Volatiles of male and female flowers of *Cucumis sativus* are differentially affected by *Tetranychus urticae* leaf herbivory

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Voorwoord

Zo vluchtig als geurstoffen, zo snel ging de tijd voor het schrijven van mijn thesis. Op een dieet van suikerbrood, gedroogde worst en koffie heb ik heel wat uren op Radix kunnen vertoeven om het werk van afgelopen half jaar op papier te krijgen. Hoewel ik best nog wat langer aan het onderzoek en de resultaten had willen werken ben ik toch ook blij dat ik het werk nu af mag ronden; een stevige stok achter de deur is een goede ervaring. Nog twee maanden studeren en dan hoop ik het diploma in ontvangst te nemen waar ik heel trots op zal zijn. Wat ik daarna ga doen, daarover ben ik nog druk aan het prakkeseren.

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Of mijn resultaten veelzeggend zijn of niet; het blijft komkommernieuws!

Sigrid Dassen

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ABSTRACT

Leaf herbivory is known to induce certain leaf volatiles and thus cause an altered volatile blend and affects expression of terpene synthases genes next to other genes. It was found that altered leaf volatile blend increases attraction of predators to the damaged plant. In this research it was studied how flower volatiles, gene expression and flower ratios were affected upon a spider mite infestation and what consequences leaf herbivory has on pollinator attraction.
Cucumber variety Corona was treated with plant hormones salicylic acid and jasmonic acid and it was observed that jasmonic acid treated plants had a reduced number of female flowers per axil. The plant hormone treatments were found to have limited effect on flower volatile composition. Flower per axil ratio in cucumber variety Chinese long 9930, infested with spider mites, was found to be increased. Also a clear effect of leaf herbivory was found in flower volatile samples. Plants suffering from spider mite herbivory release more intense- and also different volatile compounds from the flower tissue. Furthermore, male and female flowers produce different volatile blends; differences between male and female flowers was found to be larger in flowers from spider mite infested plants.
Expression of a number of Cs-TPS-genes was analysed in leaf and flower tissue of spider mite infested cucumber plants. Cs-TPS-5, -19, and -21 were induced in leaf tissue, whereas expression of Cs-TPS-22 was declined. In flower tissue expression of Cs-TPS-5, -15, and -19 was induced in female flowers but reduced in male flowers. Cs-TPS-21 showed different expression between sepal and petal and expression of Cs-TPS-22 was reduced both in male and female flowers of spider mite infested cucumber plants.
In a Y-tube experiment volatile preference of pollinators was observed by offering bumblebees flowers from spider mite infested plants and a non-infested plant. No clear preference could be observed and it was presumed that external factors or directional cues induced by the bumblebees themselves did influence the choice of direction.

CONTENTS

1. INTRODUCTION

An attractive topic: volatile organic compounds of plants. The volatiles released by a plant, via its leaves, flowers and fruits, are important signals in plant-insect communication. Insects need plants as food source, directly for herbivorous insects and nectar seeking insects and indirectly for carnivorous insects. Vice versa plants need insects for pollination and will benefit from carnivorous insects that feed on herbivorous insects. A well-studied example is the interaction between cabbage plants and parasitic wasps. The parasitic wasps find their prey by using the altered volatile cues induced by the plant, herbivore induced plant volatiles (HIPVs), upon herbivory damage (Steinberg *et al.* 1992). Several other plant-insect interactions have been identified and studied: *Tetranychus urticae* infested lima bean plants attract the predator *Phytoseiulus persimilis* (Dicke & Sabelis, 1988), cabbage plants damaged by *Pieris brassicae* (L.) caterpillars attract parasitizing *Cotesia glomerata* (L.) wasps (Steinberg *et al*., 1992). It is the subtle change in volatile blend and composition that influence the insects search behaviour.

Herbivory activates the plant defence mechanism which is mediated by an increase in the phytohormones jasmonic acid (JA) and ethylene; in case of pathogen attack the defence mechanisms is mediated by increased levels of salicylic acid (SA) (Bari and Jones 2009). Exposure to SA and JA can mimic respectively pathogen infections and herbivory. JA treatment has not the same effect on the released volatile blend as natural herbivory; JA treatment is a reflection of the plants potential to release HIPVs, whereas natural herbivory induces a selection of HIPVs at different intensities too (Kappers *et al.* 2010).

Beside vegetative volatiles, alterations in floral volatiles is also likely to influence insect behaviour. The herbivore induced volatiles released by the plant may deter pollinators in its fight to repel the herbivore or it may increase its attractiveness to pollinators to speed up the life cycle of the plant. In a study by Kessler *et al.* it was found that the number of pollinator visitations was reduced when wild tomato (*Solanum peruvianum*) was treated with MeJA (to mimic herbivory) and they proofed that it was the altered flower volatiles and not visual cues nor pollinator reward that caused the change in pollinator visitations (Kessler *et al.* 2011). It has also been reported that male and female flowers are differently affected by herbivory. Enhanced leaf herbivory by *Acalymma vittatum* reduced the number of male flowers in *Curcubita moschata*, whereas female flower number was unaffected (Hladun 2008). Similar results were found for continuous herbivory by *Helix aspersa* on *C. sativus* where the number of male flowers was reduced and female flower number was not altered (Thomson 2004). Another report on herbivory of *C. sativus* found a reduced number of female flowers after root herbivory; male flower number was neither affected by root nor leaf herbivory (Barber 2011).

Intensity and ratio differences, addition of a novel compound and the context in which the volatiles are presented can theoretically all influence insect search behaviour (Raguso 2008). Insects can learn to associate cues with rewards but as volatiles are highly different, among species and also within varieties of the same species, is it the precise composition of the complex floral scent that pollinators and carnivorous insects do recognise or do they associate specific odours within the blend with a reward? Interesting is to find out which compounds really play a major role in attracting carnivorous insects and pollinators. One of the major compounds of HIPVs are terpenoids (Dicke & Baldwin 2010). Terpenes are also among the major chemicals that compose floral scents, beside aromatics and fatty acids (Schiestl 2010). At the Plant Physiology group of Wageningen UR several studies have been conducted in an attempt to unravel the mechanisms of plants signalling by terpenoids (i.a. Aharoni *et al*. 2003, Mercke *et al.* 2004, Kappers *et al*. 2005, Kappers *et al*, 2008). Plants of study have been *Arabidopsis thaliana* and cucumber (*Cucumis sativus*). In general, cucumber has been a model plant in several studies among which are volatile research and flower sex determination studies. Its recently sequenced genome will

support us with a better understanding of metabolic processes. Beside the scientific value of this crop, cucumber is also a vegetable which is produced and consumed all over the world. In 2010 the world production reached 57.559.836 tonnes of which 71% was produced in China, the Netherlands accounts for 1% of total cucumber production (FAO, 2011).
One of the common insects encountered in cucumber cultivation is spider mite (*Tetranychus urticae*), which feed on the leaves and are found both in field and glasshouse grown cucumbers (Robinson 1999). Herbivory by spider mites is known to alter the volatile composition released by cucumber leaves. This alteration in volatiles is known to influence the plants attractiveness for predatory mites (*Phytoseiulus persimilis*), a natural enemy of spider mites (Kappers *et al*. 2010). Limited research has been conducted on differences in floral scents of male and female cucumber flowers and their relation to insect behaviour. Although in seedless cucumber cultivation pollination is not needed natural pollination would occur by honeybees and bumblebees (*Bombus* spp.) visitation (Robinson and Decker-Walters 1999). Bumblebees find their nectar sources by both visual and olfactory cues, therefor alterations in volatile blend are likely to influence pollinator visitation too (Goulson 2009).

