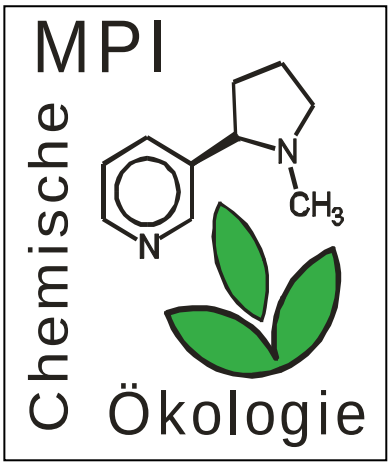

Diplomarbeit

Multivariate analysis of volatile emission from wild tobacco *Nicotiana attenuata* using GCxGC-ToFMS

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eingereicht von

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Abstract

The release of volatile organic compounds (VOCs) is one of the plant specific responses to insect attack. The specificity of this physiological and adaptative response is based on the perception of elicitors contained in the oral secretions (OS) of insect larvae. Fatty acid amino acid conjugates (FACs) are the best characterized class of VOCs elicitors but to which extend they control the total OS-induced VOC emission is only partly understood. To that attempt, we produced sub-fractions of *Manduca sexta* larvae oral secretions (OS), purified by ion exchange chromatography (FACs-free OS) or simply boiled to denature their protein content, and monitored, as well as that of synthetic FACs, 2-hydroxyoctadecanoid and β-glucosidase (two OS constituents), their capacity to induce VOCs production in the wild tobacco *Nicotiana attenuata*. VOCs extracts obtained at different time-point post-treatment were analyzed following an unbiased approach combining comprehensive resolution of those mixtures by GCxGC-TOFMS analysis and the use of multivariate statistics. The work-flow developed for this study allowed us to partly identify VOCs markers discriminating samples at the treatment level. If our results confirm the central role fulfilled by FACs; they also show that FACs do not fully explained the reconfiguration VOCs production herbivory and highlight for instance the effect of β-glucosidase in the emission of eucalyptol during the dark phase. In parallel, this work also provides a conceptual and reliable approach of analysis GCxGCTOFMS-generated data.

Abstrakt

Die Freisetzung flüchtiger organischer Verbindungen (VOCs) ist ein pflanzenspezifischer Abwehrmechanismus gegen natürliche Fraßfeinde, im speziellen gegen Insektenlarven. Die Ausprägung dieser physiologischen und adaptiven Fraßfeindabwehr basiert auf der Zusammensetzung des Larvensekrets, das verschiedenste Substanzen enthält, die den Abwehrmechanismus auslösen. Eine der am besten charakterisierten Substanzklassen ist die der Fettsäure-Aminosäure Konjungate (FACs), aber inwieweit diese an der, durch Larvensekret, induzierten VOC-Emission beteiligt sind, ist bis jetzt nur teilweise verstanden.
Für eine detaillierte Untersuchung haben wir aus dem Sekret des Tabakhornwurms, *Manduca sexta*, verschiedene Fraktionen hergestellt und ihren jeweiligen Einfluss auf die induzierte VOC-Synthese im wilden Tabak, *Nicotiana attenuata*, beobachtet. Wir verwendeten im Einzelnen folgende Fraktionen: durch Ionenaustauschchromatographie von FACs befreites Larvensekret, gekochtes Larvensekret, in dem enthaltene Proteine denaturiert sind, außerdem eine synthetisch hergestellte FAC Lösung, sowie weitere im Larvensekret enthaltene Induktoren. Die nach der Pflanzenbehandlung entstandenen VOCs, wurden über verschiedene Zeiträume gesammelt und anschließend mit Hilfe zweidimensionaler Gaschromatographie, gekoppelt mit einem Flugzeitmassenspektrometer, analysiert. Die Auswertung der erhalten Daten erfolgte unter Zuhilfenahme multivariater statistischer Methoden.
Der Versuchsaufbau unsere Studie ermöglichte uns teilweise, unseren Fraktionen spezifische VOC-Marker zuzuordnen. Obwohl unsere Ergebnisse nahe legen, das FACs die zentrale Rolle bei der VOC-Emission spielen, erklären sie jedoch nicht vollständig die veränderte VOC-Synthese nach Fraßfeindbefall. Beispielweise ist die Emission von Eukalyptol während der Nachtphase von ß-Glucosidase abhängig.
Zusätzlich bietet diese Arbeit eine grundlegende und verlässliche Methode zur Auswertung von GCxGC-ToF Daten.

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Abbreviations:

DMAPP	dimethylallyl diphosphate
GPP	geranyl diphosphate
IEX-OS	Ion-exchanged purified oral secretion
IPP	isopentenyl diphosphate
FACs	fatty acid amino acid conjugates
FPP	farnesyl diphosphate
2-HOT	2-hydroxyoctadecatrienoic acid
HPL	hydroperoxide lyase
HR	hypersensitive response
JMT	jasmonic acid carboxyl methyl-transferase
LOX	lipoxygenase
OS	oral secretions
PI	proteinase inhibitors
VOC	volatile organic compounds
ToFMS	time-of-flight mass spectroscopy

1. Motivation

The aim of this work was to investigate the eliciting activity of the different constituents of the oral secretion of *Manduca sexta* larvae to wounded leaves of *Nicotiana attenuata*.
When attacked by *M. sexta*, *N. attenuata* plants release a mixture of volatiles attracting natural enemies of this phytophageous insect. FACs (fatty acid amino acid conjugates) are well characterized elicitors contained in *M. sexta* oral secretions (OS) which are responsible for the induction of both direct, but also indirect defense responses.
Nevertheless, a characterization of volatiles specifically induced by FACs has never been exhaustively detailed. It is therefore worth to as the following questions.

i) Are the bouquets of volatiles emitted after FACs treatment and natural herbivory really the same?
ii) Could others spit factors trigger emission of volatiles?
iii) If so, would these mixtures of volatiles be qualitatively and quantitatively similar, and release in a comparable time scale

Other components recognized as defense elicitors by other plant systems have been described in insect oral secretion. For instance, β-glucosidase is contained in *P. brassicae* regurgitant and triggers volatiles emission when it contacts wounded leaves from cabbage plants [1]. The case of this elicitor is highly interesting since the activity of this enzyme has been measured in *M. sexta* regurgitant [2].

In the same way, 2-hydroxyoctadecatrienoic acid (2-HOT) is accumulated in *M. sexta* OS and potentiates herbivory-induced signalling cascades when it contacts damaged *N. attenuata* leaves.

Using different treatments containing these synthetic elicitors and combinations of biochemically modified OS, we would like to decipher the individual role of these different OS components when they contact damaged leaves during *M. sexta* herbivory.

An unbiased approach of this question should allow resolution of volatile mixtures coupled to statistical analysis, with the aim of assigning a specific elicitor component of *M. sexta* OS to the different subsets which compose the bouquet of volatiles released during herbivory. Two-dimensional comprehensive gas chromatography coupled to time-of-flight mass spectroscopy provides mass spectra with quality and sensitivity largely improved by enhanced resolution and zone compression, which are features of two-dimensional gas chromatography.

Therefore this analytical tool is used to obtain peak tables reaching the level of information necessary for statistical analysis. A data processing method, using the ChromaTOF (LECO, St. Jospeh, MI, USA) software, was adapted to provide comparability between the obtained data of all plant treatments. Multivariate statistical analysis will be applied to highlight volatile markers discriminating groups of treatments.

2. Plant-Insect Interaction

Plants have not only to fight with other competitors for nutrients, but also to defend efficiently against attacking heterotrophs, like phytophagous insects. The defensive mechanisms should be effective against a great number of possible enemies, ranging from microbes to insects.

To defeat microbes, plants have developed the hypersensitive response (HR). Cells surrounding an infection site die rapidly and fill afterwards with antimicrobial components. This prevents the spread of the pathogen and is one of the best-studied examples of plant defense. However, such a response requires a sophisticated recognition system and is not effective against free-living and -moving insects. Many herbivorous larvae complete development on plants after oviposition and are fully equipped with mandibles to break through protective coverings, and a digestive tract build to break down and assimilate plant parts after feeding. Because of these well-prepared and mobile enemies, plants are forced to evolve a different way to handle their attack.

2.1 Direct Defense

Defensive responses can be divided into two different types: direct and indirect. Direct defense includes thorns, and trichomes but also primary and secondary metabolites. There plant defenses directly affect the attacking herbivore without any mediators. Secondary metabolites can be categorized by the way they influence insects. Proteinase inhibitors (PI) are proteins which inhibit the same insect's digestive enzymes [3],[4]. Polyphenol oxidases decrease the nutritive value of the wounded plant by converting nutrients to indigestible compounds [5,6]. Other compounds like alkaloids or terpenoids are toxic for generalists and force specialists to invest resources in detoxification.

2.2 Indirect Defense

In contrast to the direct defense, is indirect defense the attraction of predators or parasitoids of herbivores and the increase in carnivore's foraging success by plants [7]. Volatile organic compounds (VOCs) released by herbivore-attacked plants are known to be attractive to predators and parasitoids in laboratory experiments [8] and have recently been shown to have a defensive function under natural conditions. [9].

The release of VOCs can be highly specific. Parasitic wasps use the emitted blend to locate the particular host plant or even a specific herbivore on the plant [10]. There are examples in the literature which show the ability of predators to distinguish the different bouquets emitted after different herbivore attacks [11]. Studies also indicate a different response of infested plants to different types of enemies. Emitted VOCs can also function as a direct defense by repelling ovipositioning herbivores [12],[9].

