that were subjected to both pollination and infestation from 24 h treatment (Wilcoxon signed rank test, P = 0.049). For 120 h treatment, results showed that the total flavonoid content was larger in leaves of control plants than leaves of plants subjected to herbivore infestation and pollination-only treatments (Wilcoxon signed rank test, P = 0.001).

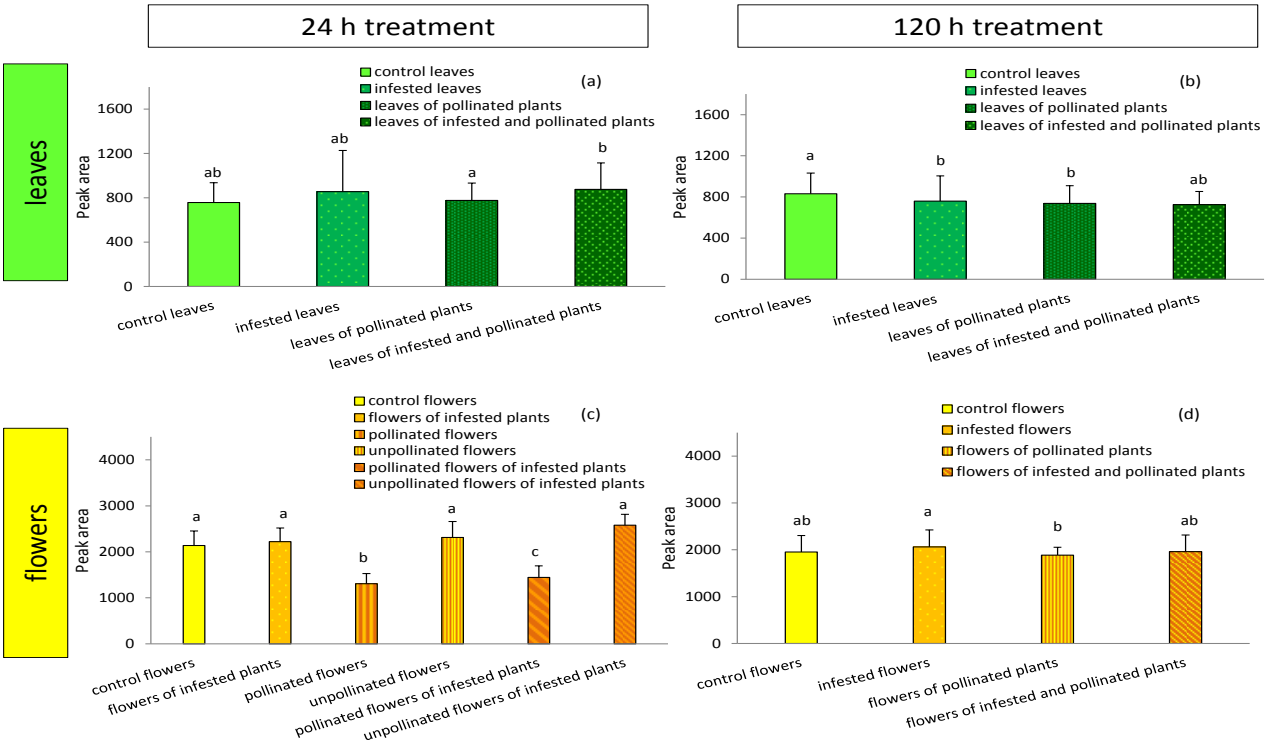

Figure 11. Total amount of flavonoid compounds of extracts of leaves and flowers of *Brassica nigra* plants subjected to different treatments. Total flavonoid content is shown for leaves of control plants, infested plants, pollinated plants and for plants that had been pollinated and infested with *Pieris brassicae* caterpillars at two time points: 24 h (a) and 120 h (b) after plants had been treated. Total flavonoid content is shown for flowers of control plants, infested plants, pollinated plants and for plants that had been pollinated and infested with *P. brassicae* caterpillars 24 h (c) and 120 h (d) after plants had been treated. Different letters (a,b,c) above each column indicate significant difference between medians at the 0.05 level (Wilcoxon signed rank text and Kruskal-Wallis test).

Identification of flavonoid compounds

Brassica nigra plants exposed to herbivory and pollination differed in terms of their flavonoid composition; several individual flavonoid compounds were produced in larger amounts after exposure to one of the treatments (Table A 1 and Table A 2). Data regarding identified flavonoids from leaves and flowers of plants that were subjected to different treatments is shown in the appendix (Table A 1 and Table A 2). Data for identification of individual flavonoid compounds were collected in the negative mode and positive mode (Table 2).

Table 2. Identified flavonoid compounds. Retention time (RT), molecular formula, the exact mass and maximum UV-Vis absorbance are shown for compounds identified in leaves and flowers of *Brassica nigra* plants.

Compounds	RT (min)	Formula	$[M+H]^+/[M-H]^-$	UV-Vis Abs (nm)	Plant part
Quercetin dihexoside	4.08	$C_{27}H_{30}O_{17}$	-/625.1	-	flowers
Kaempferol trihexoside	4.82	$C_{33}H_{40}O_{21}$	-/771.2	-	leaves/flowers
Isorhamnetin dihexoside	6.27	$C_{28}H_{32}O_{17}$	641.2/639.2	226, 255, 354	leaves/flowers
Quercetin dihexoside	6.86	$C_{27}H_{30}O_{17}$	-/625.1	-	flowers
Kaempferol dihexoside	8.26	$C_{27}H_{30}O_{16}$	-/609.1	-	flowers
Isorhamnetin hexoside	11.70	$C_{22}H_{22}O_{12}$	479.1/477.1	226, 255, 355	flowers

UV-Vis absorbance spectra of *B. nigra* leaves and flowers

In order to see the general effects of herbivore infestation and pollination on the flavonoid content of *B. nigra* plants, the UV absorbance spectra of the extracts of leaves and flowers subjected to different treatments were recorded. The changes in UV absorbance spectra can be correlated to changes in colour of leaves and flowers. The UV-Vis profile of flowers that have been subjected to pollination is different from that of control plants and of plants that have been exposed to herbivore infestation only (Figure 12). A shift was observed in terms of wavelength and absorbance. In ultraviolet A (UVA) range (315–400 nm), pollinated flowers of non-infested plants (wavelength/nm: Abs_{max} 343:0.47) and pollinated flowers of infested plants (wavelength/nm: Abs_{max} 336:0.53) had lower and shifted absorption than flowers exposed to the other treatments (about wavelength/nm: Abs_{max} 330:0.82). Leaf-extract of plants that were exposed to both pollination and infestation with *P. brassicae* caterpillars (wavelength/nm: Abs_{max} 326:0.49) had higher absorption than the others (about wavelength/nm: Abs_{max} 327:0.39).