The genome of the cucumber variety *C. sativus* L. var. Chinese long 9930 was sequenced (Huang *et al*, 2009) and screened for terpene candidates (unpublished, He and Kappers). For each *C. sativus* terpene candidate primer sets were developed and RNA expression was observed in all plant parts of *C. sativus*. Among the terpene candidates that showed expression in both male and female flowers five genes were selected for further analysis. Cs-TPS-5 is involved in linalool synthesis, a monoterpenes. Cs-TPS-15, 19, 21 and 22 are (putative) sesquiterpene synthases. Cs-TP-S21 is responsible for the production of β-caryophyllene and Cs-TPS-22 is involved in τ-cadinol, a putative precursor of α-muurolene (unpublished Kappers and He). Cs-TPS-19 has a dual function: with substrate geranyl pyrophosphate (GPP) it produces β-ocimene, a monoterpene, whereas with substrate farnesyl pyrophosphate (FPP) Cs-TPS19 produces α-farnesene, a sesquiterpene.

The research questions in this study

- Are flower volatiles of cucumber affected upon treatment of the leaves with SA, JA or a spider mite infestation?
- Is the ratio between female and male cucumber flowers affected upon treatment of the leaves with SA, JA or a spider mite infestation?
- Do bumblebees have a preference for flower volatiles from spider mite infested cucumber plants or for flowers of non-infested cucumber plants?
- Can expression of terpene synthesis genes be related to terpenes present in the volatile blend of flowers from spider mite infested plants?

Expression of terpenoid synthesis genes in cucumber (Cs-TPS's) and floral volatiles were analysed in two different experiments. In the first experiment *C. sativus* L. var. Corona was treated with plant hormones salicylic acid, jasmonic acid and both salicylic and jasmonic acid (50/50%)(Chapter 3). In the second experiment *C. sativus* L. var. Chinese long, 9930 was infested with spider mites (Chapter 4). The results of both experiments were jointly discussed (Chapter 5).

2. MATERIAL AND METHODS

Plant material C. sativus L. var. Corona was used for the hormone treatment experiment. Seeds were sown in a greenhouse compartment at Wageningen University, The Netherlands. Daily average temperature was 20-18^{o}C for day and night-time respectively and relative humidity 40-80% (February-April). Photoperiod was 16 hours and supplemented with SON-T agro lamps if intensity fell below 250 W m^{-2}. Plants were transplanted to 2 litre pots when bearing 2 mature leaves.

For the spider mite treatment cucumber plants (*C. sativus* L. var. Chinese long, 9930) were sown in a climate chamber at Wageningen University, The Netherlands. Daily average temperature was 28-25^{o}C for day and night-time respectively. Photoperiod was 11 hours and relative humidity 50-70%. Plants with two full grown mature leaves were split in two separate greenhouse compartments. 8 control plants were grown in 2 litre pots at average daily temperature of 24^{o}C and night temperature of 18^{o}C, and a relative humidity of 30-90% (July-August). Photoperiod was set at 16 hours and daylight was supplemented with SON-T agro lamps if intensity fell below 250 W m^{-2}. Another 8 plants was grown in a greenhouse suitable for insect infestations. Day and night temperature was set at 22^{o}C, with relative humidity of 60% and a photoperiod of 16 Hours.

Plant treatments In the hormone experiment leaves of the cucumber plants were treated with either 50mM jasmonic acid, salicylic acid, or jasmonic- and salicylic acid (50/50%). Stocks for each treatment were dissolved in 96% EtOH. The treatment was applied by dissolving the hormone into a 1% agarose gel, the gel was then cut into squares of ± 1.8g and applied onto the leaf. Control plants were treated with agar containing 1% EtOH. At the start of the treatment the first three full grown leaves were treated with 50mM hormone. The treatment was continued by alternating the treatment every other leaf with 25mM for 40 days.

For the spider mite experiment 2x10 spider mites were introduced on the top two mature leaves when the plant was bearing two to four leaves. Ten days after the first infestation on each leaf of every plant a spider mite infested lima bean (*Phaseolus lunatus*) leaf was placed to speed up the infestation level.

Observations To observe changes in plant composition and flower number the final number of male and female flowers per axil and total number of axils was counted for each plant. Statistical analysis on flower distribution was conducted in Excel (Microsoft).

Hormone analysis Jasmonic acid, salicylic acid, and abscisic acid levels of the leaf were analysed by taking leaf samples from each treatment and the control group and freeze them in liquid nitrogen. Samples were stored at -80°C. For analysis of JA, SA and ABA levels 250 mg of frozen leaf was grinded with pestle and mortar. Deuterated abscisic acid (D6-ABA), 0.5 nmol/ml, was used as internal standard. 1 ml MeOH including the internal standard was added to the frozen sample and homogenized in an ultrasonic bath (Ultrasonic cleaner, Branson 200) for 15 min. 0.9 ml EtOAc was added to the pellet and the combined supernatants were dried in a speedvac (Labconco centrifuge + Savant condensation trap RT-100). Dried samples were dissolved in 250 µl ACN:H2O:Fa (25:75:0.1), filtered through 0.45µm filters (Minisart SRP4, Sartorius Stedim Biotech) and eluted in 0.3 ml LC-MS vial with insert (Short thread micro vial + open hole cap, Grace). Analysis of JA, SA and ABA in cucumber leaves was performed by comparing retention times and mass transitions with those of standards using ultraperformance liquid chromatography (UPLC) coupled to MS/MS. Chromatographic separation was obtained on an Acquity UPLC BEH C18 column (150 × 2.1 mm, 1.7 µm; Waters) by applying a water/acetonitrile gradient to the column, starting from 5% acetonitrile for 2.0 min and rising to 50% (v/v) acetonitrile in 8.0 min,

followed by a 1.0-min gradient to 90% (v/v) acetonitrile, which was maintained for 0.1 min before going back to 5% acetonitrile using a 0.2-min gradient, prior to the next run. Operation temperature and flow rate of the column were 50°C and 0.4 mL min−1, respectively. Sample injection volume was 20 µL. Multiple reaction monitoring (MRM) was used for identification of JA, SA and ABA by comparing retention times and MRM mass transitions with those of standards. For JA, MRM transitions m/z 211>151 and 211>133 were selected; for SA m/z 139>121 and 139>65 and for ABA 271>234 and 271>253 were selected. Data output was analysed for differences between treatments using the t-test in Excel (Microsoft).

Head space analysis Head space was conducted on female flowers at day 34, 35 and 36 after hormone treatment was started. Each day one flower per treatment was picked and placed in a 0.5 L glass jar connected to an inlet and outlet Tenax liner. For the spider mite experiment head space was conducted on both male and female flowers 20 to 26 days after the first spider mite infestation. Per measurement volatiles of five flowers (e.g. five male flowers of control plants) plus one empty jar was trapped.

Volatiles were trapped for 1.5 hours and collected on Tenax® sorbent tubes combined with micro air samplers (PAS-500, Spectrex). Volatile samples were analysed with a Thermo Trace GC Ultra connected to a Thermo Trace DSQ USA) quadruple mass spectrometer (Thermo Fisher Scientific, Waltham, MA) according to Kappers et al, 2011. In short: Tenax cartridges were dry-purged with nitrogen at 30 ml min^{-1} for 30 min at ambient temperature to remove water before desorption of the volatiles. Volatiles were desorbed from the Tenax cartridges using a thermal desorption system at 250°C for 3 min with a helium flow of 30 ml min^{-1}. Volatiles were transferred in split mode (1:5) to the analytical column (Rtx-5 ms, 30 m, 0.25 mm i.d.,1.0 µm film thickness) by rapid heating of the cold trap to 250°C. The GC was held at an initial temperature of 40°C for 3.5 min followed by a linear thermal gradient of 10°C min^{-1} to 280°C, and held for 2.5 min with a column flow of 1 ml min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 45–400 m/z with a scan rate of 3 scans s^{-1}. Compounds were identified by using the Xcalibur (Thermo Scientific) software in combination with the NIST 98 database libraries and by comparing their retention indices with those from the literature (Adams 1995; www.pherobase.com). For quantification, characteristic quantifier ions were selected for each compound. Metalign software (PRI-Rikilt, Wageningen, the Netherlands) was used to align peaks of chromatograms of all samples and to integrate peak areas for the quantifier ions. GeneMath was used for normalisation (log-transformation) of the whole dataset before multivariate analysis was done via Principal Component Analysis based on Pearson correlations. T-test statistic was conducted in Excel (Microsoft).