2.3 Elicitors from Herbivores

As mentioned above, plant response to an attack can differ depending on the type of attacking herbivore. Thus, plants are able to discriminate among insects, which indicates a presence of different elicitors in different herbivores. Any compound from herbivores that interacts with plants on a cellular level is a potential elicitor. Herbivore-specific elicitors have been isolated from oral secretions of different lepidopteran (moth and butterfly) species. Oral secretions come in contact with plant wounds, when herbivores attack. Two classes of elicitors, both of which elicit direct defense, have been isolated. The first class contains lytic enzymes, the second fatty-acid-amino-acid conjugates (FACs).

β-Glucosidase is such a lytic enzyme and was shown to trigger the emission of terpenoids from cabbage plants [1]. Many other enzymes such as glucose oxidases, alkaline phosphatase and watery digestive enzymes were found in the saliva of many lepidopteran species. Their roles as elicitors have not been established yet.

Fatty-acid-amino-acid conjugates have been found in the reguritant of many herbivores [13-15]. One of these conjugates, isolated in the oral secretions of *Spodoptera exigua,* is volicitin (N-(17-hydroxylinolenoyl)-L-glutamine). This elicitor induces excised maize seedlings to release a bouquet of volatile terpenoids, that is similar to the one released by caterpillar feeding.

The early steps in the herbivore elicitation process are still unknown. Some mechanisms have been suggested but there was not found similar a receptor comparable to the recognition of pathogens. The only system that demonstrates a link from specific elicitors to signaling to the subsequent response is the *Nicotiana attenuata – Manduca* system. [16]

2.4 Volatile Organic Compounds

Emission of VOCs of many plant and plant-caterpillar systems has been examined. In all systems the released wound- and herbivore-inducible bouquets of volatiles function as signals in tritrophic interactions. Analysis of the released volatiles has shown similarities between different plant species, but there are also many species- and herbivore-specific elicited compounds. There are at least three biosynthetic pathways from, which volatiles are derived.

2.4.1 Green Leaf Volatiles (GLVs)

So called green leaf volatiles, C6 volatiles, are produced in the octadecanoid pathway from α–linolenic acid. After some earlier steps in wound response, linolenic acid is released from the plasma membrane. This is the initial step of the octadecanoid pathway (figure 1).

LOX is a nonheme iron-containing dioxygenase and catalyzes the oxygenation of linolenic acid to the 9- and 13-hydroperoxides. One of these peroxides, 13(S)-HPOT, is a substrate for several other enzymes including HPL. This lytic enzyme cleaves the hydroperoxides into C6-aldehydes and C12-oxoacids.

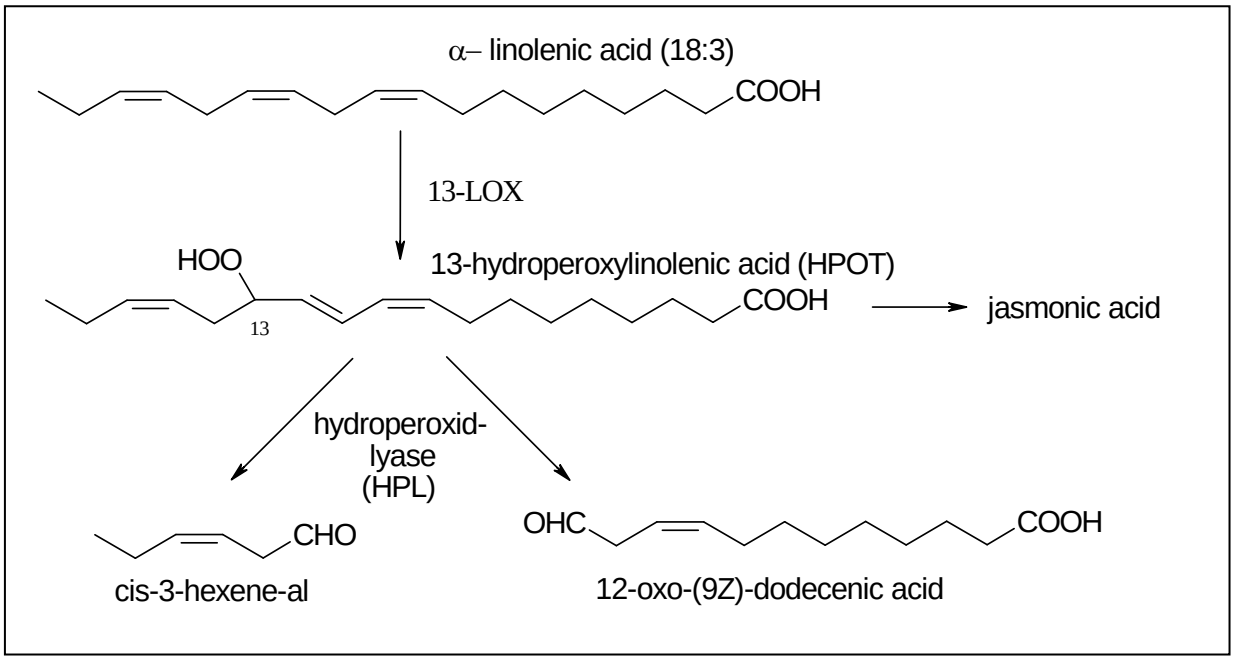

Figure 1: octadecanoid pathway

These C6 aldehydes and their derivatives, C6 alcohols and a great number of esters, so called green leaf volatiles may function in both direct and indirect defense or as signals with information content. Green leaf volatiles play a major role in defense by protecting the wound site from microbial protecting or acting as a direct defending agent. One of the key compounds that is found after herbivore attack is *cis*-3-hexen-1-ol [17,18]. Another volatile, which is emitted after herbivore attack and functions as a biocide, is *trans*-2-hexenal [12]. This volatile elicits the accumulation of sesquiterpernoid phytoalexins in wounded cotton and *Arabidopsis*, suggesting a role in intra- and interplant signaling. Other products derived from 13-hydroperoxy linolenic acid can further be processed to produce jasmonic acid, an important phytohormone, by enzymatic cyclization, reduction, and β-oxidation.

2.4.2 Tepernoids [19]

The second large group of volatiles that are emitted as a response to herbivore feeding is represented by terpenes. These compounds are produced by the two isoprenoid (mevalonate and nonmevalonate) pathways. The initial compound for the mevalonate pathway (figure 2) is mevalonic acid, which is retrieved from acetyl-CoA. After some phosphorylation steps isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are formed. Both condense to form higher order terpenoid precursors, including the monoterpenoid precursor geranyl diphosphate (GPP) and the sesquiterpenoid precursor farnesyl diphosphate (FPP).

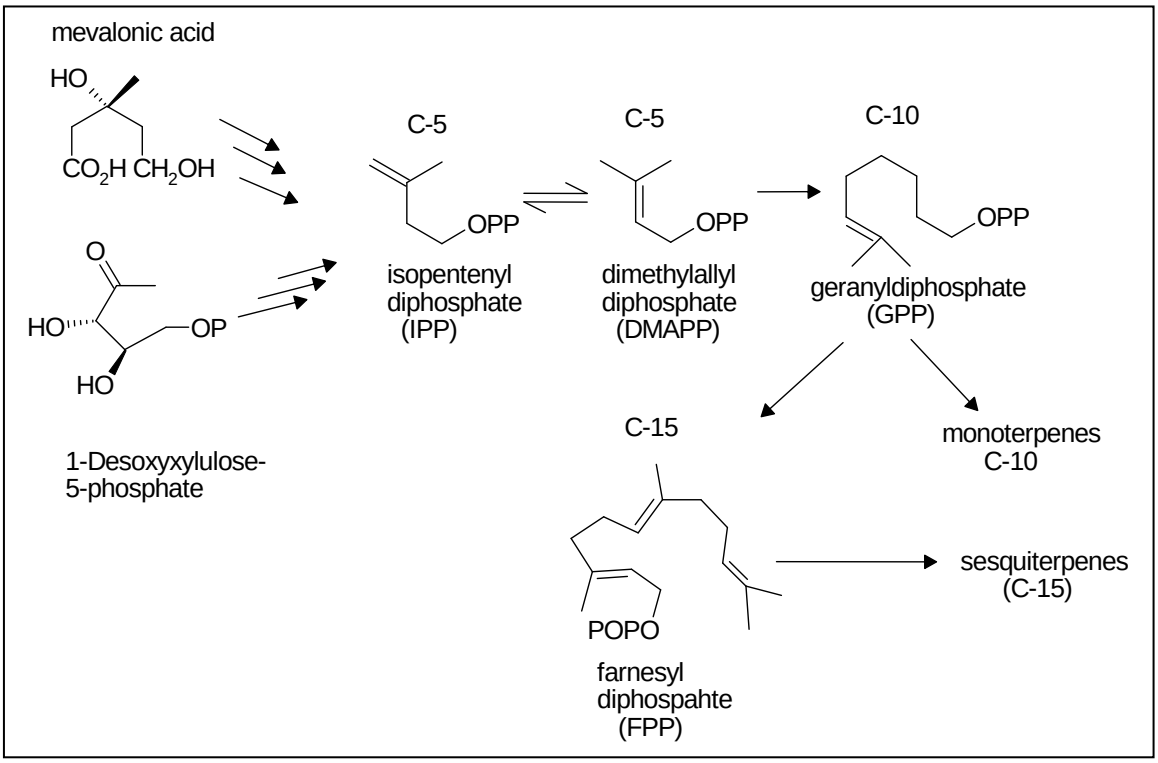

Figure 2: mevalonic acid pathway

Mono- and sesquiterpenes play a major role in attracting predators and parasitoids [18] and function as phytoalexins [20]. Many terpenoids are emitted transiently and systemically after herbivore wounding [8,17,18]. For example, naphthalene-containing terpenoids are selectively activated by the FAC volicitin, which is derived from caterpillar regurgitant

2.4.3 Shikimate derived volatiles

The third group of volatiles detectable in the headspace of wounded plants is derived from the shikimate pathway. This pathway (figure 3) starts with shikimic acid, which is converted into two amino acids phenylalanine and tryptophan. Salicylic acid and the esterification product methyl salicylate are derived from phenylalanine, whereas indole is derived from tryptophan.