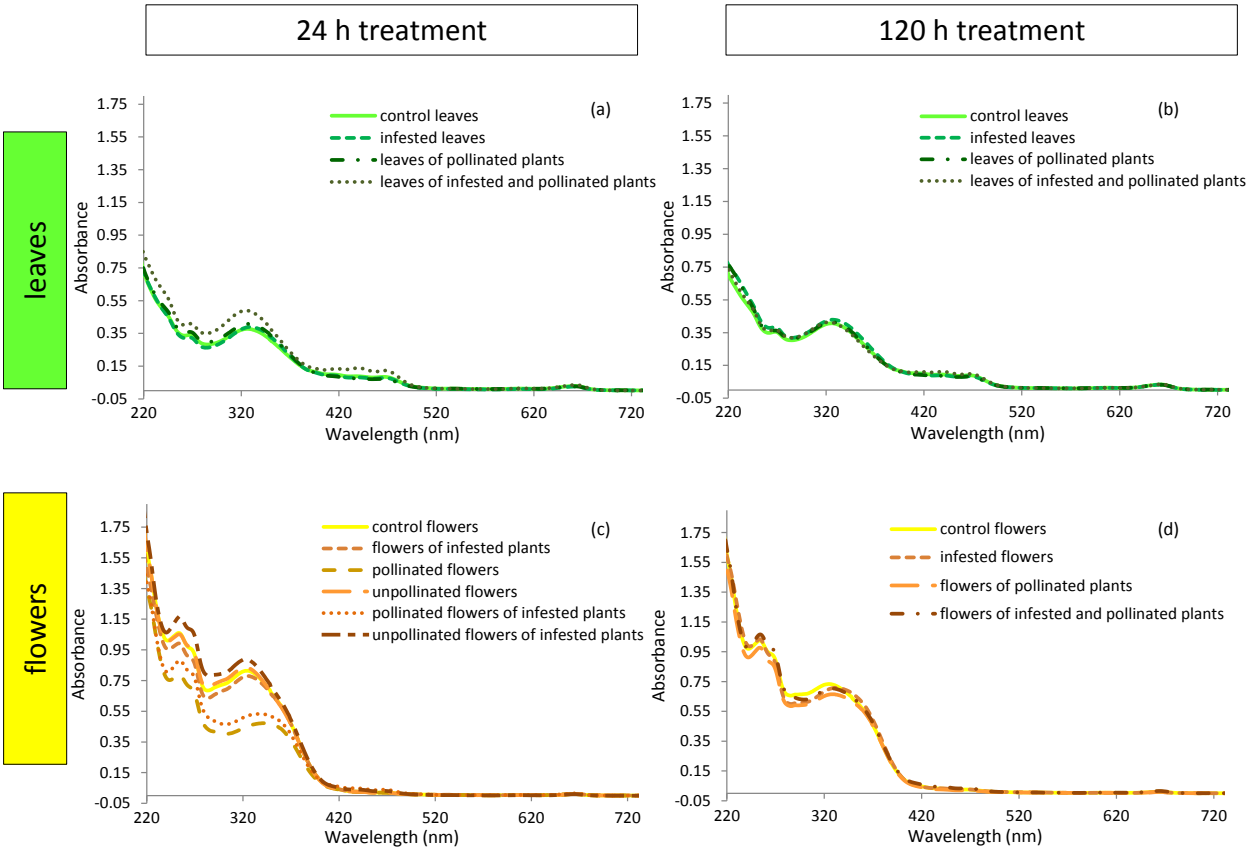

Figure 12. Ultraviolet-visible (UV-Vis) absorbance spectra of the extracts of leaves and flowers of *Brassica nigra* plants subjected to different treatments. UV-Vis spectra are shown for leaves of control plants, *Pieris brassicae*-infested plants, pollinated plants and of plants that were pollinated and infested with *P. brassicae* caterpillars at two time points: 24 h (a) and 120 h (b) after plants had been treated. UV-Vis spectra of flowers of control plants, *P. brassicae*-infested plants, pollinated plants and of plants that were pollinated and infested with *P. brassicae* caterpillars of 24 h (c) and 120 h (d) after plants had been treated. Absorbance is shown from 220 nm to 720 nm.

Effects of hand-pollination and insect-pollination

No difference was observed between hand-pollination and insect-pollination by male butterflies of *P. brassicae*. PLS-DA results show that samples that were subjected to hand-pollination clustered well with samples that were pollinated by the butterflies (Figure 13).

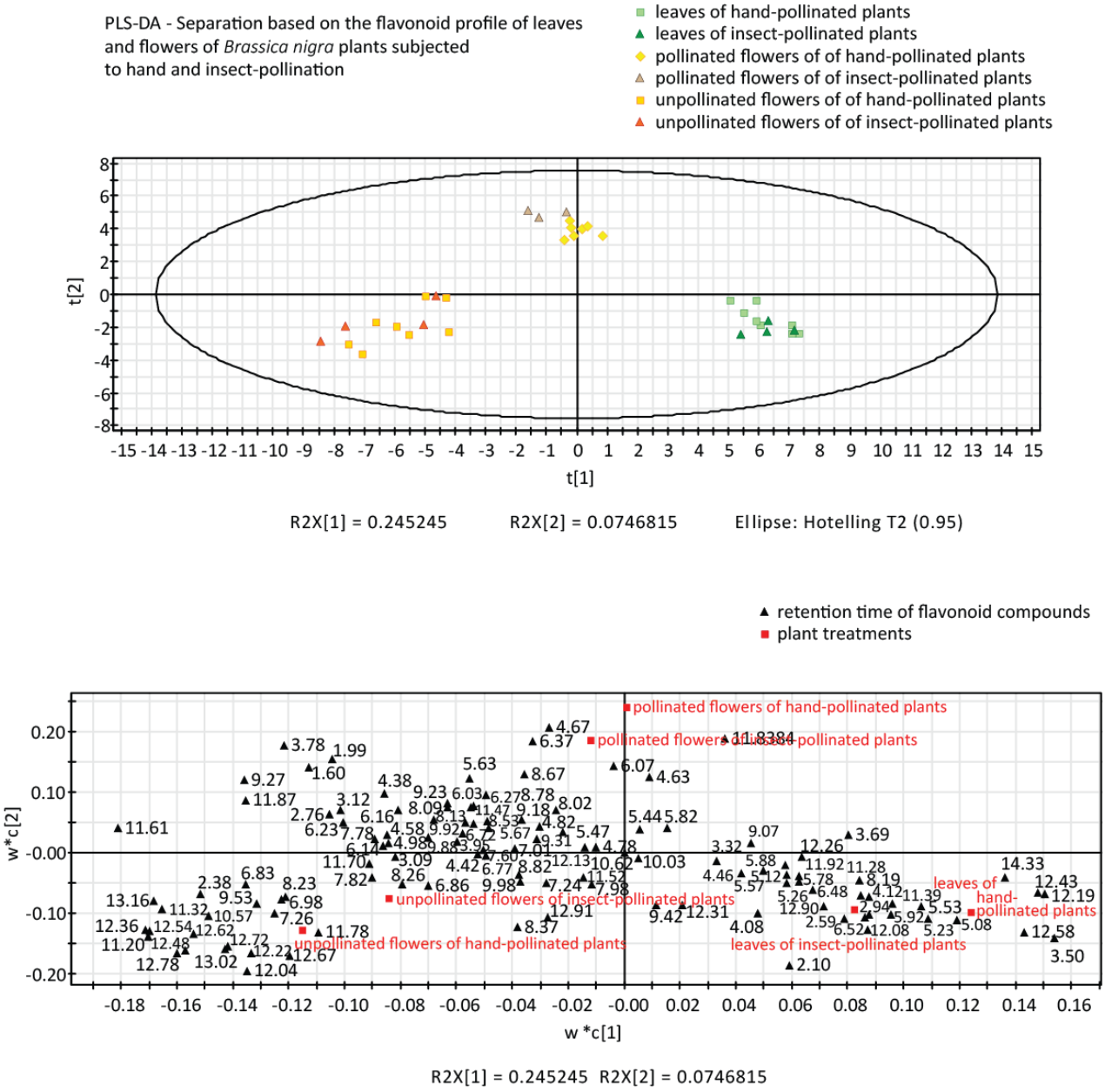

Figure 13. PLS-DA on flavonoids compounds extracted from pollinated, unpollinated flowers and leaves of hand-pollinated *Brassica nigra* plants and of insect-pollinated plants by male butterflies *Pieris brassicae*. Projection to Latent Structure Discriminant Analysis (PLS-DA) on the peak area of flavonoids compounds from leaves and flowers of *B. nigra* plants. (a) Grouping pattern of samples based on the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot; (b) Loading plot of the PLS-DA components shows the contribution of individual flavonoids compounds to the first two principal components.

Discussion

Results of this study show that the flavonoid profile of *B. nigra* leaves is different from that of *B. nigra* flowers. A larger number of flavonoid compounds were quantified in flowers than in leaves of *B. nigra*. The total flavonoid content of flower extracts were about three times larger than the total flavonoids content of leaf-extracts. Remarkably, *B. nigra* plants responded to herbivore infestation as well as to pollination with changes in the flavonoid content of leaves and flowers.

Brassica nigra plants responded to herbivore infestation mainly with changes in flavonoid content of flowers rather than that of leaves. This was observed at the two time points tested. Herbivore infestation had relatively small effect on total flavonoid content of flowers, at both time points tested. This indicates the differences observed in the PLS-DA tests in flowers were caused by the changes in ratios of individual flavonoid compounds. Interestingly, although at 24 h time point tested, *P. brassicae* caterpillars were all in first larvae stage and they feed on leaves, the changes of flavonoid content were observed on flowers rather than on leaves. This phenomenon may be explained by systemic changes on flavonoid content upon herbivore infestation. For instance in *Petunia* flowers, the flavonol compound kaempferol was accumulated continuously in stigma for at least 48 h, which happened 4 h after flowers of plants were subjected to mechanical damage on distal tissues of the plant (Vogt et al., 1994). Perhaps, the reason for plants to respond systemically to herbivores in terms of changes on flavonoid content of flowers is to protect flowers. It is known that many herbivores prefer feeding on the flowers (McCall and Irwin, 2006), including *P. brassicae* caterpillars that were used in this study (Lucas-Barbosa et al., 2013; Smallegange et al., 2007). Because *P. brassicae* caterpillars are specialists of *B. nigra* plants, they are unlikely to be deterred from feeding on the flowers (Lucas-Barbosa et al., 2013; Smallegange et al., 2007), but the responses by *B. nigra* plants with changes in the flavonoid content of flowers might prevent generalist herbivores from feeding from the reproductive tissues of these plants (Erhard et al., 2007; Simmonds, 2001). Actually, induced responses (48 h herbivory) did reduce the preference and performance of many generalist herbivores, but not of the specialist herbivores (Agrawal, 1999). Indeed, plants with higher levels of secondary compounds in flowers are considered to be more resistant to florivores than those with lower levels of chemical defence (McCall and Irwin, 2006). Larger amount of flavonoid in flowers associated with stronger effects due to the herbivore infestation may indicate flowers are better protected from florivory by investing more nutrients from leaves to flowers. The leaves of *B. nigra* plants also responded to the herbivore infestation in terms of changes on the total flavonoid content, as well as in terms of changes in ratios among individual flavonoid compounds. However, it only occurred when plants were exposed to herbivory for a longer period of time (five days after caterpillars have hatched from the eggs). Long-term induced response caused by herbivory may have little effect on the initial attackers themselves, but may suppress the feeding behaviour of subsequent herbivores (Karban and Myers, 1989).