Cs-TPS expression Plant material was taken for analysis of gene expression. From each hormone treatment and the control plants flowers were taken in triple and the petals were separated from the sepal, receptacle and pedicel prior to freezing and storage at -80°C. In the spider mite experiment also leaf samples were taken for gene expression and flowers were further separated by keeping the pistil and stamen apart from the other flower tissues.

Plant material samples were grinded with pestle and mortar to facilitate RNA isolation. RNA from 80 mg plant material samples was isolated using TriPure isolation reagent (Roche) and chloroform. A DNAse treatment was applied (Invitrogen) and RNA samples were washed with RNeasy Minikit (QIAGEN), according to manufactures protocol. RNA concentrations were checked with Nanodrop and cDNA was synthesized with iScript (Biorad), according to manufactures protocol. RT-PCR was conducted with MyIQ (BioRad) in 96-well plates and samples were measured in duplo. Samples composed of 10 µl SYBRgreen

(BioRad), 2 µl forward primer, 2 µl reverse primer, 2 µl cDNA template and 4 µl MilliQ. The reference gene used was actine, primer sequences are given in appendix I. RT-PCR data was processed with the iQ5 programme (BioRad) and statistical analysis (T-test, Log_2Ratio) was conducted with Excel (Microsoft). For the T-test the Ct-values minus Actine Ct-value was used and for calculating the Log_2Ratio the formula: Log_2 (Normalised expression/Mean Normalised expression Control group) was applied.

Pollinator preference To analyse the pollinators' flower volatile preference (flowers from spider mite infested plants vs. non-invested) a Y-tube olfactory experiment was conducted (Fig. 1). Clean air was blown (3L min^{-1}) over a single flower and led into the short arms of a glass Y-tube; one end holding a flower picked from a spider-mite infested plant and at one end a flower from a non-infested cucumber plant. Bumblebees, *Bombus terrestris* (Koppert, The Netherlands), were observed for their volatile preference. The bumblebees were unable to see the flower as sight was strongly reduced by coverage of the Y-tube with a black cover and access to the flower was blocked by a fine mesh. Bumblebees were placed into the long arm of the Y-tube one by one and the first choice for the left or right arm was noted as their preference. Volatile preference was observed for male and female flowers separately, for male and female flowers 50 bumblebees were observed each, excluding bumblebees that did not make a choice. To minimize the effect of attractive cues from the surrounding on direction choice of the bumblebees the flowers (infested/non-infested) were switches position every five observations. Flowers were kept on water to reduce wilting. Observations for male flowers was held three times (14, 22, 21 bumblebees respectively), observations for female flowers was held four times (13, 13, 12, 21) bumblebees respectively).

Fig. 1 *Bombum terrestris* flower volatile preference observation in a Y-tube experiment. During observations the long arm of the Y-tube was covered by the black sheet.

3. EFFECTS OF PLANT HORMONES (JASMONIC ACID AND SALICYLIC ACID) ON FLOWER CHARACTERISTICS IN CUCUMBER

Agar plugs with salicylic acid did cause local cell death on the leaves within two days, no such strong reaction was visible in jasmonic acid treated plants. This fast reaction was only observed after the first few treatments, speed of reaction and reaction size did reduce after several treatments with salicylic acid Eight weeks after sowing a mildew infection appeared on the leaves of both the control group and all treatment groups. Interesting was that the mildew infection showed minor infection spots only on the plants from the salicylic acid and SA+JA treated plants. There were no measures taken to calculate precise infection severity. Growth of JA and JA+SA treated plants was reduced compared to the SA treated plants and control plants. The number of leaves and internode length was reduced in JA treated plants, however exact measurements on growth were not taken (Appendix II).

Analysis of SA and JA levels in the leaves showed that in none of the treatments the level was significantly altered compared to the control group (data not shown). Also the level of abscisic acid (ABA), a plant hormone induced in stress situations, was not increased in leaves of plants treated with SA, JA or SA+JA.

Appearance of flowers on the jasmonic acid treated plants was 7 days delayed compared to control and salicylic acid treated plants. Plants treated with both salicylic and jasmonic acid did produce flowers three days after the control group. In the setup of the experiment it was not taken into account that the Corona variety only produced female flowers; this was found out when the plants started producing flowers. Number of female flowers per axil on jasmonic treated plants was reduced compared to the control group, the salicylic acid- and SA+JA treatment (P <0.01) (Fig. 2).

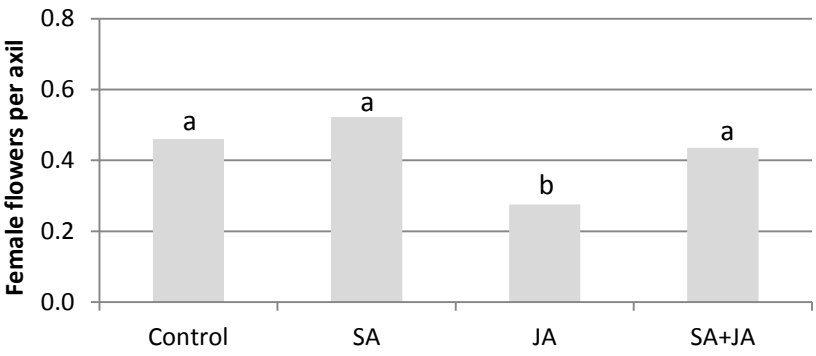


Fig. 2 Number of Female flowers per axil on Dutch cucumber variety Corona (*Cucumis sativus* L. var. Corona). Different letters indicate significant differences (α = 0.05, LSD).

Expression of TPS genes was performed using RT-PCR. Results are given without standard error because replicates were not always reliable, sometimes resulting in single measurements (Fig.3). Expression of Cs-TPS-5 in SA treated plants was increased in both petal and sepal and in SA+JA treated plants expression was decreased in petal and sepal. In petals of JA treated plants expression of Cs-TPS-5 was decreased in petals and in sepals only minor expression was observed. Cs-TPS-15 showed similar expression in JA and SA+JA treated plants where a decrease was observed in petals and an increase in the sepals. In SA treated plants the expression of Cs-TPS-15 was limited. Cs-TPS-19 was hardly induced in petals but upregulated in sepals of SA and SA+JA treated plants, however in sepals of JA treated plants a decrease in expression of Cs-TPS-19 was observed. In petals expression of Cs-TPS-21 and -22 was downregulated in all treatments, whereas in the sepals of JA and SA+JA treated plants Cs-TPS-21 and -22 were upregulated and in sepals of SA treated plants the expression was downregulated too.