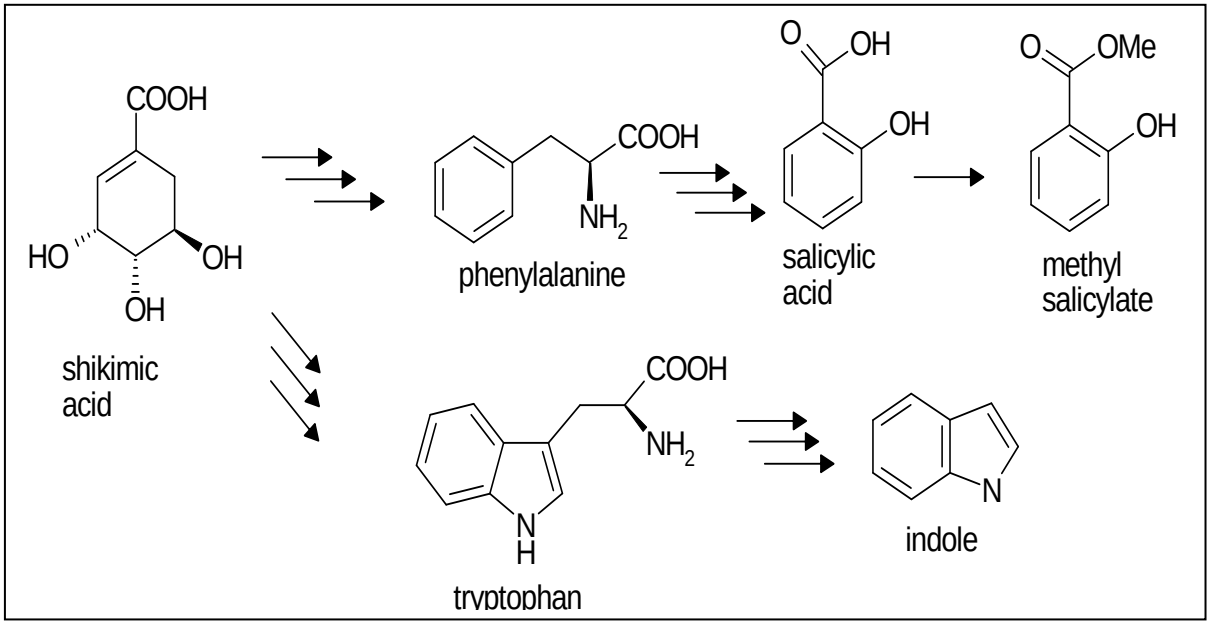

Figure 3: shikimate pathway

Methyl salicylate is produced in this pathway and is part of the VOCs emission after herbivore damage, but not after mechanical wounding in lima beans and wild tobacco. Application of methyl salicylate to lima bean attracted foraging predatory mites [21] but did not attract predatory bugs foraging on wild tobacco in a field experiment [9]. Indole is another shikimate-derived metabolite, which is released from maize seedlings after attack by beet armyworm caterpillars but not after mechanical damage. The released bouquet of maize, containing terpenoids and indole, is attractive to an endoparasitic wasp, which attacks larvae of several lepidoptera species [18].

It has been demonstrated in nature that VOCs attract predators and parasitoids and reduce the herbivore load of the plant, but a direct proof of fitness benefits for plants is still lacking. Understanding how VOCs are released after herbivore attack and manipulating these mechanisms will provide such a proof. Agricultural plants engineered to release specific volatiles after herbivore attack could provide the basis for enhanced biological methods to control agricultural pests.

2.5 Plants and Insects used

2.5.1 *Nicotiana attenuata*

Nicotiana attenuata, part of the Solanaceae family, is an annual wild plant native to the Southwestern USA. Large populations germinate from long-lived seed banks after fires in desert habitats. The germination takes place as a response to factors in wood smoke [22-24]. This 'fire-chasing' behavior forces the plant's herbivore community, to reestablish itself with every new population. As a consequence of this particular germination behavior,

Figure 4: *Nicotiana attenuata* in natural habitat [insituts ordner)]

N. attenuata is thus attacked by a large number of different herbivore species and has evolved a plethora of induced responses against herbivore attack. Moreover, this germination behavior ensures that the plant will compete primarily with conspecifics (due to the large synchronized germinations that occur after fires) and in turn selects for rapid growth in the post-fire nitrogen-rich soils.

The same pressures have selected for strong growth-defense tradeoffs, as the plants adjust their phenotypes to the environment they find themselves in.

2.5.2 *Manduca Sexta*

Manduca sexta L. is a moth of the family Sphingidae present through much of the American continent. It is commonly known as the tobacco hornworm. The larvae feed on the foliage of various plants from the family *Solanaceae*. *M. sexta* has mechanisms for selectively sequestering and secreting the neurotoxin nicotine present in tobacco.[25]

M. sexta is a common model organism, especially in neurobiology, due to its easily accessible nervous system and short life cycle. It is used in a variety of biomedical and biological scientific experiments.

Figure 5: *Manduca Sexta* larvae feeding

3. Methods and Material

3.1 Plant material

We used an inbred line of *N. attenuata* Torr. *ex* Watson (synonymous with *N. torreyana* Nelson and Macbr.; *Solanaceae*) derived from field-collected seeds [26]. Germination of plants occurred as described below.

3.1.1 Germination media:[27]

1x strength of Gamborg's B5 (Duchefa, http://www.duchefa.com) was dissolved in 980mL distilled water. pH was adjusted to 6.80 and afterwards 6g plant agar was added. The solution was autoclaved at 121ºC for 20 min. The media was poured into 100x20mm petri dishes.

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3.1.2 Germination procedure:

The seeds were incubated for 5 min. in 5 ml of a freshly prepared sterilization solution containing 0.1g dichloroisocyanuric acid an 50µl of 0,5% Tween-20 detergent stock solution. After 5 min. the solution was decanted and seeds were 3 times rinsed in sterile water.

50µl of a 0.1 M gibberellic acid solution and 5 ml of a 50:1 solution of "Liquid Smoke" (House of Herbs, Passaic NJ) were added to the seeds. They were incubated for 1 hour.

After 1 hour, the solution was decanted and the seeds were rinsed in sterile water three times. Afterwards they were placed gently on the germination media. The petri dish was sealed and incubated with the following regime set: 27ºC/16hrs 100% light, 24ºC/8hrs dark. After 6 days seedlings could be clearly seen. Seedlings were transffered to 1 L individual pots and grown in the glasshouse at 26-28 ºC under 16 h of light supplied by Philips Sun-T Agro 400- or 600-W sodium lights.

3.2 VOCs eliciting treatments

All experiments were performed on plants in the rosette stage. Six replicates for each treatment were randomly assigned into nine groups (eight treatment groups, one control group). The second fully expanded leaf (+1 position) from each plant was used. Plants were either not treated (control), or wounded using a fabric pattern wheel to punch three rows of holes on each side of the mid vein. 20 µL of the eliciting solution was pipetted directly onto the wounded leaf and gently dispersed across the surface.

Figure 6: wounding technique on *solanum nigrum* plants.

3.2.1 Buffer

Volatile emissions strictly induced by the mechanical wounding and/or the alkaline pH of oral secretion (OS) were assessed by application of a 0.1 M Tris pH 9 buffer solution containing 0.1 % *v/v* Triton X-100. This non ionic surfactant, which was used for the preparation of the fatty-acid-amino-acid-conjugate (FAC) solution, was added to monitor its potential eliciting effect.

3.2.2 *M. sexta* Oral secretion

OS were collected from 3rd - to 4th -instar *M. sexta* larvae reared on WT plants by treating them gently with a small tubing and collecting the regurgitant via a vacuum pump. The collected reguitant was centrifuged at 4° and the supernatant was collected. The centrifuge tube was flushed with argon and stored at -20ºC.

3.2.3 Boiled oral secretion, β-glucosidase, FAC, and 2-HOT

To evaluate the influence of enzymatic activities present in OS, an aliquot from the OS batch was heated at 100°C for 20 min.

Synthetic N-linolenoyl-l-glutamine (C18:3-Gln) and N-linolenoyl-l-glutamate (C18:3-Glu), the two dominant FACs, 2-HOT (2-hydroxyoctadetrienoic acid), (Larodan Fine Chemicals, Malmö, Sweden) and commercial almond, *prunus dulcis*, β-glucosidase (Sigma, Steinheim, Germany) were diluted in the buffer solution respectively at 0.12 nmol*μL^{-1}, 0.34 nmol*μL^{-1}, 0.170 nmol*μL^{-1} and 25 U*μL^{-1}. These concentrations were similar to those found in *M. sexta* OS.

3.2.4 Ion-exchanged oral secretion (IEX-OS) [14]

IEX-OS, that were FAC- and (2-HOT)-free, were prepared by filling six Millipore columns (0.45µm pore size) with 300mg Amberlite IRA-400 resin (Sigma, Steinheim, Germany). Columns were 3 times conditioned with 400µl distilled water. 400 µl of the supernatant from OS collection were added and columns were centrifuged for 10 min. Afterwards the supernatant was transferred to the next Millipore column and again centrifuged for 10 min. This was done for all columns and at least six purification steps were performed. The obtained IEX-OS was stored at -20ºC and diluted 1:1 *v/v* with a two times concentrated buffer solution before using.

3.3 Volatile Collection

Treated leaves were enclosed, 1 hour after elicitation (time needed to complete all the treatments), in two 50 mL food-quality plastic containers (Huhtamaki, Espoo, Finland) secured with miniature claw-style hair clips. Ambient air flowed into the cage primarily through a charcoal filled glass tube secured in a clipped-off P1000 pipette tip inserted into the bottom container, and was pulled out through a self-packed glass tube (ARS Inc., Gainsville, FL, USA) containing glass wool and 20 mg of SuperQ (Alltech, Deerfield, Il, USA), secured in a second clipped-off P1000 pipette tip inserted into the top container.