Similarly, *B. nigra* plants responded to pollination mainly with changes on flavonoid content of flowers rather than on that of leaves, both in terms of total flavonoid content and individual flavonoid compounds. Furthermore, the response of plants upon pollination seemed relatively stronger than that upon herbivore infestation, because the difference caused by herbivore infestation was only shown on the individual flavonoid compounds, but pollination caused differences on both individual flavonoid

compounds and the total flavonoid content. The largest differences were observed between pollinated flowers and unpollinated flowers, regardless of herbivore infestation, especially at 24 h time point. The total flavonoid content of flowers dropped when flowers were pollinated. This effect seems occurred locally because the flowers from the same plant individuals that had not been pollinated and they had the similar total flavonoid content, compared to that of control flowers. This sharp decrease on the total flavonoid content influenced the absorbance of the extracts of pollinated flowers. When this is translated in changes in colour of flowers, I expect that pollinator behaviour can be affected, Indeed, insect pollinators can respond to changes in colours (Eisikowitch and Lazar, 1987; Niesenbaum et al., 1999; Weiss, 1991). These cues might be exploited by pollinators to increase their efficiency, guiding them to unpollinated flowers. At 120 h time point, all flowers harvested were unpollinated as pollinated flowers start to form seeds 4–5 days after they have been pollinated. In terms of total flavonoid content, little difference was observed among flowers samples of different treatments at 120 h time point, except the infested flowers and the flowers of pollinated plants. This may indicate herbivore infestation as well as pollination has minor effect on unpollinated flowers since all flower samples in 120 h time point had not been pollinated, although there was different between infested flowers and flowers of pollinated plant. This may imply that herbivore infestation and pollination influence the flavonoid profile of flowers differently. Besides, the flavonoid content of leaves was also changed upon pollination. At 24 h time point, difference was observed in the result of PLS-DA test but not in the comparison of total flavonoid content, which indicated the difference may be caused by the variation of individual flavonoid content upon pollination. At 120 h time point, difference between leaves of control plants and leaves of the plants that had been pollinated was also shown in terms of total flavonoid content. I assumed that the reduction in total flavonoid content of leaves might indicate that resources are being reallocated into reproduction. However, this hypothesis is needed to be proved.

When the plants were subjected to both herbivore infestation and pollination for 24 h time point tested, herbivore infestation seemed to have an effect on pollinated flowers but not on unpollinated flowers; the total flavonoid content of pollinated flowers of infested plants was larger than that of pollinated flowers of non-infested plants. Besides, the increase of total flavonoid content was also observed in leaves when the plants were subject to both pollination and infestation and compared to plants that were only subjected to pollination. Probably, only when plants were subjected to both herbivore infestation and pollination instead of either one or the other, the synthesis of flavonoid would be up-regulated. This regulation was not observed among 120 h treatments perhaps because pollination was a relatively short-term effect on the flavonoid content of leaves and had little effect on unpollinated flowers. However, the underline mechanism of regulation of flavonoid biosynthesis upon pollination plus herbivore infestation is still needed to be investigated.

It seems that there is a synergistic effect between herbivore infestation and pollination. A larger amount of flavonoids were detected in pollinated flowers that were exposed to herbivory when compared to plants that were not exposed to herbivory. Perhaps, plants that were induced by herbivory will start to accumulate flavonoids in pollinated flowers, and later on in seeds. Indeed, *B. nigra* plants can develop seeds faster upon herbivore infestation (Lucas-Barbosa et al., 2013), and it is known that seeds of

Brassicaceous plants, including *B. nigra,* contain flavonoids (Fang et al., 2012; Obi et al., 2009). Probably accumulation of flavonoids in seeds could protect the seeds from predators or pathogens.

The main differences in terms of UV-Vis absorbance were found at 24 h time point, especially among flower samples subjected to different treatments. Lower absorbance and shift in wavelength of the maximum absorbance were observed mainly in pollinated flowers. Flavones and flavonols are important pigments to attract insect pollinators (Iwashina, 2003). Their typical UV absorbance bands were in UVA, which can be detect and distinguish by some insects pollinators, especially bees (Iwashina, 2003; Obi et al., 2009; Tsimogiannis et al., 2007). The shift of the maximum absorption wavelength as well as the decrease of total flavonoid content can result in changes in colour of flowers, which could be used as visual cues for insect pollinators to search for unpollinated flowers rather than re-visit pollinated flowers. However, the increase of flavonoid content due to the infestation was not as pronounced as the decrease of flavonoid content due to the pollination. This may indicate that pollination was the main driving force that caused the change in flavonoid content. Interestingly, the change in UV absorbance was also observed in leaves of the plants that were subjected to both pollination and 24 h herbivore infestation. The maximum UV-Vis absorbance of leaves of such plants was higher than that of infested-only plants and pollinated-only plants. This may also suggest that there is a synergetic effect between herbivore infestation and pollination. This increase in flavonoid content may protect the plants from further feeding damage.

The derivatives of kaempferol, quercetin and isorhamnetin were the main flavonoid compounds identified during this study. It is know that in most of the plants in Brassicaceae family contain mainly the glucosides of three flavonols: kaempferol, quercetin and isorhamnetin (Aguinagalde, 1988; Francisco et al., 2009; Lin et al., 2011; Schmidt et al., 2010). Results of UHPLC separation showed that flavonoid compounds that varied upon herbivore infestation were mostly eluted within 10 min, in where the more polar flavonoid compounds were eluted, such as dihexosides and trihexosides. Flavonoid compounds that varied upon pollination were mostly eluted between 10 and 15 min, which were shown more among flower-extracts rather than leaf-extracts, in where less polar compounds eluted, such as aglycones (quercetin and isorhamnetin). Some flavonols, flavones, flavavones are feeding deterrents to against herbivores, such as isoquercetin in cotton plants that acted as deterrent against pink bollworm (*Pectinophora gossypol*) (Iwashina, 2003; Shaver and Lukefahr, 1969). Flavonoid compounds that varied due to herbivore infestation eluted before 10 min may indicate that the aglycones derivatives, quercetin and isorhamnetin with two or three sugars, might play a role in plant defence against herbivores. Whereas flavonoid compounds that varied due to pollination mainly eluted after 10 min may indicate for instance, aglycones such as flavanones, may be responsible for the change of UV pattern of flowers to act as visual cues for insect pollinators.

In conclusion, herbivore infestation as well as pollination influenced the flavonoid content of leaves and flowers of *B. nigra* plants. It seems that there is synergistic effect between herbivore infestation and pollination. The total flavonoid content of pollinated flowers of infested plants was greater than that of pollinated flowers of non-infested plants. Additionally, when the plants were subject to both pollination and infestation, an increase on total flavonoid content was also observed in leaves when compared to plants that were only subjected to pollination. Herbivore infestation and pollination affected the

flavonoid content of flowers and influenced the UV-Vis absorbance of flower-extracts. Change in UV-Vis absorbance can be correlated with change in colours, which may influence the behaviour of insect pollinators. It is known that the insect pollinators usually associate both visual and odour cues with the quality of nectar and pollen offered by flowers (Harborne and Grayer, 1993; Milet-Pinheiro et al., 2012; Weiss, 1991). Pollinator behaviour can be influenced by the changes in flower traits (Ollerton et al., 2007). Pollinators are able to distinguish pollinated flowers and unpollinated flowers using odours (Rodriguez-Saona et al., 2011) and visual cues (Weiss, 1991). Herbivore infestation can have positive and negative effects on pollinator behaviour (Kessler and Halitschke, 2009). However, whether pollinators of *B. nigra* plants can distinguish between the pollinated and unpollinated flowers, and how synergistic effects of herbivory and pollination affect the behaviour of pollinating insects needs to be investigated. Additionally, whether the discrimination between pollinated and unpollinated flowers could be associated with changes in colours and odours and which group of specific compounds mainly affected by pollination should be further investigated. Synergist effects observed in this study may bring new insights to the increasing number of studies that address a potential trade-off between plant defence and plant reproduction (Dicke and Baldwin, 2010; Lucas-Barbosa et al., 2011).