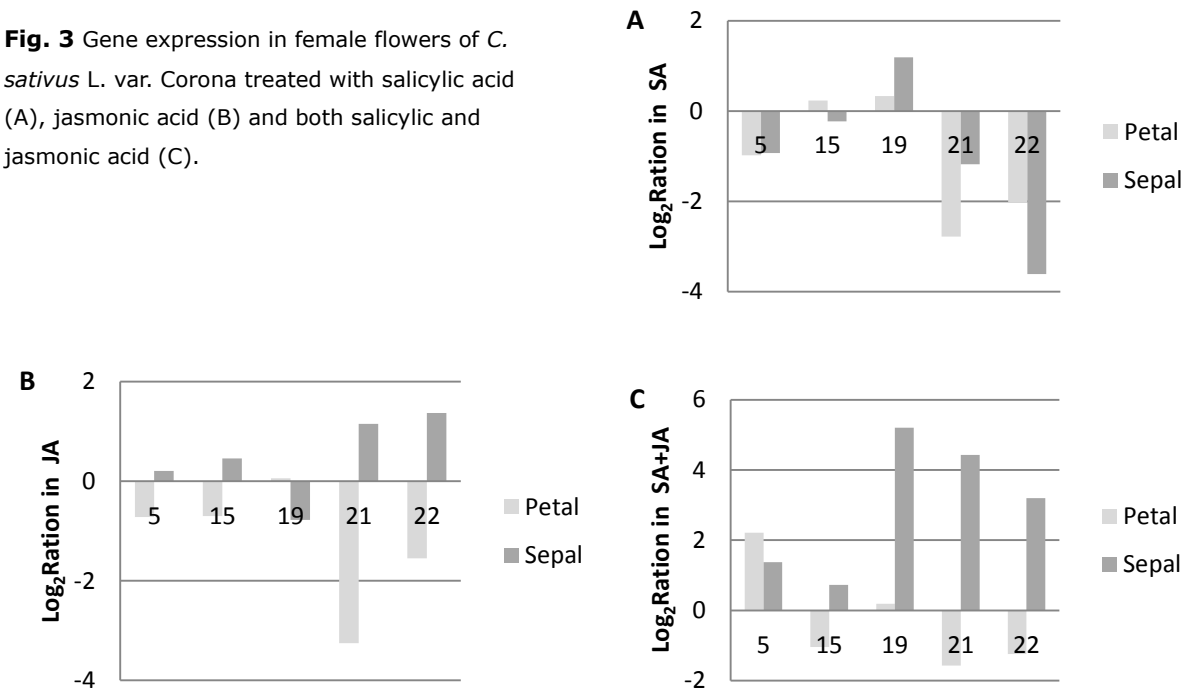


Fig. 3 Gene expression in female flowers of *C. sativus* L. var. Corona treated with salicylic acid (A), jasmonic acid (B) and both salicylic and jasmonic acid (C).

Visual assesment of the head space data shows minor alterations between the hormone treatments; at lower abundance more differences are visible (Fig.4; Table1). The two major peaks are benzaldehyde and benzyl alcohol.

Table 1 Major compounds of the volatile analysis of female flowers from JA and SA treated cucumber plants.

Peak number	Retention time (min)	Compound
1	9.50	2-cyclopenten-1-one
2	12.20	Benzaldehyde
3	12.50	6-methyl 5-Hepten-2-one
4	12.82	Octanal
5	13.50	Benzyl alcohol
6	14.53	Linalool
7	16.22	Decanal

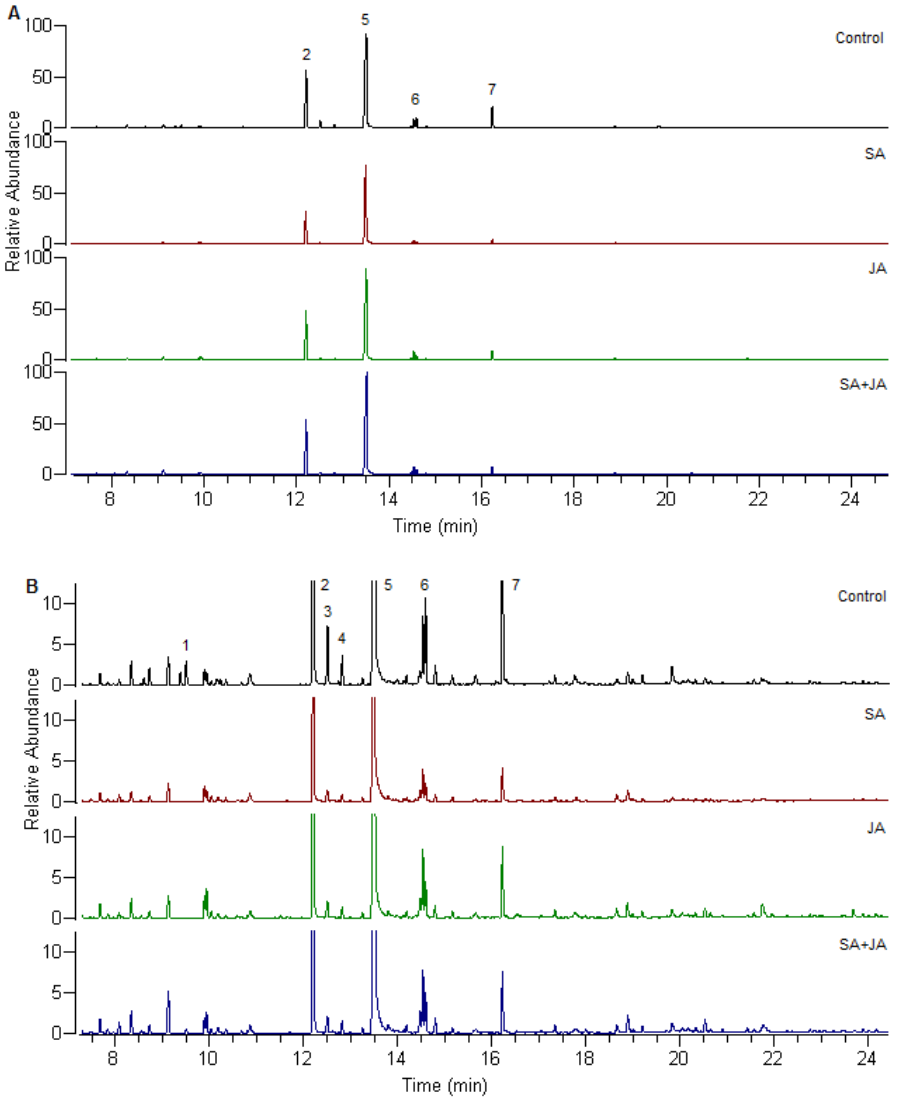


Fig. 4 Volatile compounds of female cucumber flowers from plants treated with SA, JA or SA+JA. (A), at 100% abundance, (B), at 10% abundance. The compounds corresponding to the numbered peaks are listed in Table 1.

4. EFFECTS OF SPIDER MITE INFESTATION OF LEAVES ON FLOWER CHARACTERISTICS IN CUCUMBER

The level of jasmonic acid in leaves of spider mite infested plants was increased (P=0.015) by 5.5 pmol/mg compared to the levels in control leaves. The level of salicylic acid was not significantly affected but seems higher in the control group. The ABA level in leaves of the spider mite infested plants was nearly four times higher than in the control plants (P<0.001)(Fig 5).

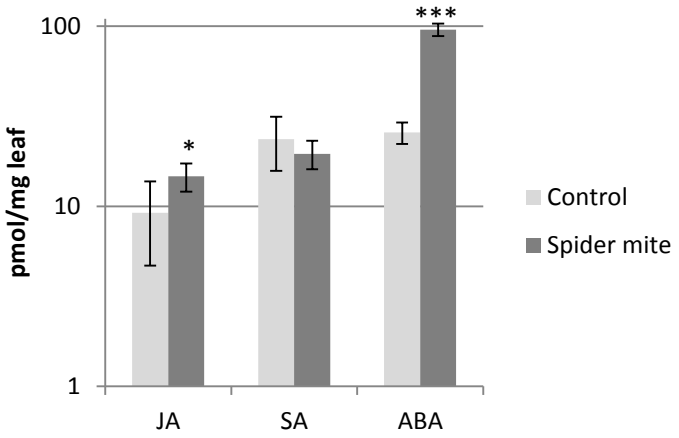


Fig. 5 Hormone levels in spider mite infested cucumber plants. * indicates P<0.05 and *** P<0.001 in two-sided T-test.