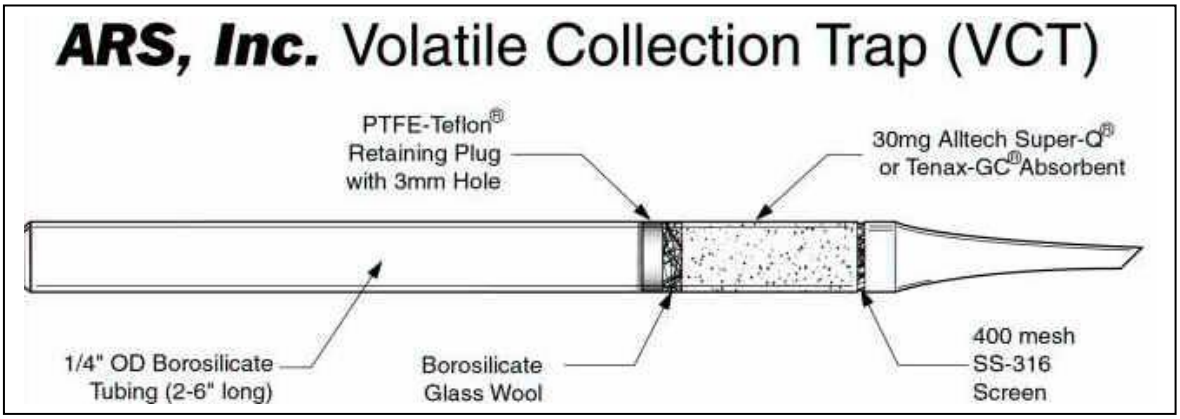

Figure 7: schematic design of a trap for volatile collection
[http://www.ars-fla.com/_fpclass/fp_volatile.html 15.1.2008 10.30]

Airflow was powered by a manifold vaccum pump (model DAA-V114-GB; Gast Mfg., Benton Harbor, MI, USA) as described in [17]. Plant volatiles were collected during a 24 h period with12 hours, the second one occurring during the glasshouse dark time. Background contaminants present in ambient air were also collected using empty trapping containers.

Figure 8: experimental design

Immediately after collection, traps were spiked each with 400 ng tetraline as an internal standard (IS) and eluted with 250 µL dichloromethane into a GC vial containing a glass insert.

3.4 Instrumental parameters

An Agilent 6890N gas chromatograph equipped with an Agilent 7683 auto-injector (Agilent Technologies, Santa Clara, CA, USA) coupled with a LECO Pegasus III time-of-flight mass spectrometer with a 4D thermal modulator upgrade (LECO, St. Joseph, MI, USA) was used to collect the 3D GCxGC-TOFMS data. The GC inlet and transfer line were held constant at 250 ºC. Splitless injections of 1 µL were made onto an unpolar RTX-5MS column of the GC x GC (20 m x 250 µm i.d. x 0.5 µm, Restek, Bellefonte, PA, USA). The collected first column effluent was transferred to an midpolar DB-17 column (0.890 m x 100 µm i.d. x 0.1 µm J&W Scientific, Folsom, CA USA) every 6 s (modulation time). The first column was held at 40 ºC for 5 min and then the temperature increased 5 ºC per min up to 190 ºC and finally 25 ºC per min to 250 ºC, where it was held for 5 min. The second column was initially set at 45 ºC and followed the same temperature program as C1, giving a total run of 45 min. The modulator was kept 30 ºC higher than column 1. The ion source was set to 250 ºC. Data were collected, after a solvent peak delay of 120 s, in the *m/z* range 50 to 400, at a rate of 200 spectra per second.

4. An introduction to comprehensive two-dimensional gas chromatography

Because of the great number of volatiles released by wounded *N. attenuata* it is necessary to use an analytical tool, which is able to detect and to resolve them in a proper way. Due to the measurement of volatile substances gas chromatography is the method of choice.

The separation of compounds by gas chromatography is affected by two factors: the interaction with the stationary phase influenced by functional groups, and the volatility caused by vapor pressure. When analyzing plant volatiles, a large number of compounds with different chemical properties such as alcohols, esters and terpenoids must be included. Comprehensive two-dimensional gas chromatography coupled to time of flight mass spectroscopy (GCxGC-ToFMS) provides an excellent tool for combining the two factors polarity and volatility by using an orthogonal separation system.

To create an orthogonal separation, it is necessary to combine two independent retention mechanisms. This means selection of a non-polar column where separation is achieved by differences of volatility, for the first dimension. The subsequent second-dimension separation occurs in only a few seconds and therefore under isothermal conditions. Hence retention in the second column is controlled by the interactions of the analytes with the stationary phase. The separation is indeed orthogonal. [28]

It is not only necessary to use independent separation mechanisms but also analysts have to assure that they preserve the separation from the first dimension through the second one. To solve this problem an interface between both columns is needed. This interface, or modulator, is a main part of a GCxGC system. There have been many approaches to create such a modulator, which are well described in the literature [29,30].

Figure 9 describes the problem when carrying out a two-dimensional separation without a modulating interface. It is possible, that obtained resolution in the first dimension is lost in the second dimension. Former separated peaks could reunite on the second column. Also a change of the elution order compared to the first column could occur. An interface solves these problems by subdividing second dimension separation into modulation cycle.

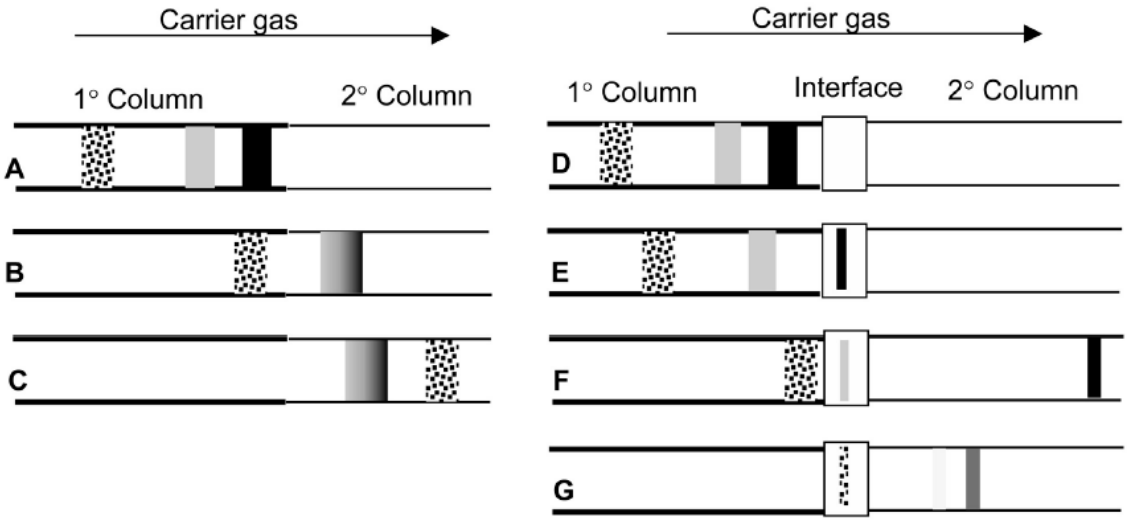

Figure 9: modulator function [31]

In this section I only focus on the dual stage cryogenic jet modulator because this modulator is used in our system. As mentioned above, the main function of a modulator is to preserve the separation reached in the first column. The cryogenic dual-stage jet modulator is placed on the beginning of the second column and is working with two jets, which can alternate. The first jet is for a cold flow using liquid nitrogen and the second jet for a hot flow, using compressed air, heated by the column oven. ***Figure 10*** shows operation of the modulator. If the downstream jet is on while the upstream jet is off compounds are trapped in a narrow band within the start of the second column (B). When the upstream is activated, substances from the first dimension column are focused. At the same time the downstream jet switches to heated air and the first fraction is injected as a short pulse into the second dimension column (C). After that the downstream jet switches back to liquid nitrogen and the next eluting compounds are focused (D). The span of time between two cooling periods of the downstream jet is called the modulation time.

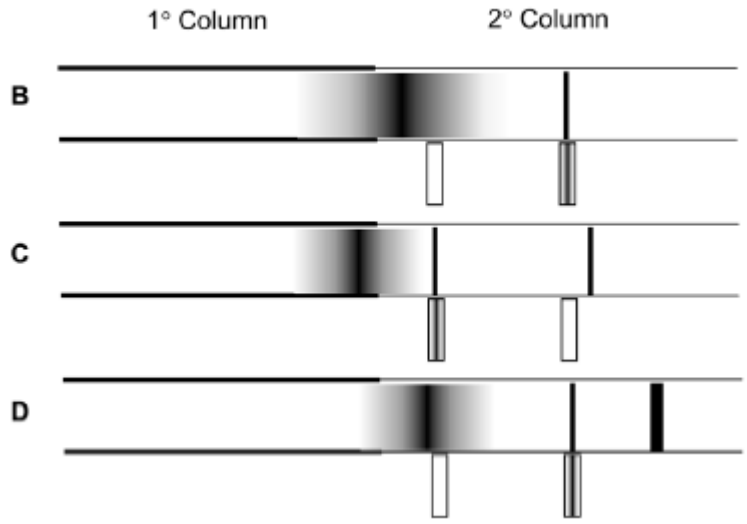

Figure 10: operation of a dual-stage cryogenic modulator [31]

The modulation time has a major influence over second dimension resolution. A modulation cycle should last longer than the retention times of the compounds most strongly retained in the second column. Otherwise there will be a so-called wrap around and compounds may elute together with them of next modulation cycle.