Acknowledgements

During these last six-months of work on my thesis, I had a very nice time in Organic Chemistry group and Entomology group, with nice people and a variety of cakes during breaks. All kind of nice activities that were organized by party committee in both groups, such as Labuitje, The End of World party, dinner with phytochemistry team, leave me lots of delightful memories. Apart from that, I was so glad that I was able to do research in such a wonderful topic. I felt very comfortable everyday dealing with live creatures and colourful plant extracts, although a bit tired, but full with surprise and happiness. It was especially amazing when I first observed a butterfly successfully eclosing. It felt like watching the start of a new life!

There are many people whom I would like to express my gratitude. First of all, I would like to thank my daily supervisor, Dani Lucas-Barbosa. She helped me really a lot on different aspects, not only on the thesis itself, but also in my daily life. She always listened to me, and was willing to discuss whatever, questions that I had. She was the one that was always able to understand what I wanted to express although, I could not even understand what I was talking about. She was the one with whom I could share my happiness, excitement, confusion and sadness. She was tolerant towards my stubbornness and tried to guide me back to the right way. Dani was the one that always supported me and kept me company whenever I needed to work during the weekends. She also helped me to improve a lot my English and writing skills. She was the one that encouraged me and gave me confidence to express myself to others and, she also made me fall in love with the cute *Pieris brassicae* caterpillars and their mothers, beautiful butterflies, as well as the nice flowering *Brassica nigra*. Second, I would like to thank my other supervisor, Teris van Beek. He always provided me with very useful suggestions. In my opinion, he is a talent man, full of amazing points. He always can come up with inexpectable nice suggestions, capable to calculate numbers by heart, and have comprehensive and super careful mind. Besides, I would also like to thank Alexandre Villela, Elbert van der Klift and Frank Claassen. They offered me very nice tutorials of HPLC, UHPLC and LC-MS as well as lots of help on the machine operation, and nice ideas for experiments. I also would like to thank Barend van Lagen for offering me the UV-Vis spectrophotometer for consecutive 3 days. Furthermore, I would like to thank the insect rearing group in Entomology for providing me fine conditions for my experiments. Finally, I would like to thank all the people in Organic chemistry and Entomology for their company in these last six-months. Especially I would like to thank Yavanna, Twan, Marijn and Martina. Thanks Yavanna for selling drinks with me during the Insect Society lectures and for sharing happiness and problems with me; thanks Twan for the nice statistic syllabus, and help on me with statistics in general; thanks Marijn for your company when having dinner with Teris' family, and for guiding me back to Wageningen; thanks Martina for your company during the practical work at ORC.

References

Agrawal, A. A., 1999. Induced responses to herbivory in wild radish: effects on several herbivores and plant fitness. Ecology 80, 1713-1723.

Aguinagalde, I., 1988. Flavonoids in *Brassica nigra* (L.) Koch, *B. oleracea* L., *B. campestris* L. and their natural amphidiploids. Botanical Magazine 101, 55-60.

Burghardt, F., Fiedlert, K., Proksch, P., 1997. Uptake of flavonoids from *Vicia villosa* (Fabaceae) by the lycaenid butterfly, *Polyommatus icarus* (Lepidoptera: Lycaenidae). Biochemical Systematics and Ecology 25, 527-536.

Conner, J. K., Neumeier, R., 1995. Effects of black mustard population size on the taxonomic composition of pollinators. Oecologia 104, 218-224.

Dicke, M., Baldwin, I. T., 2010. The evolutionary context for herbivore-induced plant volatiles: beyond the cry for help Trends in Plant Science 15, 167-175.

Echeverri, F., Cardona, G., Torres, F., Pelaez, C., Quiñones, W., Renteria, E., 1991. Ermanin: an insect deterrent flavonoid from *Passiflora foetida* resin. Phytochemistry 30, 153-155.

Eisikowitch, D., Lazar, Z., 1987. Flower change in *Oenothera drummondii* Hooker as a response to pollinators' visits. Botanical Journal of the Linnean Society 95, 101-111.

Erhard, D., Pohnert, G., Gross, E., 2007. Chemical defense in *Elodea nuttallii* reduces feeding and growth of Aquatic Herbivorous Lepidoptera. Journal of Chemical Ecology 33, 1646-1661.

Fang, J., Reichelt, M., Hidalgo, W., Agnolet, S., Schneider, B., 2012. Tissue-specific distribution of secondary metabolites in rapeseed (*Brassica napus* L.). PLoS ONE 7, e48006.

Ferreres, F., Fernandes, F., Pereira, D. M., Pereira, J. A., Valentão, P., Andrade, P. B., 2009. Phenolics metabolism in insects: *Pieris brassicae–Brassica oleracea* var. *costata* ecological duo. Journal of Agricultural and Food Chemistry 57, 9035-9043.

Francisco, M., Moreno, D. A., Cartea, M. E., Ferreres, F., Garcia-Viguera, C., Velasco, P., 2009. Simultaneous identification of glucosinolates and phenolic compounds in a representative collection of vegetable *Brassica rapa*. Journal of Chromatography A 1216, 6611-6619.

Gaspar, H., Moiteiro, C., Turkman, A., Coutinho, J., Carnide, V., 2009. Influence of soil fertility on dye flavonoids production in weld (*Reseda luteola* L.) accessions from Portugal. Journal of Separation Science 32, 4234-4240.

Harborne, J. B., Grayer, R. J., 1993. Flavonoids and insects. In: Harborne, J. B. (Ed.), The Flavonoids: advances in research since 1986. Chapman & Hall, London, pp. 589-618.

Haribal, M., Renwick, J. A. A., 1996. Oviposition stimulants for the monarch butterfly: flavonol glycosides from *Asclepias curassavica*. Phytochemistry 41, 139-144.

Harper, F. R., Berkenkamp, B., 1975. Revised growth-stage key for *Brassica campestris* and *B. napus* Canadian Journal of Plant Science 55, 657-658.

Iwashina, T., 2003. Flavonoid function and activity to plants and other organisms. Biological Sciences in Space 17, 24-44.

Karban, R., Myers, J. H., 1989. Induced plant responses to herbivory. Annual Review of Ecology and Systematics 20, 331-348.

Kessler, A., Halitschke, R., 2009. Testing the potential for conflicting selection on floral chemical traits by pollinators and herbivores: predictions and case study. Functional Ecology 23, 901-912.

Lin, L.-Z., Harnly, J. M., 2007. A Screening Method for the Identification of Glycosylated Flavonoids and Other Phenolic Compounds Using a Standard Analytical Approach for All Plant Materials. Journal of Agricultural and Food Chemistry 55, 1084-1096.

Lin, L. Z., Sun, J. H., Chen, P., Harnly, J., 2011. UHPLC-PDA-ESI/HRMS/MSn analysis of anthocyanins, flavonol glycosides, and hydroxycinnamic acid derivatives in red mustard greens (*Brassica juncea* Coss Variety). Journal of Agricultural and Food Chemistry 59, 12059-12072.

Lucas-Barbosa, D., van Loon, J. J. A., Dicke, M., 2011. The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. Phytochemistry 72, 1647-1654.