Numbers of male and female flowers per axil was counted to observe a possible shift in male and female flower ratio (Fig. 6). Total number of flowers per axil was increased in spider mite infested plants (P<0.05). Major increase was caused by a higher number in male flowers per axil (P<0.05). Number of female flowers in spider mite infested plants was increased (P<0.001).

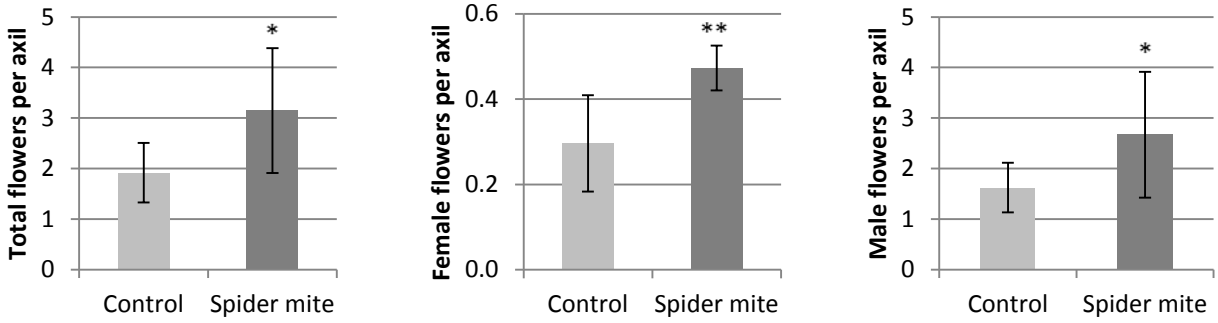
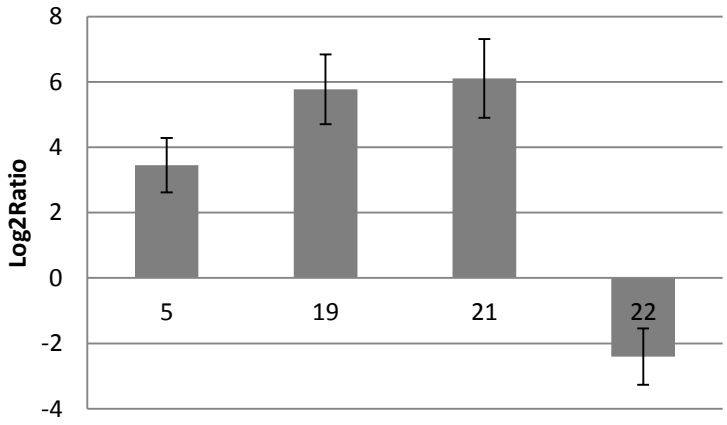


Fig. 6 Flowers per axil. Error bars indicate SE of 8 replicates. * indicates P<0.05 and ** P<0.01 in two-sided T-test.

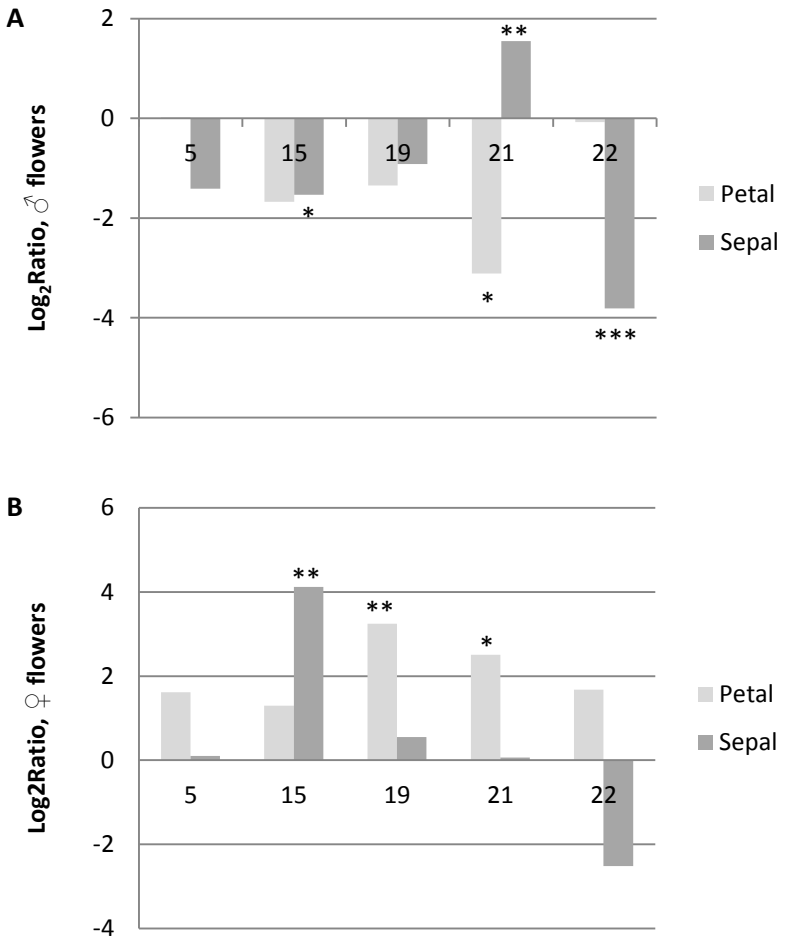
Gene expression on Cs-TPS's was analysed in both leaf and flowers of spider mite infested and non-infested cucumber plants. In leaves of spider mite infested cucumber plants expression of Cs-TPS-5, 19, and 21 was increased compared to the control group (P<0.001) and expression of Cs-TPS-22 was reduced (P<0.001) (Fig. 7).

Fig. 7 Gene expression of TPS's in spider mite infested *C. sativus* leaves compared to control group.



Upon spider mite infestation in cucumber leaves a difference in gene expression of male and female flowers was observed (Fig. 8). Expression of Cs-TPS-15 was reduced in male flower sepals (P=0.019), whereas expression of this gene was increased in female flower sepals (P=0.004). In female flower petals expression on Cs-TPS-19 was increased (P=0.003); in male flowers Cs-TPS-19 was reduced in both petals and sepals, however the reduced gene expression in male flowers was not significant. In male flower sepals and in female flower petals the expression of Cs-TPS-21 was increased (P=0.003 and P=0.043 respectively) and a reduction in Cs-TPS-21 expression was observed in male flower petals (P=0.045). In male flower sepals a reduction in Cs-TPS-22 expression was found (P<0.001). The observed expression of Cs-TPS-5 was not significantly different in all treatments.

Fig. 8 Gene expression in flowers of spider mite infested *C. sativus* plants. (A), male flowers. (B), female flowers. * indicates P<0.05, ** P<0.01 and *** P<0.001 in two-sided T-test.



Due to the limited amount of flowers that were available for RNA isolation and the small size of the carpel and stamen it was decided to combine all collected stamen and carpels for the spider mite infested group and the control plants. Without replicates the results of the gene expression in the carpel and stamen of *C. sativus* are considered as in indication of possible gene expression. Comparing the results of gene expression in carpel and stamen with male and female flowers it was observed that the expression pattern of stamen was comparable to expression in male sepals (Fig. 9). The expression pattern of carpels did match the expression of female petal except for Cs-TPS-22, which seems up regulated in female petals but down regulated in carpels.