Due to the modulation the peaks eluting from the second column have a very narrow peak width. Hence a detection system, which is able to recognize all compounds, is needed. In the past it has been shown that coupling of mass spectroscopy to chromatographic separations is a powerful tool to analyze multi component systems. It provides not only a separation of chemical substances but also structural information. Normal sequential mass spectroscopic methods (e.g. quadrupol mass spectroscopy) do not offer acquisition rates which are fast enough. A change to a simultaneous method is necessary. The time-of-flight mass spectroscopy (ToFMS) is a method which provides appropriate acquisition rates. It is well established and allows rates up to 500 spectra per second. Thus the coupling of the ToFMS to a two-dimensional GC system is the best choice for a detection system.

5. Results and Discussion

5.1 Data processing and mining work flow

The LECO ChromaTOF software v2.21 (LECO, St. Jospeh, MI, USA) was used throughout the analyses to control the instrument, as well as to acquire and process the data (including automatic peak deconvolution). Chemometric analysis of comprehensive two-dimensional separations has the potential to extend the information gained from GC x GC [32-34]. However, there is still work required for such approaches to fully utilized, especially in the development of retention time alignment strategies.

The first goal of this study was to develop a reliable data processing work-flow allowing selection of the relevant information from each individual chromatogram and comparison of the effect of the different treatments. Figure 11 summarizes the data processing work-flow.

5.1.1 Peak deconvolution

Chromatograms were deconvoluted using the LECO ChromaTOF software. The time-window RT 1D: 150 -1500 s; RT 2D: 1.5 - 6 s, since earlier experiments showed that most of the VOC blend was resolved in this window, was selected for processing. After exclusion of known artefact peaks, such as column bleed, plasticizers, or reagent peaks, data matrixes contained approximately 500-700 compounds.

However, the inconsistencies in the peak tables made it difficult to place the peak information into a suitable matrix format, where rows represent the individual peaks, columns the individual samples and values the peak intensities.

5.1.2 Matrix aligment

As previously shown by Shellie et al. [35], the comparison feature in the LECO ChromaTOF software simplifies the task of sorting the peak tables, since all chromatograms, compared to a unique reference contain post-processing the same numbers of peaks. To this attempt, each individual sample was compared to a reference chromatogram issued from the analysis of single sample from an oral secretion treated plant, assuming the highest induction of volatiles. Comparison takes place in a time-window (RT 1D: 150 -1500 s; RT 2D: 1.5 - 6 s), because earlier experiments showed that most of the VOC blend was resolved in this window. Retention time information and the full mass spectrum of the selected peaks were automatically stored as reference data. This reference peak list was checked prior the comparison processing for known artefact peaks. Processed peaks were reported at a signal to noise ratio of 10. A mass spectrum similarity threshold of 500/1000 was used. Retention time window parameters for peak-comparison were set as one modulation period (6s) for the first dimension retention time and 0.2 s for the second dimension retention time, since this was of the order of the typical peak widths of non-tailing peaks

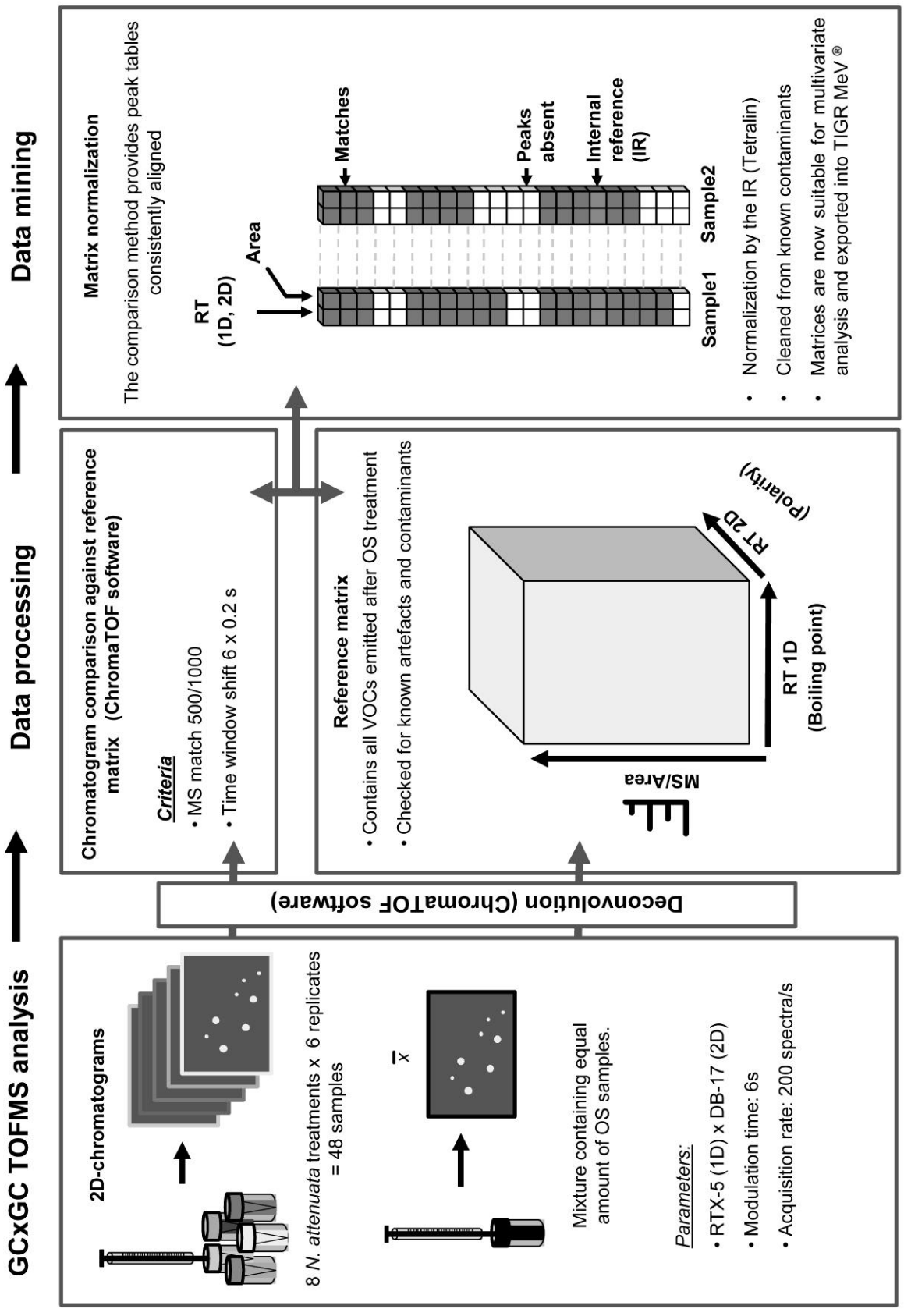

Figure 11: data processing procedure

5.1.3 Handling of missing values and normalization

Post-processing, analytes which were not found in the reference (*i.e.* not emitted during OS treatment) were excluded, using the Filter feature form the ChromaTOF software. The resulting peak tables, consistently ordered, were cut-and-pasted into an Excel spreadsheet. In terms of minimizing the effect of zero-substitution, 1 was uniformly added to all the areas and the raw data were normalized by the tetralin peak value as follows:

$$\frac{(\textit{analyte peak area}) \times 100}{(\textit{tetralin peak area})}$$

5.1.4 Data mining

The obtained data of the Excel alignment were exported into the TIGR MeV Software and log_2-transformed to achieve a normal distribution. A one-way ANOVA was performed by selecting 8 groups based on different treatments and setting the *p-value* threshold to 0.05. The intensity VOCs markers successfully filtered from the ANOVA analysis was standardized [36] (X_{auto}) as follows:

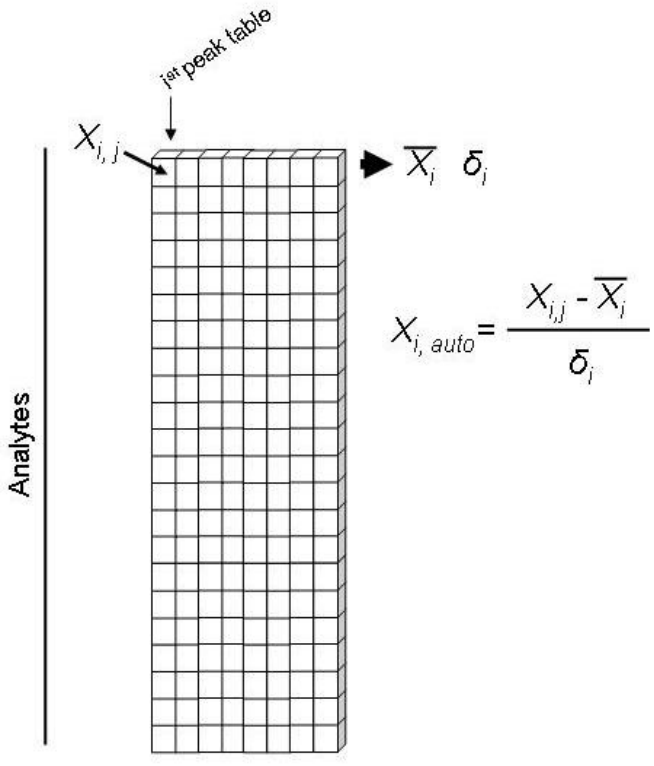

Figure 12: standardization

Cluster analyses were performed using on the autoscaled data (X_{auto}) as inputs. The cluster analysis is hierarchical agglomerative classification method resulting in a dendrogramm distribution of the tested objects. In a hierarchical agglomerative cluster analysis, objects are displayed as points in a vector space with each vector representing one variable. In this work, the Euclidean distance and complete linkage algorithm were chosen as clustering informations.