Lucas-Barbosa, D., van Loon, J. J. A., Gols, R., van Beek, T. A., Dicke, M., 2013. Reproductive escape: annual plant responds to butterfly eggs by accelerating seed production. Functional Ecology 27, 245-254.
Marais, J. P. J., Deacours, B., Dixon, R. A., Ferreira, D., 2006. The stereochemistry of flavonoids. In: Grotewold, E. (Ed.), The science of flavonoids. Springer, Ohio, pp. 1-46.
Mathpal, P., Punetha, H., Tewari, A. K., Agrawal, S., 2011. Biochemical defense mechanism in rapeseed-mustard genotypes against *Alternaria* blight disease. Journal of Oilseed Brassica 2, 87-94.
McCall, A. C., Irwin, R. E., 2006. Florivory: the intersection of pollination and herbivory. Ecology Letters 9, 1351-1365.
Milet-Pinheiro, P., Ayasse, M., Schlindwein, C., Dobson, H. E. M., Dötterl, S., 2012. Host location by visual and olfactory floral cues in an oligolectic bee: innate and learned behavior. Behavioral Ecology 23, 531-538.
Niesenbaum, R. A., Patselas, M. G., Weiner, S. D., 1999. Does flower color change in *Aster vimineus* cue pollinators? American Midland Naturalist 141, 59-68.
Obi, R. K., Nwanebu, F. C., Ndubuisi, U. U., Orji, N. M., 2009. Antibacterial qualities and phytochemical screening of the oils of *Curcubita pepo* and *Brassica nigra*. Journal of Medicinal Plants Research 3, 429-432.
Ollerton, J., Grace, J., Smith, K., 2007. Pollinator behaviour and adaptive floral colour change in *Anthophora alluadii* (Hymenoptera: Apidae) and *Erysimum scoparium* (Brassicaceae) on Tenerife. Entomologia Generalis 29, 253-268.
Rodriguez-Saona, C., Parra, L., Quiroz, A., Isaacs, R., 2011. Variation in highbush blueberry floral volatile profiles as a function of pollination status, cultivar, time of day and flower part: implications for flower visitation by bees. Annals of Botany 107, 1377-1390.
Schmidt, S., Zietz, M., Schreiner, M., Rohn, S., Kroh, L. W., Krumbein, A., 2010. Identification of complex, naturally occurring flavonoid glycosides in kale (*Brassica oleracea* var. *sabellica*) by high-performance liquid chromatography diode-array detection/electrospray ionization multi-stage mass spectrometry. Rapid Communications in Mass Spectrometry 24, 2009-2022.
Shaver, T. N., Lukefahr, M. J., 1969. Effect of flavonoid pigments and gossypol on growth and development of the bollworm, tobacco budworm, and pink bollworm. Journal of Economic Entomology 62, 643-646.
Simmonds, M. S. J., 2001. Importance of flavonoids in insect–plant interactions: feeding and oviposition. Phytochemistry 56, 245-252.
Smallegange, R. C., Loon, J. J. A., Blatt, S. E., Harvey, J. A., Agerbirk, N., Dicke, M., 2007. Flower vs. leaf feeding by *Pieris brassicae*: glucosinolate-rich flower tissues are preferred and sustain higher growth rate. Journal of Chemical Ecology 33, 1831-1844.
Stobiecki, M., Kachlicki, P., 2006. Isolation and identification of flavonoids. In: Grotewold, E. (Ed.), The science of flavonoids. Springer, Ohio, pp. 47-69.
Tabashnik, B. E., 1987. Plant secondary compounds as oviposition deterrents for cabbage butterfly, *Pieris rapae* (Lepidoptera: Pieridae). Journal of Chemical Ecology 13, 309-316.
Treutter, D., 2006. Significance of flavonoids in plant resistance: a review. Environmental Chemistry Letters 4, 147-157.
Tsimogiannis, D., Samiotaki, M., Panayotou, G., Oreopoulou, V., 2007. Characterization of flavonoid subgroups and hydroxy substitution by HPLC-MS/MS. Molecules 12, 593-606.
Villela, A., van der Klift, E. J. C., Mattheussens, E. S. G. M., Derksen, G. C. H., Zuilhof, H., van Beek, T. A., 2011. Fast chromatographic separation for the quantitation of the main flavone dyes in *Reseda luteola* (weld). Journal of Chromatography A 1218, 8544-8550.
Vogt, T., Pollak, P., Tarlyn, N., Taylor, L. P., 1994. Pollination- or wound-induced kaempferol accumulation in petunia stigmas enhances seed production. The Plant Cell Online 6, 11-23.
Weiss, M. R., 1991. Floral colour changes as cues for pollinators. Nature 354, 227-229.

Appendix

Table A 1. Individual flavonoid compounds from leaves of control plants, infested plants, pollinated plants and of *Brassica nigra* plants that were pollinated and infested with *Pieris brassicae* caterpillars.

Compounds	RT (min)	Control leaves (Peak area ± SD)	Infested leaves (Peak area ± SD)	Leaves of pollinated plant (Peak area ± SD)	Leaves of infested and pollinated plant (Peak area ± SD)
Unknown	2.10	1.0 ± 1.1^{ab†}	0.3 ± 0.8^{a}	1.6 ± 1.0^{b}	1.8 ± 1.1^{b}
Unknown	2.59	7.3 ± 4.3	9.4 ± 5.7	6.8 ± 2.1	9.3 ± 5.5
Unknown	2.94	7.8 ± 5.8^{ab}	11.9 ± 5.8^{a}	5.0 ± 6.4^{b}	5.8 ± 2.5^{b}
Unknown	3.32	3.5 ± 4.0	4.1 ± 8.2	5.7 ± 5.7	3.4 ± 3.8
Unknown	3.50	52.1 ± 33.7^{a}	83.6 ± 54.1^{b}	48.0 ± 12.3^{ab}	55.0 ± 39.7^{ab}
Unknown	3.68	18.6 ± 12.0	19.6 ± 17.1	16.0 ± 12.7	15.0 ± 12.3
Unknown	3.78	0.7 ± 1.5	0.0 ± 0.0	0.3 ± 0.9	1.8 ± 1.9
Unknown	4.12	16.5 ± 26.1^{a}	3.3 ± 8.0^{b}	11.0 ± 16.3^{ab}	4.6 ± 6.5^{ab}
Unknown	4.42	3.4 ± 6.8^{a}	14.6 ± 17.6^{b}	7.8 ± 6.9^{ab}	5.3 ± 5.2^{ab}
Unknown	4.78	4.3 ± 9.6^{a}	22.0 ± 22.9^{b}	21.0 ± 15.0^{b}	17.0 ± 19.8^{ab}
Kaempferol trihexoside	4.82	20.2 ± 15.2^{a}	7.2 ± 13.5^{b}	3.8 ± 10.7^{b}	10.0 ± 16.6^{ab}
Unknown	5.08	17.4 ± 41.1^{a}	117 ± 117.7^{b}	98.0 ± 84.9^{b}	96.0 ± 105.9^{ab}
Unknown	5.12	118 ± 86.2^{a}	41.9 ± 75.7^{b}	41.0 ± 59.6^{b}	83.0 ± 119.2^{ab}
Unknown	5.23	26.6 ± 51.2	64.8 ± 52.0	66.0 ± 72.1	89.0 ± 83.3
Unknown	5.26	74.2 ± 65.9^{a}	42.6 ± 63.0^{ab}	20.0 ± 29.6^{b}	40.0 ± 66.5^{ab}
Unknown	5.43	3.7 ± 14.7^{a}	13.5 ± 13.4^{b}	3.9 ± 5.9^{ab}	4.8 ± 4.8^{ab}
Unknown	5.47	9.9 ± 9.5^{a}	4.0 ± 9.1^{b}	3.2 ± 6.8^{b}	3.4 ± 5.0^{ab}
Unknown	5.53	21.8 ± 58.9	29.0 ± 37.6	62.0 ± 69.3	81.0 ± 138.3
Unknown	5.57	40.4 ± 48.2	29.6 ± 61.2	25.0 ± 42.4	15.0 ± 23.6
Unknown	5.63	2.1 ± 5.1	3.5 ± 6.7	6.0 ± 6.6	5.3 ± 8.8
Unknown	5.67	4.1 ± 6.5	3.1 ± 6.3	1.9 ± 2.6	2.4 ± 4.5
Unknown	5.88	2.3 ± 5.6^{a}	11.6 ± 10.0^{b}	12.0 ± 12.9^{b}	12.0 ± 11.0^{b}
Unknown	5.92	16.1 ± 11.5^{a}	9.4 ± 13.8^{ab}	4.4 ± 6.5^{b}	5.8 ± 12.4^{b}
Unknown	6.23	11.9 ± 32.1^{a}	73.8 ± 114.9^{b}	39.0 ± 33^{ab}	37.0 ± 53.7^{ab}
Isorhamnetin dihexoside	6.27	43.1 ± 39.3^{a}	25.5 ± 39.8^{ab}	31.0 ± 55.6^{ab}	11.0 ± 17.9^{b}
Unknown	6.48	0.8 ± 2.0^{a}	3.3 ± 2.9^{b}	2.6 ± 2.5^{ab}	2.8 ± 2.6^{ab}
Unknown	6.52	3.6 ± 2.2^{a}	2.3 ± 3.3^{ab}	1.5 ± 2.3^{b}	0.9 ± 1.8^{b}
Unknown	7.24	1.0 ± 2.2^{a}	4.6 ± 4.4^{b}	4.8 ± 4.1^{b}	3.8 ± 4.2^{ab}
Unknown	7.28	6.4 ± 4.2^{a}	2.2 ± 3.5^{b}	1.7 ± 3.1^{b}	3.7 ± 5.8^{ab}
Unknown	7.98	0.4 ± 1.2	0.9 ± 1.3	1.8 ± 2.2	1.4 ± 2.4
Unknown	8.19	1.5 ± 4.3	3.1 ± 3.8	4.8 ± 5.5	4.2 ± 6.1
Unknown	8.23	4.5 ± 3.2^{a}	1.8 ± 4.6^{b}	1.2 ± 2.4^{b}	4.4 ± 8.2^{ab}