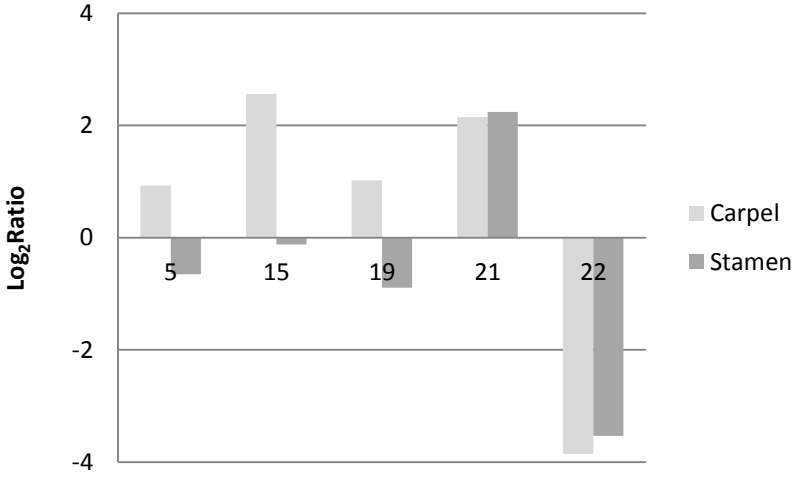


Fig. 9 Gene expression of TPS's in carpel and stamen of *C. sativus*.

Bumblebees were observed for their volatile preference. Bumblebees offered male flower volatiles from spider mite infested and non-infested plants did choose for the non-infested flowers in 56% of the cases (Table 2). When choice was between volatiles from female flowers 56% of the bumblebees chose to fly to the flower picked from a spider mite infested plant. Variation between the different sets of observations did not indicate a significant difference between preference for flower volatiles from infested on non-infested plants, this was the case for both male and female flower volatiles.

During the experiment it was suspected/ observed that the bumblebees preferred to choose the left arm over the right arm, regardless of the volatiles offered. Choice for flight direction was counted for the whole experiment (Table 3). If male flowers from infested plants were offered on the left, then 50% of the bumblebees did fly to the left and 50% of the bumblebees did fly to the flower from the non-infested plant on the right. Offering the flower from the non-infested plant on the left made 63% of the bumblebees fly to the left and 37% to the flower from the infested plant on the right. In the case of female flowers, offering flowers from infested plants on the left made 80% of the bumblebees chose for the left and 20% for the flower from the non-infested plant on the right. When the flower from the non-infested plant was offered on the left 93% of the bumblebees chose for left and 7% for the flower from the infested plant on the right.

Table 2 Bumblebee volatiles preference for flowers from plants infested by spider mites and flowers from non-infested plants.

Flower sex	Infested (%) Mean ± SE	Control (%) Mean ± SE	No choice (%)	Total # bumblebees
Male	43 ± 7.6	56 ± 7.6	12	57
Female	56 ± 16.0	44 ± 16.0	15	59

Table 3 Bumblebee preference for the left and right arm of the Y-tube.

Flower sex	Infested (%)		Control (%)	
	Left	Right	Left	Right
Male	50	37	63	50
Female	80	7	93	20

In GeneMath, a principal component analysis (PCA) plot was made based on all mass fractions of the compounds in the volatile blend (Fig. 10). On the X-axis the volatile blends of control plants and the spider mite infested plants are separated and on the Y-axis the volatile blends of male and female flowers are divided when infested with spider mites. The results indicate that differences in volatile composition can be expected between flowers from infested and non-infested plants and also between male and female flowers from infested plants. The circle enclosing VS2, VS4 and MS3 represent flowers that were open for a longer time (close to senescence).

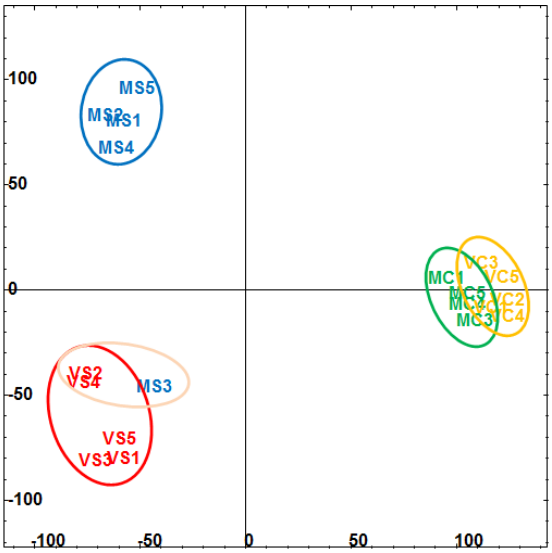


Fig. 10 PCA plot based on all mass fractions. MS, male spider mite; MC, male control; VS, female spider mite; VC, female control.

The chromatograms of the flowers confirm the differences between the treatment and control (Fig. 11). Most remarkable is the difference in volatile intensity between the flowers from control plants and spider mite infested plants. The NIST 98 database library was used to identify the putative compounds for each peak. By calculating and comparing the KRI-values with references (Adams, 1995; www.pherobase.com) the probability of the given compounds was checked (Table 4). Selecting peaks for presence of m/z (mass to charge ratio) 93, a typical component in terpenoids, also showed differences in the presence and intensity of terpene compounds between flower volatiles of the spider mite infested plants and non-infested plants (Fig 12).

Table 4 Major compounds of the volatile analysis of flowers from spider mite infested cucumber plants.

Peak number	Retention time (Min)	Compound
1	8.89	Hexanal
2	9.28	cyclotrisiloxane[1]
3	10.11	3-Hexen-1-ol
4	11.00	methoxy-phenyl Oxime-
5	12.42	Benzaldehyde
6	12.64	6-methyl 5-Hepten-2-one
7	12.98	Octanal
8	13.42	2-ethyl 1-Hexanol
9	13.80	Benzyl alcohol
10	14.73	Linalool
11	16.39	Decanal
12	18.82	Propanoic acid
13	19.05	Propanoic acid
14	20.01	(Z)-β-Farnesene
15	20.68	(E,E)-α-Farnesene
16	21.44	(E)-nerolidol
17	24.11	Benzyl benzoate

[1] Compound from column

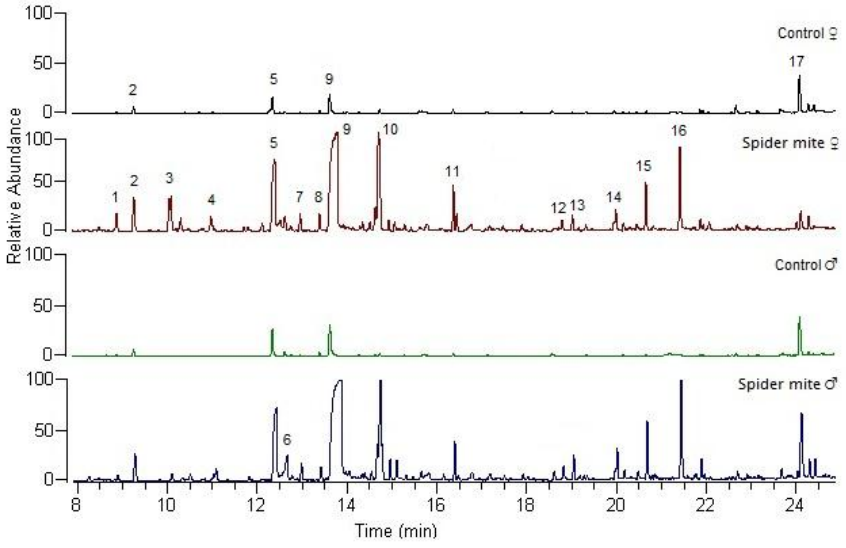


Fig. 11 Volatile compounds of cucumber flowers, from plants infested or not with spider mites.