Data were processed by principal component analysis (PCA). PCA is an exploratory multivariate statistical technique for simplifying complex data sets. The goal of PCA is the reduction of dimensionality of the data set by finding *r* new variables out of *m* given observations with *n* variables. The *r* new variables are called principal components and representing linear combinations of the original variables. Therefore it is possible to ascribe meaning to what the components represent.

5.2 Analysis of early released VOCs blends

5.2.1 Hierarchical Cluster Analysis

When the normalized data is imported into the TIGR MeV software and cluster analysis is performed you can see three groups that are different from the other. The first group represents the unwounded control samples, ignoring one 2-HOT treated sample. This shows that wounding has an effect on volatile emission, which has been described extensively in the literature[9]. Second cluster consist of the samples treated with oral secretion. All FAC-treated plants are grouped in the third cluster while all other treatments seem to be summarized in the fourth cluster. For showing the similarity between these four clusters, the same cluster analysis was performed in STATISTICA 6.0.

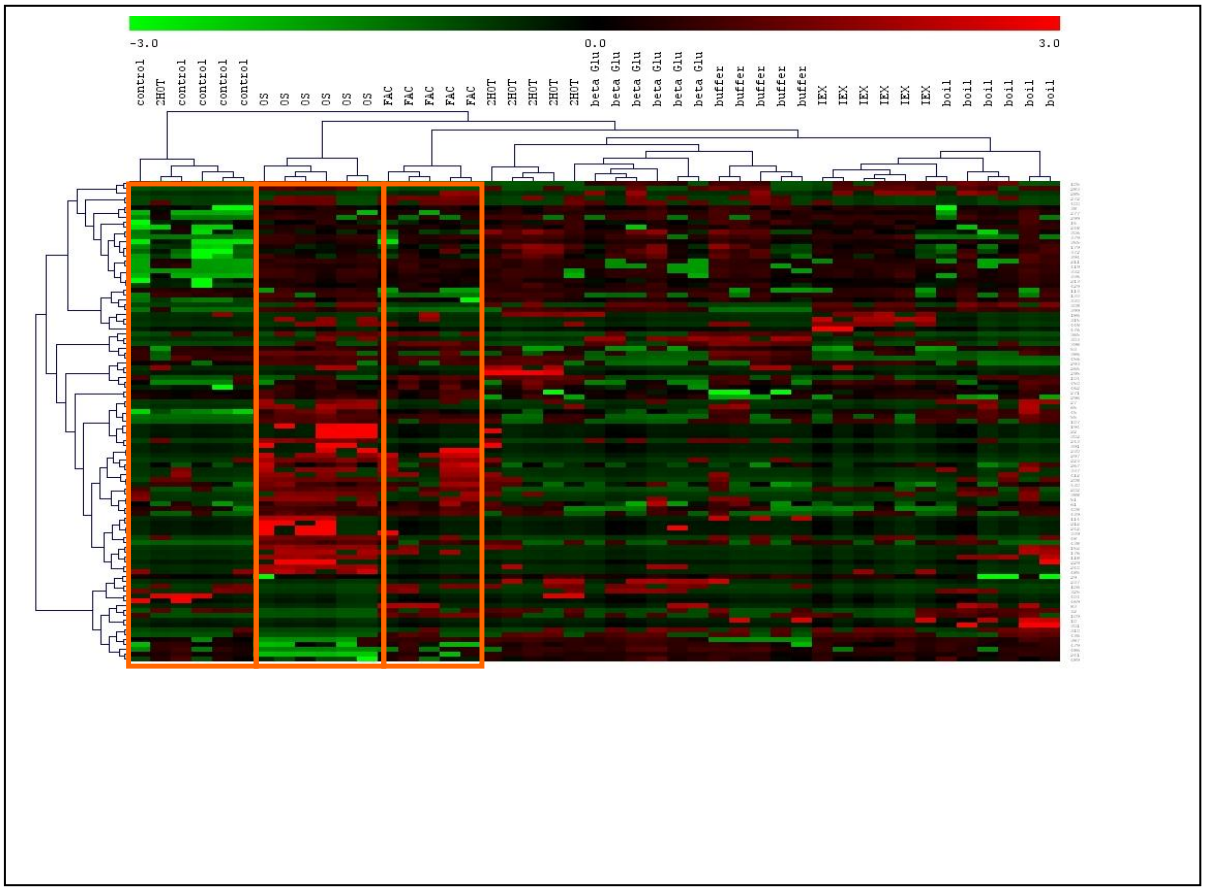

Figure 13: hierarchical cluster analysis of significant peaks (ANOVA)

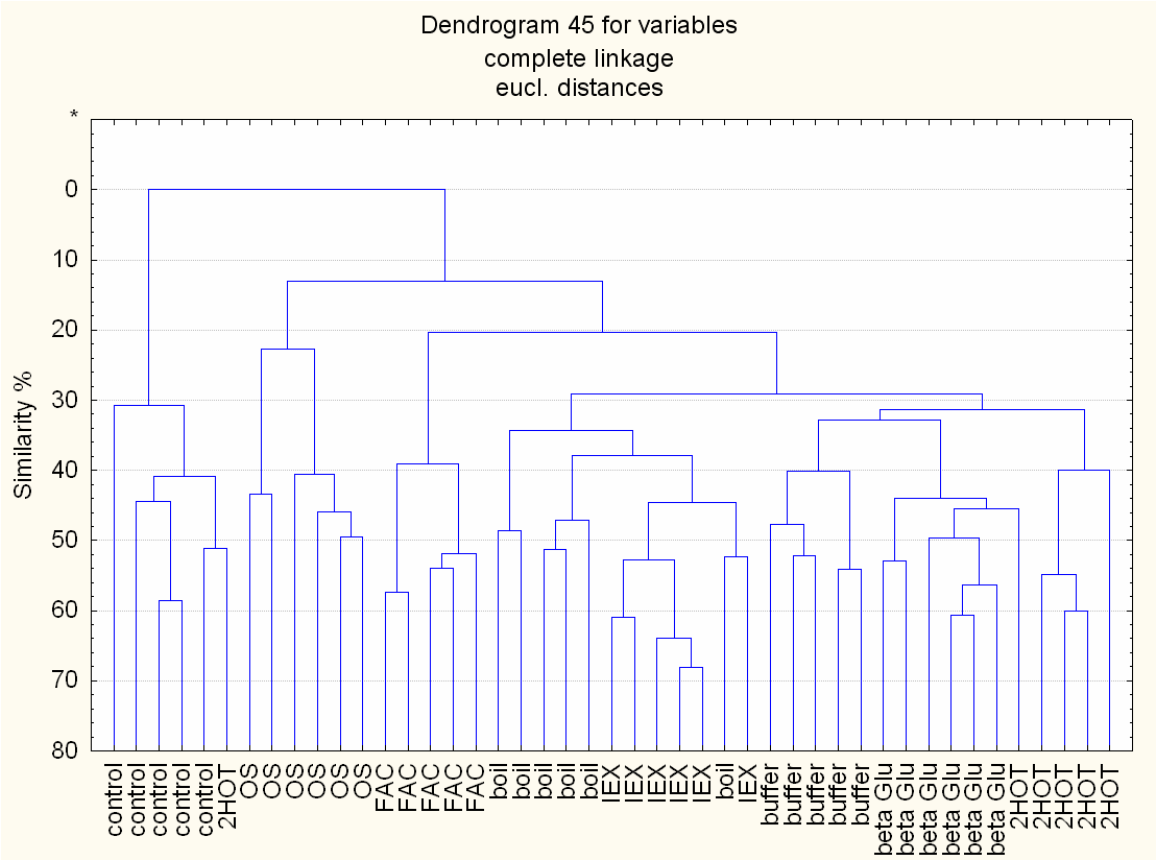

Figure 14: cluster analysis showing the similaritie

By separating clusters at a similarity close to 20 % in the obtained dendrogram, the former mentioned four clusters are well recognized. The high similarity between all samples is due to biological diversity, meaning that even with the same treatment VOC emission must not behave in a similar way.

5.2.2 Principal Component Analysis (PCA)

PCA was performed to confirm the grouping obtained from cluster analysis. This was also done by the TIGR MeV [37] software. PCA results in 45 principal components (PC). Components extracted by PCA are normally chosen by the Kaiser criterion, which means all components with an eigenvector greater than one are interpreted. Another way, of choosing components for interpretation, could be a screeplot (figure 14). Here, all the eigenvalues are plotted in their decreasing order and components are interpreted up to the point where the slope of this graph is decreasing.