Compounds	RT (min)	Control leaves	Infested leaves	Leaves of pollinated plant	Leaves of infested and pollinated plant
		(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)
Unknown	8.37	13.5 ± 15.8	10.6 ± 11.7	13.0 ± 14.0	26.0 ± 24.4
Unknown	9.98	0.6 ± 1.3[a]	0.8 ± 1.9[ab]	2.0 ± 2.4[ab]	3.3 ± 3.2[b]
Unknown	10.03	2.1 ± 2.3	0.5 ± 1.3	0.7 ± 1.4	0.9 ± 2.5
Unknown	11.28	1.9 ± 2.0	2.1 ± 2.3	1.9 ± 1.7	2.3 ± 4.7
Unknown	11.39	0.9 ± 1.5	1.3 ± 1.6	0.9 ± 1.0	0.7 ± 2.1
Unknown	11.51	1.1 ± 1.6	0.3 ± 1.0	0.8 ± 1.2	2.1 ± 1.7
Unknown	11.61	3.7 ± 2.2	4.5 ± 3.6	6.6 ± 4.3	5.4 ± 7.5
Unknown	11.78	6.5 ± 3.0	5.8 ± 3.1	6.4 ± 1.9	9.0 ± 4.8
Unknown	11.84	1.4 ± 2.1[a]	2.0 ± 3.5[ab]	0.5 ± 1.0[a]	3.7 ± 1.8[b]
Unknown	11.92	23.9 ± 14.3	24.6 ± 25.0	28 ± 14.3	26.0 ± 33.5
Unknown	11.98	4.5 ± 2.5	4.6 ± 3.8	3.8 ± 2.5	6.4 ± 5.8
Unknown	12.04	8.9 ± 6.3	9.6 ± 6.3	12.0 ± 5.3	13.0 ± 8.9
Unknown	12.08	2.7 ± 4.2	1.4 ± 1.9	1.3 ± 1.6	2.7 ± 4.1
Unknown	12.13	0.3 ± 0.9	0.9 ± 1.3	0.5 ± 1.0	1.1 ± 1.2
Unknown	12.18	3.6 ± 2.0	4.0 ± 4.1	4.7 ± 2.4	3.2 ± 2.7
Unknown	12.26	2.6 ± 7.1	4.6 ± 8.5	2.0 ± 2.3	1.9 ± 2.4
Unknown	12.31	69.3 ± 41.9	56.3 ± 38.6	69.0 ± 43.2	85.0 ± 52.2
Unknown	12.43	21.1 ± 11.1	17.0 ± 10.9	23.0 ± 14.5	17.0 ± 6.8
Unknown	12.48	13.0 ± 6.6	10.8 ± 6.5	14.0 ± 8.3	11.0 ± 4.8
Unknown	12.58	3.4 ± 2.6	2.4 ± 2.2	4.0 ± 2.4	2.1 ± 1.4
Unknown	12.67	0.6 ± 1.0	0.0 ± 0.0	0.5 ± 0.9	1.1 ± 1.2
Unknown	12.89	6.7 ± 3.8[a]	7.5 ± 3.6[a]	3.8 ± 5.1[b]	6.8 ± 4.5[ab]
Unknown	14.33	21.0 ± 16.2[a]	21.1 ± 9.6[a]	20 ± 10.9[a]	9.5 ± 10.9[b]
Total flavonoids		**758.2 ± 177.9[ab]**	**856.3 ± 371.4[ab]**	**777.1 ± 156.0[a]**	**876.9 ± 238.3[b]**

† Different superscripts ([a,b]) indicate significant difference between means at the 0.05 level (Wilcoxon signed rank test and Kruskal-Wallis test).

Table A 2. Individual flavonoid compounds from flowers of control plants, infested plants, pollinated plants and of *Brassica nigra* plants that were pollinated and infested with *Pieris brassicae* caterpillars.

Compounds	RT (min)	Control flowers	Flowers of infested plants	Pollinated flowers	Unpollinated flowers	Pollinated flowers of infested plants	Unpollinated flowers of infested plants
		(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)
Unknown	1.60	2.1 ± 1.6	1.6 ± 1.6	2.1 ± 1.5	2.0 ± 1.9	2.8 ± 2.7	3.0 ± 1.9
Unknown	1.99	4.1 ± 2.5	3.9 ± 3.3	3.5 ± 1.5	2.5 ± 2.7	5.9 ± 4.5	4.7 ± 3.7
Unknown	2.10	1.0 ± 1.5	1.4 ± 1.5	0.0 ± 0.0	0.9 ± 1.0	0.2 ± 0.6	0.7 ± 1.3
Unknown	2.37	10.9 ± 3.5^{a†}	9.9 ± 3.3^{a}	5.0 ± 2.8^{b}	16.8 ± 9.9^{ac}	6.3 ± 2.0^{b}	15.3 ± 4.4^{c}
Unknown	2.59	3.9 ± 3.3^{a}	6.5 ± 3.7^{b}	3.4 ± 1.2^{a}	3.6 ± 1.2^{a}	4.6 ± 3.3^{ab}	3.6 ± 2.5^{ab}
Unknown	2.75	4.6 ± 4.1^{ab}	7.0 ± 4.6^{b}	2.9 ± 3.5^{a}	4.2 ± 4.0^{ab}	2.3 ± 2.2^{a}	3.7 ± 2.2^{a}
Unknown	3.09	2.5 ± 6.4	8.1 ± 8.4	6.2 ± 7.0	8.9 ± 10.3	8.3 ± 9.8	3.7 ± 6.8
Unknown	3.12	15.1 ± 7.9^{a}	7.2 ± 8.9^{b}	5.4 ± 7.0^{b}	7.6 ± 8.4^{b}	12.9 ± 15.9^{ab}	17.0 ± 12.5^{ab}
Unknown	3.32	5.5 ± 5.2	6.1 ± 6.4	4.5 ± 4.6	3.8 ± 3.8	6.2 ± 5.9	3.1 ± 2.7
Unknown	3.50	0.8 ± 1.6	2.1 ± 2.1	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 1.9	0.4 ± 1.3
Unknown	3.68	10.2 ± 4.5^{a}	7.5 ± 5.1^{ab}	12.8 ± 5.4^{ac}	4.3 ± 4.0^{b}	17.1 ± 8.9^{c}	9.0 ± 5.7^{ab}
Unknown	3.78	12.3 ± 4.7^{a}	10.9 ± 4.0^{a}	16.4 ± 3.6^{b}	12.1 ± 3.4^{ab}	13.0 ± 7.0^{ab}	13.8 ± 3.4^{ab}
Unknown	3.95	2.2 ± 4.5	2.4 ± 2.8	1.7 ± 2.8	1.8 ± 2.1	1.1 ± 2.0	1.2 ± 2.3
Quercetin dihexoside	4.08	5.8 ± 3.4^{ab}	7.8 ± 3.7^{a}	4.0 ± 3.1^{b}	5.0 ± 2.8^{ab}	7.9 ± 5.7^{ab}	6.6 ± 2.2^{ab}
Unknown	4.38	15.8 ± 27.3^{a}	37.0 ± 33.1^{ab}	48.8 ± 27.4^{b}	32.7 ± 30.8^{ab}	28.9 ± 24.4^{ab}	32.1 ± 28.7^{ab}
Unknown	4.42	41.1 ± 30.3^{a}	20.4 ± 28.4^{ab}	3.8 ± 10.1^{b}	24.0 ± 38.8^{ab}	23.6 ± 33.7^{ab}	29.8 ± 42.1^{ab}
Unknown	4.58	3.0 ± 5.0	2.2 ± 2.9	3.6 ± 3.7	3.5 ± 2.9	2.1 ± 3.3	2.9 ± 4.4
Unknown	4.63	1.7 ± 2.2^{acd}	4.1 ± 4.8^{bc}	4.5 ± 3.3^{be}	1.0 ± 1.1^{def}	4.9 ± 4.3^{abf}	1.9 ± 1.8^{abf}
Unknown	4.67	3.1 ± 2.3^{a}	0.7 ± 1.3^{b}	1.7 ± 3.1^{ab}	0.4 ± 1.0^{b}	3.7 ± 5.3^{ab}	1.2 ± 1.6^{ab}
Unknown	4.78	9.3 ± 16.2^{a}	22.4 ± 28.5^{ab}	29.1 ± 20.4^{b}	23.4 ± 19.7^{ab}	18.8 ± 20.9^{ab}	15.0 ± 20.9^{ab}
Kaempferol trihexoside	4.82	30.6 ± 21.9^{a}	20.2 ± 23.5^{ab}	6.2 ± 16.4^{b}	6.5 ± 12.1^{b}	19.2 ± 22.0^{ab}	25.6 ± 22.4^{ab}
Unknown	4.93	0.7 ± 2.1	2.1 ± 3.5	0.0 ± 0.0	0.9 ± 1.3	0.8 ± 1.5	1.9 ± 2.0
Unknown	4.98	3.1 ± 3.8^{a}	1.4 ± 2.0^{ab}	0.5 ± 1.2^{ab}	1.3 ± 1.9^{ab}	0.0 ± 0.0^{b}	0.8 ± 1.5^{ab}
Unknown	5.08	2.0 ± 4.5^{a}	14.4 ± 13.5^{b}	16.5 ± 13.5^{b}	9.9 ± 9.1^{b}	13.3 ± 16.8^{ab}	9.2 ± 11.3^{ab}