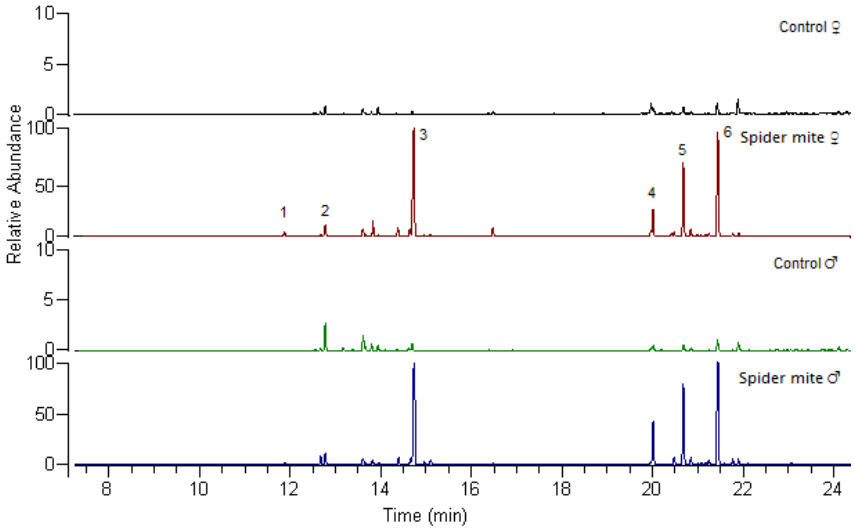


Fig. 12 Volatile compounds containing m/z 93, an indicator of terpenoids. Mind the variation on the y-axis. 1, α-pinene; 2, β-pinene?; 3, Linalool; 4, (Z)-β-farnesene; 5, (E,E)-α-farnesene; 6, (E)-nerolidol.

Beside observing visual differences in VOC emissions between the treatments also statistical analysis was performed to identify the peaks which could not be visualised. Over 700 centrotypes were found; VOC's induced over a 50-fold were considered as interesting for further investigation (Table 5). Not all centrotypes could be related to a compound with certainty. At Rt 11.69 a compound was found which is either 3-carene or thujene and is expressed in male flowers of infested and non-infested plants and in female flowers of infested plants. Other compounds with similar induction patterns are the terpenes (E)-linalool oxide, iso-caryophyllene and a unknown terpene at Rt 14.84. Compounds found to be induced both in male and female flowers after spider mite infestation were α–pinene (?), benzyl alcohol, (Z)-linalool oxide, indole, (E,E)-α–farnesene, (Z,E)-α–farnesene, (E)-nerolidol, and TMTT. 6-Methyl-5-heptene-2-one was found to be induced mainly in female flowers of spider mite infested plants but in lower levels also in male flowers of spider mite infested plants and was present in female flowers of the control group. Linalool and α-farnesene were present in flowers of the control plants but highly increased in both male and female flowers of spider mite infested plants.

Among the compounds listed in table 4 differences in VOC emission between female and male flowers of spider mite infested plants were found to be an unknown compounds at Rt 7.16 and α–Pinene, which was induced a 13-fold times higher in female flowers compared to male flowers. Increased VOC's in male flowers compared to female flowers of the control group were 3-carene or thujene, (E)-linalool oxide, an unknown terpene at Rt 14.84, and iso-caryophyllene. Vice versa, an increase of 6-Methyl-5-heptene-2-one was observed in female flowers of the control group compared to male flowers of the control group.

Table 5 Induced volatiles of flowers from spider mite infested cucumber plants.

Retention time (min)	Abundance [1]				Induction/ increase		Putative identification
	VC	VS	MC	MS	Fold (VS/VC)	Fold (MS/MC)	
7.16	-	+++	-	-	2993	1	?
7.65	-	+++	-	++	1842	1009	?
10.35	+	+++	++	+++	50	12	?
11.69	-	++	++	++	990	3	3-Carene/Thujene [2]
11.87	-	+++	-	++	3673	266	Monoterpene/α-Pinene [2]
12.64	++	+++	-	++	6	1025	6-Methyl-5-heptene-2-one
13.70	-	++	-	++	375	369	Benzyl alcohol
14.35	-	++	+	++	810	12	Trans linalool oxide [2]
14.63	-	++	-	++	631	910	Cis linalool oxide [2]
14.69	++	++++	+++	++++	23	50	Linalool
14.84	-	++	+	++	555	23	Unknown terpene [2]
17.54	+	+++	+	+++	63	64	?
17.62	-	+++	-	+++	1733	1630	?
17.88	-	+++	-	+++	3157	7171	Indole?
19.19	+++	++++	+++	+++++	43	66	?
20.01	+++	+++++	+++	+++++	34	68	α-farnesene
20.69	-	+++	-	+++	1464	2353	(Z,E)-β-farnesene [2]
20.78	-	++	+	+++	1016	37	Iso-caryophyllene [2]
21.00	-	+++	-	++++	10569	13098	(E,E)-α-farnesene [2]
21.52	-	+++	-	+++	3046	5349	Nerolidol [2]
21.58	-	+++	-	+++	8130	10588	TMTT [2,3]

[1] Abundance expressed in peak surface area, each '+' is a 10-fold surface increase
[2] Terpenoids
[3] TMTT = 4,8,12-Trimethyl-1,3,7,11-tridecatetraene

Also two compounds were found which were not detected in flower volatiles of spider mite infested plants but were present in the flowers from control plants (data not shown). Limonene-6-ol pivalate (Rt: 19.97) was present in male flowers of control plants but was not detected in flowers of spider mite infested plants. In female flowers the compound limonene-6-ol pivalate was a 10-fold lower in flowers of spider mite infested plants. Trans-geranyl acetate (Rt: 19.96) was present in both male and female flowers of control plants but was not detected in male nor female flowers of spider mite infested plants.

5. DISCUSSION

The plants treated with plant hormones JA and SA did not show major alterations in volatile composition between the hormone treatments, also the expression of TPS-genes in flowers did not present clear differences. Since there was no increase in JA, SA, nor ABA levels observed in the leaves upon SA or JA treatment the outcome of the head space data and gene analysis is not surprisingly. The lack of variation between the treatments can be a result of the mildew infection in both the hormone treated plants and the control group. A mildew infection will increase the SA levels in the plant since SA is a plant signal hormone induced during microorganism infections. This can also clarify why the mildew infection areas in the SA treated plants were smaller than on the control and JA treated plants; in the SA treated plants the mechanism for pathogen defence was triggered already and thereby could reduce the infection area. Nevertheless, the data clearly suggest that there is a difference in gene expression between petal and sepal and that among the TPS-genes tested Cs-TPS-19, -21 and -22 are stronger expressed in floral tissue than Cs-TPS-5 and -15.

The effect on flower ratio of total flower number per plant upon JA treatment or herbivory by spider mites was not found to be consistently, also in literature conflicting results are found. A reduction of cucumber female flowers upon leaf herbivory was shown by Barber *et al*. 2010, yet another experiment shows no reduction in total number of female flowers upon leaf herbivory but does show a reduction in male flowers per plant during continuous herbivory (Thomson *et al*. 2004). Both experiments have contradictory outcomes compared to the findings of this report but it should be mentioned that in all experiments different cucumber varieties were used. Whether a cucumber flower will develop in a male or female flower is dependent on the selective abortion of pistil or stamen. It is known that ethylene and gibberellins (GA) play a major role in floral sex determination (Tanurdzic and Banks, 2004), measuring the ratios of ethylene and GA in different cucumber varieties, with and without induced herbivory, could give more insight on the varying results of male and female flower ratios in cucumber.