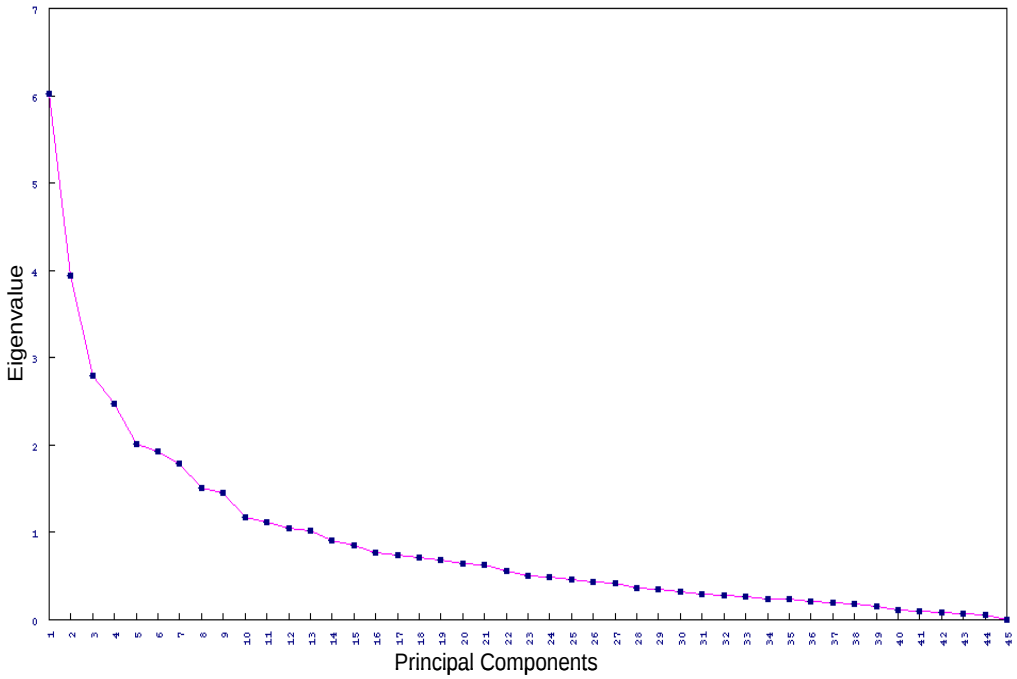

Figure 15: screeplot of principal components

According to the Kaiser criteria the first thirteen components and according to the scree plot the first five components should have been selected. The criterion which is optimized in the PCA is the variance. By applying PCA for visualization, we have to assume that the most interesting information is directly related to the highest variance in the data. Often this assumption does not hold and discriminative variables are hidden by the noise associated with biological and technical variability. Such a phenomenon was observed from our data set since the first PCA analyses performed using the raw total data (prior to one-way ANOVA filtering) as an input did not succeed in term of discriminating efficiently treatments (data not shown).

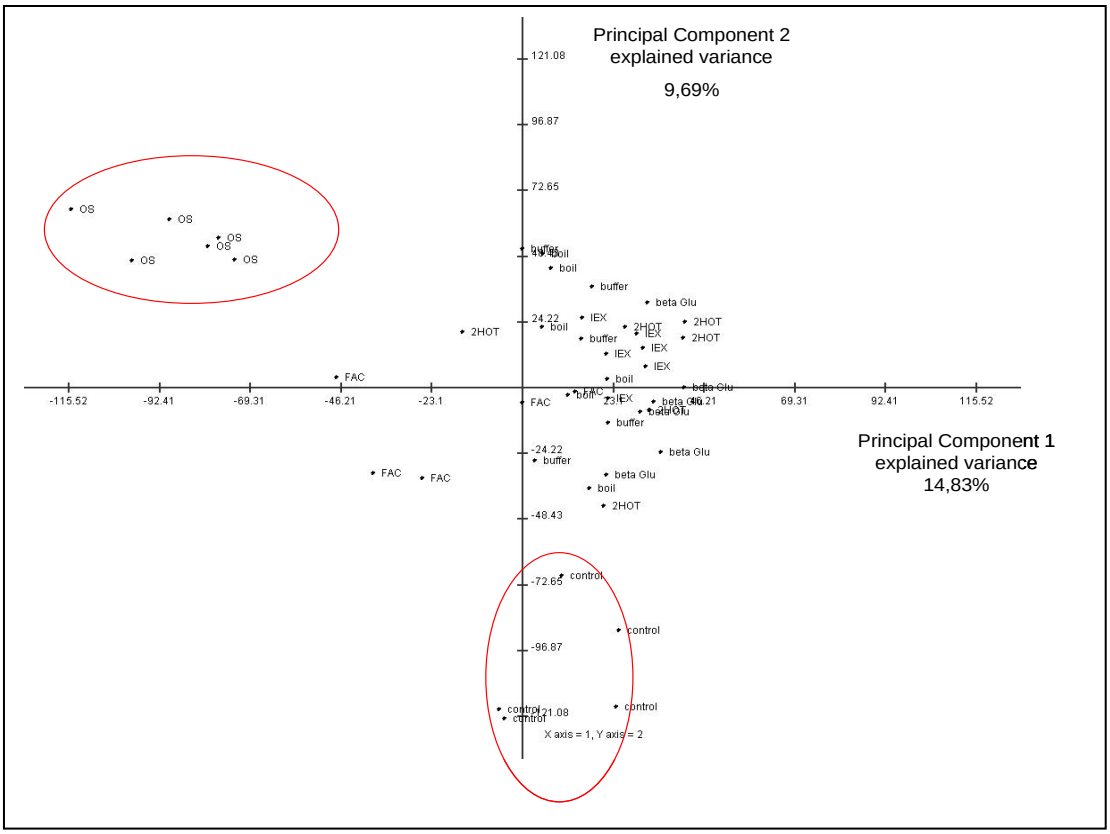

Figure 16: 2D plot of PC 1 against PC 2

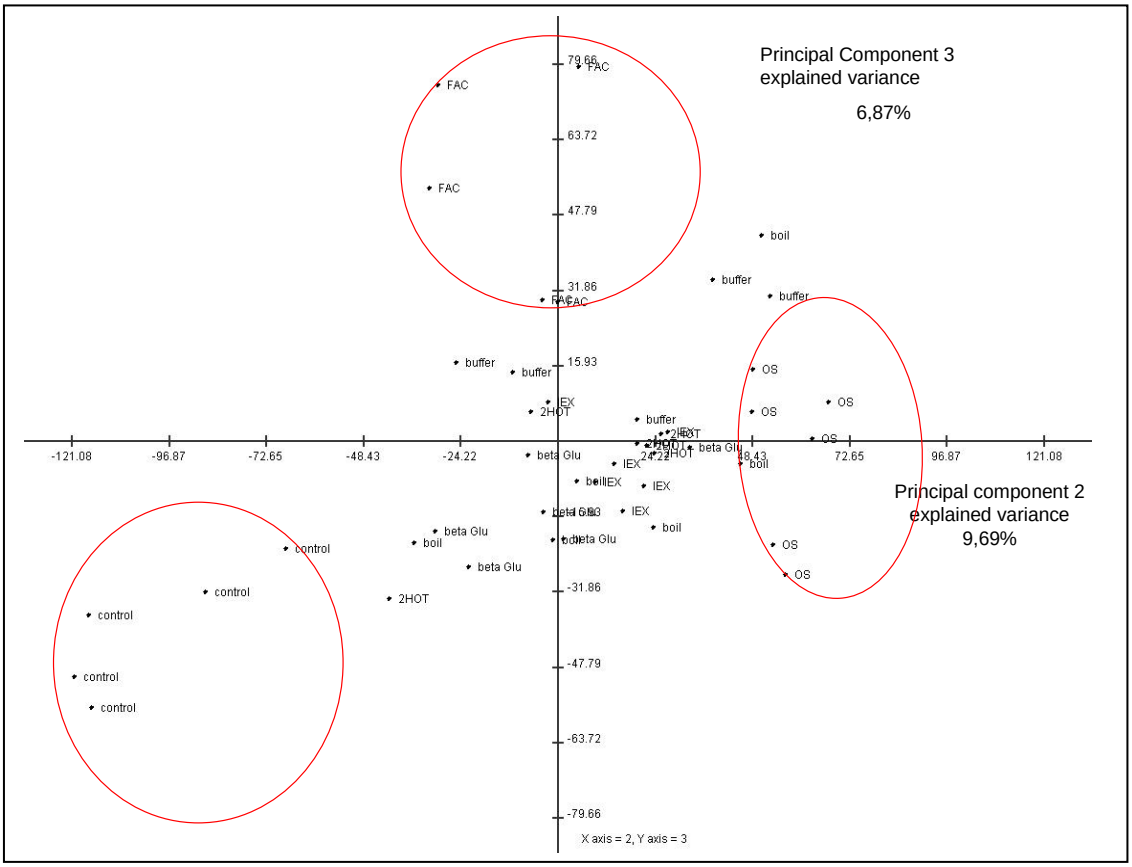

Figure 17: 2D plot of PC 2 against PC 3

Like during hierarchical clustering analysis, we achieved better results from the sub-matrixes obtained after one-way ANOVA filtering. The best projection was given by the first three PCs (figure 16,17). These results confirmed that the VOC compounds pre-selected during the ANOVA analysis were those significantly influenced by the treatments we applied to damaged *N. attenuata* leaves.

OS-treated and CONTROL plants formed two highly discriminated groups, which separated from each other as well as from the other treatments as visualized on the projection PC1 vs PC2. Groupings visualized on the projection PC1 vs PC2 (figure 16) overlapped partly those obtained during clustering analysis. In details, OS- and FAC-treated individual samples as well as CONTROL ones gated at the treatment level; whereas no clear classification was observed for the rest of the samples. PC1 and PC2 explained the main part of the comprehensive resolution obtained between OS-treated plants and the rest of the sample population. A clear discrimination between samples from treated and untreated plants was also given by VOCs highly influencing PC1. The blends of VOCs emitted by FAC-treated leaves significantly influenced PC3 (figure 17). The fact that those samples did not group with OS-treated ones supported the previously published observation that if FACs act central eliciting component of insect OS, they do not explain all the changes induced by OS-elicitation. Boiled-OS-treated samples did not groups with crude OS ones. This was probably related to the partial degradation of the FAC content or of constituents of *M. sexta*'s OS rather than to the effect β-glucosidase denaturation since that samples treated by this enzyme were not significantly grouped with the OS-ones. Similarly the neighboring localization of IEX-OS and Boiled-OS treated data sets also supported such an interpretation. This was also in agreement with Roda et al.[38] have already shown the consequences at the transcriptional level of FAC degradation during the boiling treatment of *M. sexta* OS.