Compounds	RT (min)	Control flowers	Flowers of infested plants	Pollinated flowers	Unpollinated flowers	Pollinated flowers of infested plants	Unpollinated flowers of infested plants
		(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)
Unknown	5.12	13.0 ± 10.3^{a}	7.0 ± 12.9^{ab}	3.8 ± 6.7^{b}	4.3 ± 6.0^{b}	7.8 ± 11.0^{ab}	7.7 ± 11.0^{ab}
Unknown	5.23	4.7 ± 6.5	13.4 ± 13.2	11.7 ± 10.0	9.6 ± 9.6	8.9 ± 9.3	8.8 ± 8.8
Unknown	5.26	9.0 ± 10.2^{a}	6.0 ± 8.4^{ab}	0.9 ± 2.3^{b}	3.7 ± 6.0^{ab}	4.9 ± 7.8^{ab}	5.8 ± 8.3^{ab}
Unknown	5.43	1.9 ± 4.9^{a}	8.7 ± 8.2^{b}	7.5 ± 7.7^{b}	5.2 ± 6.3^{ab}	5.0 ± 6.5^{ab}	5.3 ± 8.2^{ab}
Unknown	5.47	9.1 ± 9.6^{a}	1.8 ± 3.7^{b}	1.6 ± 3.1^{b}	1.4 ± 2.9^{b}	4.3 ± 8.4^{ab}	3.1 ± 6.0^{ab}
Unknown	5.53	2.5 ± 6.2^{a}	8.3 ± 7.5^{b}	14.9 ± 9.6^{b}	7.3 ± 6.0^{b}	21.5 ± 16.7^{b}	13.0 ± 12.7^{b}
Unknown	5.57	9.7 ± 9.6^{a}	8.2 ± 13.0^{ab}	3.7 ± 9.8^{b}	4.5 ± 7.2^{ab}	3.4 ± 5.5^{ab}	1.6 ± 3.2^{b}
Unknown	5.63	5.5 ± 11.4^{a}	15.3 ± 22.6^{ab}	26.1 ± 13.6^{b}	14.0 ± 12.6^{ab}	15.9 ± 18.6^{ab}	11.9 ± 13.9^{ab}
Unknown	5.67	15.4 ± 34.6^{a}	6.7 ± 10.1^{ab}	1.3 ± 3.4^{b}	6.5 ± 10.8^{ab}	6.6 ± 10.0^{ab}	5.9 ± 9.0^{ab}
Unknown	5.78	4.2 ± 20.5^{a}	0.8 ± 1.7^{ab}	0.6 ± 1.0^{ab}	0.2 ± 0.5^{a}	2.3 ± 1.9^{b}	1.0 ± 1.5^{ab}
Unknown	5.88	1.0 ± 2.0	1.8 ± 2.3	2.6 ± 3.8	2.2 ± 3.2	1.4 ± 2.3	0.0 ± 0.0
Unknown	6.03	0.4 ± 1.6^{a}	1.1 ± 2.0^{ab}	5.3 ± 6.1^{b}	3.7 ± 5.8^{ab}	2.6 ± 4.3^{ab}	2.6 ± 2.8^{b}
Unknown	6.07	1.7 ± 1.6^{a}	0.4 ± 1.0^{b}	0.6 ± 1.7^{ab}	0.0 ± 0.0^{b}	1.0 ± 1.4^{ab}	0.3 ± 0.9^{b}
Unknown	6.14	0.7 ± 1.9^{a}	1.5 ± 2.7^{ac}	3.8 ± 5.6^{abc}	5.5 ± 3.9^{bd}	6.2 ± 8.3^{acd}	6.1 ± 5.4^{cd}
Unknown	6.16	6.8 ± 3.9^{a}	3.6 ± 5.2^{b}	1.7 ± 3.0^{b}	2.5 ± 3.6^{b}	3.5 ± 3.9^{ab}	4.2 ± 6.4^{ab}
Unknown	6.23	138.9 ± 253.7	348.6 ± 299.0	348.9 ± 259.0	306.7 ± 279.8	235.9 ± 211.4	270.8 ± 251.4
Isorhamnetin dihexoside	6.27	328.3 ± 259.8^{a}	184.4 ± 268.2^{ab}	104.2 ± 189.6^{b}	174.5 ± 275.0^{ab}	225.4 ± 330.2^{ab}	261.3 ± 368.3^{ab}
Unknown	6.37	7.7 ± 22.6	1.3 ± 3.2	4.7 ± 4.9	1.4 ± 2.0	2.7 ± 4.2	1.1 ± 2.2
Unknown	6.48	0.3 ± 0.9	0.6 ± 1.5	0.6 ± 1.0	0.7 ± 1.0	1.5 ± 1.8	0.5 ± 0.9
Unknown	6.72	0.3 ± 0.8^{a}	1.8 ± 2.3^{b}	1.2 ± 1.8^{ab}	1.4 ± 1.7^{ab}	1.1 ± 2.4^{ab}	1.8 ± 2.4^{ab}
Unknown	6.77	1.9 ± 1.9^{a}	0.5 ± 1.1^{b}	0.0 ± 0.0^{b}	0.3 ± 0.9^{b}	0.7 ± 1.3^{ab}	1.2 ± 1.7^{ab}
Unknown	6.83	10.0 ± 13.8	22.7 ± 20.1	6.8 ± 3.5	17.3 ± 16.2	9.5 ± 7.1	22.2 ± 19.9
Quercetin dihexoside	6.86	20.4 ± 17.5^{a}	14.1 ± 19.8^{ab}	2.0 ± 5.3^{b}	17.2 ± 24.0^{ab}	2.4 ± 4.6^{b}	10.0 ± 13.8^{ab}
Unknown	6.98	7.5 ± 8.3	12.2 ± 12.0	3.0 ± 2.2	12.8 ± 12.8	2.6 ± 2.2	7.8 ± 7.7
Unknown	7.28	34.6 ± 22.4^{a}	21.7 ± 30.9^{ab}	7.5 ± 7.0^{b}	41.2 ± 35.4^{ab}	5.5 ± 6.2^{b}	24.8 ± 22.2^{ab}
Unknown	7.78	6.8 ± 15.0	20.0 ± 29.8	15.8 ± 15.8	25.1 ± 23.9	15.0 ± 16.3	27.0 ± 29.8