Introducing spider mites on cucumber leaves did increase the level of JA acid and ABA in the leaves significantly and also TPS-gene expression in the flowers and VOC emission were altered. The increased expression of Cs-TPS-19, and -21 in leaves induced by spider mite herbivory is consistently with earlier findings by Kappers *et al.* 2010 who did observe an increase in α-farnesene and β-caryophyllene leaf volatile emission both when plants were treated with JA and upon herbivory. In this report levels of α-farnesene were also found to be increased in the male and female flower volatiles of spider mite infested plants. Interesting is that expression of Cs-TPS-19 was increased in female flowers but reduced in male flowers, thus the increased gene expression of Cs-TPS-19 could not be directly linked to the emission of α-farnesene in flower VOC. The expression pattern of Cs-TPS-21 was well defined in the floral tissue of both male and female flowers, unfortunately its product, β-caryophyllene, could not be found in the volatile blend of the flowers. The most abundant m/z compounds of β-caryophyllene are 93 and 133, selecting peaks in the chromatogram containing m/z 93 or 133 did neither show presence of β-caryophyllene in the flower volatile blends. β-caryophyllene emission by flowers is common in *Cucurbitaceae* (Ferrari 2006), however in literature no reports were found that proof β-caryophyllene emission from cucumber flowers. Although the Cs-TPS-21 gene is transcribed in flower tissue a mutation in the gene may result in a protein product which is not functional or has an altered function.

Plants suffering from spider mite herbivory release more intense- and also different volatile compounds from the flower tissue (Table 5). Also male and female flowers produce different volatile blends in which some compounds were found to be either male or female related. Lucas-Barbosa *et al*. (2011) summarises that alterations in flower VOC's induced by leaf herbivory have been investigated for several plant-herbivore combinations and shows that an alteration in flower VOC is not the rule but is observed

in most cases. In one of these studies both leaf and flower volatiles of herbivore damaged *Solanum peruvianum* plants was studied and revealed that not only the VOC's of damaged plants were altered but also shows that leaf and flower both induce unique compounds upon herbivory (Kessler and Halitschke, 2009). Kessler also proofs that pollinator visitation was reduced in the damaged plants, unfortunately it was not proven whether the reduction in pollinator visitation was due to olfactory or visual cues. It is known that bees use visual cues to select their nectar sources (Goulson 2010). Differential VOC release of male and female flowers after herbivory has been shown in *Cucurbita pepo* (Theis *et al.* 2009 and Granaro *et al*. 2005).

During the Y-tube experiments it was found that bumblebees are easily triggered to feel threatened. The treated bumblebee will lie on its back for a while, ready to sting whatever comes close. This behaviour was mainly observed when the bumblebees tried to fly in the narrow Y-tube. To reduce flying behaviour the long arm of the Y-tube was covered by a black sheet. A thin layer of fine sand was used to facilitate the bumblebees to walk through the tube without sliding.

A clear preference for flower volatiles from spider mite infested or non-infested cucumber plants was not observed, neither for male nor female flowers (Table 2). However, including the data on the choice of direction there is an indication that bumblebees prefer to choose the left arm, especially when female flowers volatiles from non-infested plants are offered at left. Although it was aimed to reduce differences (ea. light intensity, wind speed) between left and right arm it is likely that an unknown attractant was present, either present in the design of the experiment or induced by the bumblebees themself. Bumblebees are known to release olfactory cues for communication and reward linkage; the secreted compounds are hydrocarbons and can be similar to compounds of volatiles found in flowers (Saleh 2007; O.E. Prŷs-Jones and S.A. Corbet 2011; Schiestl 2010). For example blends of eucalyptol, ocimene and farnesol are used by returning foraging bumblebees to stimulate other bumblebees to leave the nest too (Granero 2005). The set-up of the volatile preference experiment should be improved to reduce the possibility that bumblebees can be influence each other by the choice of direction. Cleaning the Y-tubes and refreshing the sand in between each observation will reduce the remaining of olfactory cues, but will increase the labour.

In conclusion, herbivory by spider mites affects reproduction and interaction of cucumber plants. Female flower ratios were altered and it was found that expression of terpene synthase genes and the volatile blend of the flowers changed when plants did suffer from herbivory. Although pollination in cucumber cultivation is not necessary for seedless fruit production, it is important for breeding and the results from this report proves that leaf herbivory has consequences for pollination and seed production.

6. RECOMMENDATIONS

- Plants treated with JA and SA showed differences in morphology and disease resistance compared to the control plants. Although these differences were observed there was no significant difference of internal SA and JA levels between treated- and control plants. It could be that an increase in JA and or SA did occur but that at the moment of sampling the hormone levels were already decreased. By detecting the degraded compounds of JA and SA in the leaves one can see if higher levels of SA and JA have been present in the tissue. Indicators to measure the switch off of jasmonate signaling are 12-hydroxyjasmonic acid (12-OH-JA) and its glucosylated and sulphated derivates, 12-*O*-Glc-JA and 12-HSO_4-JA respectively (Miersch *et al*. 2008). In the case of SA most of the SA synthesised by the plant is converted to salicylic acid *O*-β-glucoside (SAG), methyl salicylate or methyl salicylate *O*-β-glucoside (Vlot *et al*. 2009) and can be used to quantify the total amount of SA produced in the tissue of interest.

- The remainder of the 700 centrotypes are not identified yet; thus far it was attempted to identify the centrotypes with a 50-fold induction. In the remainder of the centrotypes more peaks can be identified that explain the outcome of the PCA (Fig. 10).

- The pollination experiment did not reveal a bumblebees' preference for flowers from spider mite infested plants or for flowers from control plants. To find out whether an unknown cue influences the choice of direction the experiment should include a positive control. This control can be conducted by placing an attractant at one of the short arms; no matter at which position (left of right) the attractant is placed a preference for the attractant must be observed. Finding a suitable attractant might be more challenging than the setup of the experiment since, as far as the author knows, no volatile attractants for bumblebees have been identified. Considering pheromones, sex pheromones produced by queens might not be an attractant during all life stages of worker bumblebees and trail pheromones are known as repelling substances instead of attracting other bumblebees (Wilms and Eltz 2008). But, perhaps a flower of any kind is more attractive that pure air and can serve as a positive control.

- Furthermore, in the pollinator preference experiment it would also be interesting to find out if there is a preference for male flowers over female flowers and does this preference, if present, also holds for male and female flowers from spider mite infested plants?

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http://faostat.fao.org/

www.pherobase.com

APPENDIX I

Table I Primers of Cs-TPS's genes and actin.

Gene	Primer forward	Primer reverse
Cs-TPS-	ACACCCGTACCGGATGAACAAAACA	ACGAGCATTTTTCTCTGAACTGCCT
Cs-TPS-	GCAGGTGGAATAATTGCCCGACT	AGCACTCAACTGCACAAACCACA
Cs-TPS-	AGCCTCCGCTGTTATTTGTAGGCT	GCAGAGGCCACATGACCTCGC
Cs-TPS-	TAGCTGAAATCTGCAGGTGGTGGA	ACACTCCACAATTCGATCCCTCGC
Cs-TPS-	TGGACGACATCGCTTCCCACA	TCCTTCCATGCATCAACCACTTCCT
Actin	GCCGAGGATATTCAGCCCCTCGT	CCAGTATGCCGGGGACGACCA

APPENDIX II

Table II Flower, axil and side branch numbers per plant of JA and SA treated cucumber plants.

Treatment	Flowers per plant, mean	Axils per plant, mean	Flower/axil ratio	Side branches
Control	8.3 ± 2.8	18.2 ± 1.0	0.46 ± 0.17	19
SA	9.5 ± 3.1	18.6 ± 0.8	0.52 ± 0.16	14
JA	5.4 ± 2.2	17.1 ± 2.2	0.28 ± 0.14	4
SA/JA	7.9 ± 1.4	18.0 ± 1.3	0.44 ± 0.07	5