Compounds	RT (min)	Control flowers	Flowers of infested plants	Pollinated flowers	Unpollinated flowers	Pollinated flowers of infested plants	Unpollinated flowers of infested plants
		(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)
Unknown	7.82	36.3 ± 28.1^{a}	29.9 ± 36.4^{ab}	6.2 ± 10.9^{b}	17.2 ± 23.7^{ab}	11.5 ± 14.4^{b}	20.0 ± 22.0^{ab}
Unknown	7.98	0.7 ± 1.6	1.3 ± 1.4	2.0 ± 1.5	1.9 ± 1.8	1.4 ± 1.2	1.4 ± 1.2
Unknown	8.02	2.3 ± 1.6^{a}	1.1 ± 2.3^{b}	0.7 ± 1.2^{b}	1.0 ± 1.5^{ab}	1.1 ± 1.5^{ab}	1.0 ± 1.5^{ab}
Unknown	8.08	0.8 ± 1.7^{a}	3.4 ± 4.5^{b}	4.2 ± 4.0^{b}	3.7 ± 3.7^{b}	3.6 ± 4.5^{ab}	3.4 ± 4.2^{ab}
Unknown	8.13	3.4 ± 2.7^{a}	1.6 ± 2.8^{ab}	1.1 ± 2.0^{b}	1.6 ± 2.4^{ab}	2.0 ± 4.6^{ab}	2.4 ± 5.3^{ab}
Unknown	8.23	20.2 ± 30.9^{a}	50.8 ± 43.1^{b}	15.6 ± 12.7^{ab}	49.8 ± 41.7^{ab}	20.6 ± 19.4^{ab}	59.3 ± 52.5^{ab}
Kaempferol dihexoside	8.26	53.7 ± 43.6^{a}	32.0 ± 44.8^{ab}	7.4 ± 13.4^{b}	37.2 ± 53.2^{ab}	5.8 ± 8.7^{b}	16.7 ± 23.3^{b}
Unknown	8.37	21.8 ± 12.4^{a}	21.1 ± 17.2^{ab}	12.4 ± 10.9^{b}	24.2 ± 13.8^{ab}	14.1 ± 10.8^{b}	28.1 ± 20.3^{ab}
Unknown	8.77	0.1 ± 0.5^{a}	1.7 ± 1.7^{b}	0.6 ± 1.0^{ab}	0.8 ± 1.5^{ab}	0.4 ± 1.2^{ab}	1.2 ± 1.4^{ab}
Unknown	9.18	1.2 ± 2.6^{a}	5.1 ± 5.2^{b}	4.8 ± 4.6^{b}	4.0 ± 5.0^{ab}	3.0 ± 3.4^{ab}	3.2 ± 3.5^{ab}
Unknown	9.23	5.7 ± 5.4^{a}	2.0 ± 4.2^{b}	1.9 ± 3.3^{ab}	1.9 ± 3.8^{ab}	1.0 ± 2.8^{b}	1.8 ± 3.4^{ab}
Unknown	9.27	7.1 ± 7.3^{a}	11.8 ± 10.3^{ab}	14.5 ± 7.8^{b}	16.3 ± 6.5^{b}	15.9 ± 12.6^{ab}	10.7 ± 9.7^{ab}
Unknown	9.53	18.4 ± 10.4^{a}	12.2 ± 14.2^{ab}	4.4 ± 4.8^{b}	22.2 ± 19.4^{ab}	5.7 ± 5.8^{b}	16.7 ± 14.9^{ab}
Unknown	9.88	27.1 ± 61.7^{a}	115.4 ± 134.2^{b}	77.3 ± 79.7^{ab}	104.2 ± 117.3^{ab}	78.0 ± 73.5^{ab}	120.4 ± 113.3^{b}
Unknown	9.92	141.8 ± 118.6^{a}	91.1 ± 134.3^{ab}	33.0 ± 63.6^{b}	36.5 ± 68.6^{b}	40.6 ± 84.2^{b}	68.7 ± 121.8^{ab}
Unknown	10.57	2.6 ± 4.3^{a}	6.4 ± 6.6^{ab}	1.4 ± 1.5^{ab}	9.5 ± 6.2^{b}	1.3 ± 1.5^{a}	7.3 ± 6.4^{ab}
Unknown	10.62	6.5 ± 6.5^{a}	3.7 ± 6.6^{ab}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
Unknown	11.32	26.7 ± 11.3^{a}	26.7 ± 13.5^{a}	13.0 ± 5.1^{bd}	43.6 ± 14.0^{c}	11.7 ± 8.7^{d}	36.4 ± 13.1^{ac}
Unknown	11.61	153.3 ± 61.2^{a}	157.2 ± 101.3^{ab}	123.9 ± 21.7^{a}	178.8 ± 28.1^{bc}	144.9 ± 45.0^{ac}	193.3 ± 34.9^{b}
Isorhamnetin hexoside	11.70	1.2 ± 1.8	0.8 ± 1.1	0.2 ± 0.6	1.4 ± 1.5	1.0 ± 1.2	1.6 ± 1.6
Unknown	11.78	9.4 ± 4.9^{ac}	8.7 ± 3.7^{ab}	5.6 ± 1.3^{b}	11.3 ± 4.7^{ad}	6.3 ± 1.5^{bc}	18.4 ± 8.1^{d}
Unknown	11.84	0.3 ± 1.3^{a}	0.3 ± 1.1^{ab}	2.3 ± 2.6^{b}	0.0 ± 0.0^{ab}	1.4 ± 2.0^{ab}	0.0 ± 0.0^{ab}
Unknown	11.87	6.0 ± 3.1^{a}	3.8 ± 1.9^{b}	4.2 ± 2.9^{ab}	4.8 ± 1.4^{ab}	6.6 ± 5.2^{ab}	5.0 ± 2.3^{ab}
Unknown	11.92	20.3 ± 8.2^{a}	20.6 ± 7.8^{a}	19.3 ± 6.7^{a}	20.3 ± 5.8^{a}	14.2 ± 6.9^{ab}	11.0 ± 5.7^{b}
Unknown	11.98	190.6 ± 60.5^{a}	187.7 ± 54.8^{a}	32.1 ± 18.1^{b}	239.0 ± 51.0^{ad}	51.3 ± 26.3^{c}	305.4 ± 94.0^{d}
Unknown	12.04	30.1 ± 10.0^{a}	26.4 ± 11.0^{a}	6.9 ± 1.4^{b}	36.9 ± 11.1^{ac}	8.8 ± 4.4^{b}	43.4 ± 12.4^{c}

Compounds	RT (min)	Control flowers	Flowers of infested plants	Pollinated flowers	Unpollinated flowers	Pollinated flowers of infested plants	Unpollinated flowers of infested plants
		(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)	(Peak area ± SD)
Unknown	12.18	0.1 ± 0.4^{a}	0.0 ± 0.0^{a}	1.1 ± 1.1^{b}	0.0 ± 0.0^{ab}	0.2 ± 0.6^{ab}	0.4 ± 1.0^{ab}
Unknown	12.22	2.2 ± 1.5^{a}	2.4 ± 1.5^{a}	0.0 ± 0.0^{b}	3.4 ± 1.7^{a}	0.4 ± 1.1^{b}	5.1 ± 2.5^{c}
Unknown	12.31	85.5 ± 54.9^{a}	64.1 ± 46.2^{abc}	44.4 ± 21.5^{bc}	63.4 ± 55.2^{abc}	45.9 ± 21.2^{b}	17.9 ± 41.9^{c}
Unknown	12.36	229.2 ± 101.3^{a}	217.5 ± 85.6^{a}	46.6 ± 28.0^{b}	302.9 ± 44.9^{c}	74.7 ± 39.2^{d}	403.3 ± 94.3^{e}
Unknown	12.43	1.7 ± 3.0^{ac}	0.4 ± 0.9^{a}	6.4 ± 3.7^{b}	0.6 ± 1.1^{a}	3.7 ± 3.5^{bc}	0.6 ± 1.7^{ac}
Unknown	12.48	69.8 ± 18.9^{a}	69.7 ± 12.0^{a}	15.3 ± 2.3^{b}	76.9 ± 19.2^{a}	17.6 ± 4.8^{bc}	76.2 ± 11.2^{a}
Unknown	12.54	83.8 ± 25.5^{a}	81.7 ± 20.0^{a}	14.0 ± 6.0^{b}	101.4 ± 17.0^{ad}	24.3 ± 7.8^{c}	129.2 ± 33.6^{d}
Unknown	12.62	11.0 ± 5.2^{a}	12.2 ± 4.2^{ae}	0.7 ± 1.3^{bc}	13.3 ± 4.9^{ad}	2.3 ± 2.0^{c}	15.7 ± 5.9^{de}
Unknown	12.67	2.0 ± 2.3^{abd}	1.1 ± 1.9^{abc}	0.0 ± 0.0^{ac}	3.2 ± 2.1^{bd}	0.0 ± 0.0^{c}	4.0 ± 2.3^{d}
Unknown	12.72	2.5 ± 2.6^{ac}	2.8 ± 2.0^{ace}	0.0 ± 0.0^{bd}	3.8 ± 1.7^{ae}	1.2 ± 1.3^{cd}	6.8 ± 4.5^{e}
Unknown	12.78	5.2 ± 1.5^{a}	5.5 ± 1.8^{ac}	0.0 ± 0.0^{b}	6.5 ± 1.7^{ac}	0.7 ± 1.3^{b}	7.1 ± 1.9^{c}
Unknown	12.89	1.0 ± 1.4^{acd}	4.0 ± 3.5^{bc}	1.1 ± 2.3^{cd}	1.5 ± 1.7^{cd}	0.3 ± 0.9^{d}	1.7 ± 1.5^{cd}
Unknown	12.91	1.5 ± 1.6^{a}	1.3 ± 1.8^{ab}	0.0 ± 0.0^{b}	0.9 ± 1.3^{ab}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
Unknown	13.02	4.8 ± 3.9^{ac}	3.5 ± 3.2^{a}	0.0 ± 0.0^{b}	7.1 ± 2.9^{cd}	0.0 ± 0.0^{b}	9.4 ± 5.7^{d}
Unknown	13.16	9.6 ± 5.3^{a}	11.4 ± 9.2^{abc}	4.6 ± 2.6^{b}	12.3 ± 3.8^{ac}	4.0 ± 1.0^{b}	14.8 ± 4.3^{c}
Unknown	14.33	6.7 ± 10.3^{ab}	1.8 ± 6.1^{a}	12.4 ± 14.3^{b}	0.0 ± 0.0^{a}	15.0 ± 13.0^{b}	0.0 ± 0.0^{a}
Total flavonoids		**2222.2 ± 297.4^{a}**	**1305.3 ± 223.2^{b}**	**2311.4 ± 347.4^{a}**	**1444.9 ± 248.3^{c}**	**2580.8 ± 235.3^{a}**	**2222.2 ± 297.4^{a}**

† Different superscripts (a,b,c,d,e,f) indicate significant difference between means at the 0.05 level (Wilcoxon signed rank test and Kruskal-Wallis test.