On the other hand, the absence of a clear separation between other eliciting solutions (2-HOT, β-glucosidase, IEX-OS and boiled-OS) and the Buffer partly ruled out that those effectors or solutions played a major role on the early release of VOCs by *N. attenuata* during *M. sexta* herbivory. We analyzed VOC calculated loadings (eigenvectors) in order to investigate their specific influence on the classifications observed following each of the three first PCs

	RT 1D	RT 2D	eigenvector	compound
	672	4.14	2.34	methyl benzyl alcohol
	1164	2.47	1.66	hexenyl ester 6
PC 1	1266	2.58	-1.23	β-duprizianene
14.83%	714	2.71	-1.41	monoterpene 2
	648	2.56	-1.95	*trans*-ocimene

	RT 1D	RT 2D	eigenvector	compound
	882	2.71	2.80	hexenyl-ester 3
	984	2.51	2.76	hexenyl ester 4
	846	2.55	2.66	hexenyl ester 2
	570	2.84	2.41	*cis*-3-hexenyl-acetate
	834	2.51	2.31	hexenyl ester 1
PC2	912	2.56	2.29	*cis*-3-hexenyl-butyrate
9.69%	1140	2.85	2.09	hexenyl ester 5
	738	2.74	1.98	monoterpene 3
	768	4.45	1.76	phenylethanol
	1302	2.28	1.55	*trans*-α–bergamotene
	1236	2.34	1.36	sesquiterpene 2
	1164	2.47	1.14	hexenyl ester 6

	RT 1D	RT 2D	eigenvector	compound
	714	2.71	1.95	monoterpene 2
PC 3	1302	2.28	1.56	*trans*-α–bergamotene
6,87%	1236	2.34	1.33	sesquiterpene 2
	834	2.51	-0.99	hexenyl ester 1

Figure 18: principal components and their corresponding compounds

Figure 18 shows identified compounds and those which could assigned to a special group of volatiles. Compounds containing a negative eigenvector for PC 1 seem to belong to the group of terpenoids such like the monoterpene *trans*-ocimene and the sesquiterpene β-duprezianene, while the most high eigenvector-containing compounds of PC 2 represent the group of hexenyl-esters (e.g. *cis*-3-hexenyl-methylbutyrate and *cis*-3-hexenyl-acetate).

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Identified compounds (based on structure elucidation and comparison with authentic standards) and those putatively assigned to a special sub-group of VOCs influencing the results of PC classifications were summarized into the figure 17. VOCs displaying a negative eigenvector for PC1 mainly belonged to the terpenoid group (e.g. the monoterpene *trans*-ocimene and the sesquiterpene β-duprezianene); while those with the highest PC2 eigenvectors were mainly hexenyl-esters (e.g. *cis*-3-hexenyl-methylbutyrate and *cis*-3-hexenyl-acetate). Terpenes- as well as hexenyl esters-related VOCs also displayed significantly high loadings on PC3. These latter VOCs were those whose emission was under the control of FACs (figure 18)..

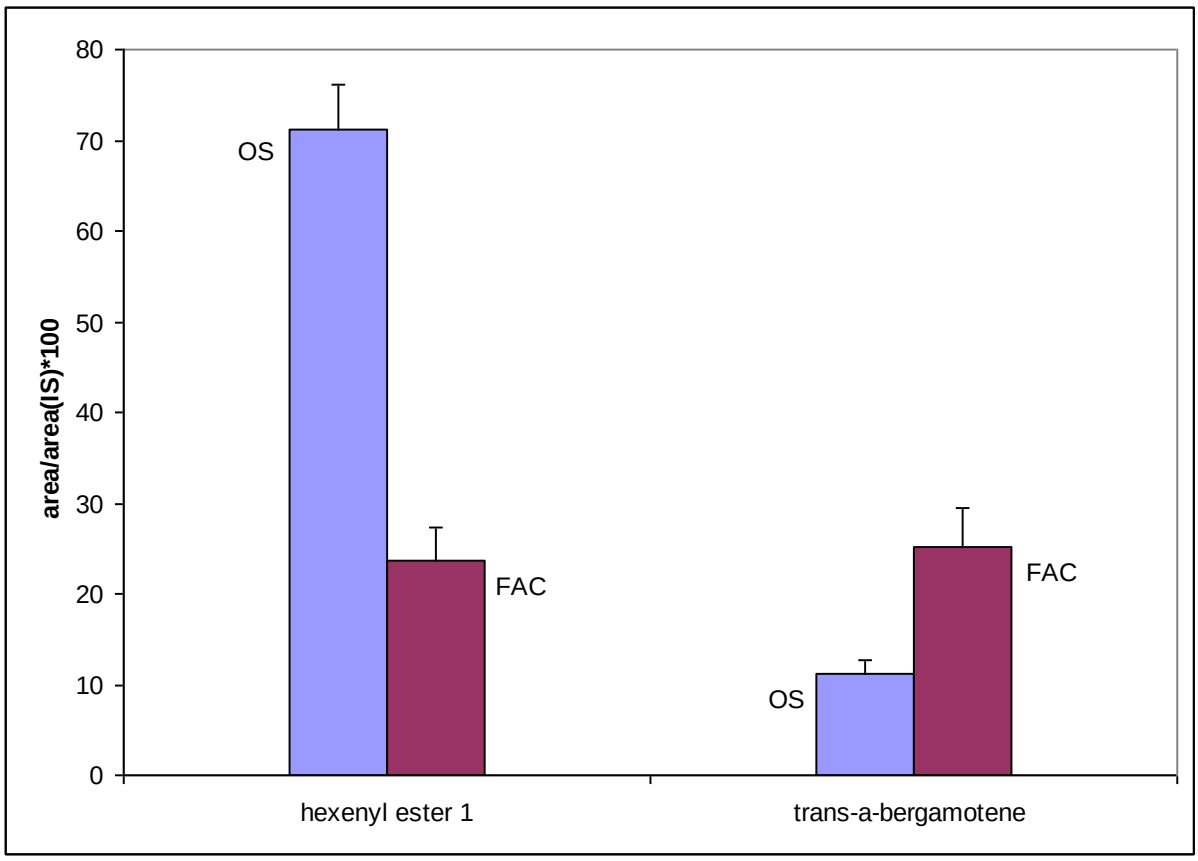

Figure 19: comparison of normalized areas of 2 compounds responsible for clustering of FAC and OS

An example of the use of eigenvector values to discriminate of VOCs regulated through FACs perception from the rest of OS-induced ones is given in figure 18. Hexenyl-ester 1 whose emission was clearly induced by OS elicitation had a negative eigenvector for PC3, while *trans*-α-bergamotene had positive eigenvector on this component suggesting its FACs-induction. This illustration also confirmed the great help provided by ANOVA pre-filtering VOCs fingerprinting, from the large-scale data-sets generated by the GCxGC-TOFMS analysis, prior the use classification methods. Those two VOCs displayed contrasting patterns of regulation. While the FACs treatment restored completely OS-induced *trans*-α-bergamotene, such a trend was not observed for hexenyl-ester 1.

5.3 Analysis of later released VOCs blends

5.3.1 Hierarchical Cluster Analysis

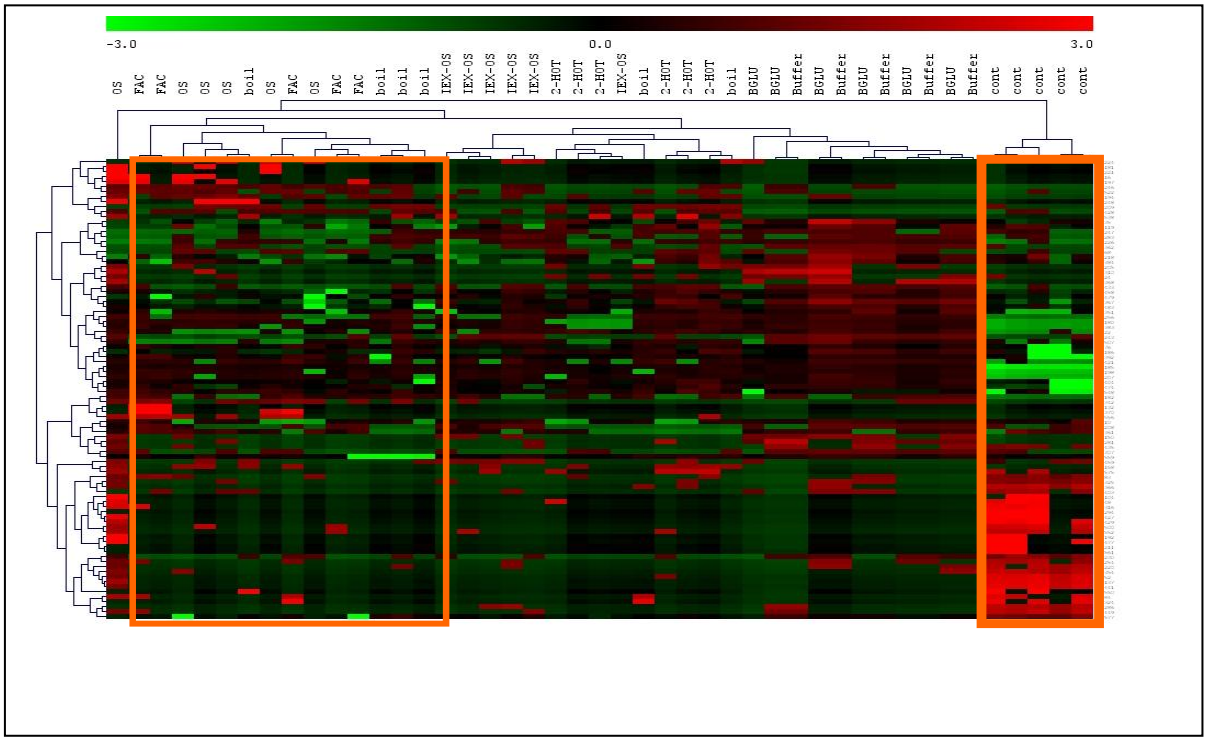

Figure 20: hierarchical cluster analysis of significant peaks (ANOVA)

For all samples of the second trapping period a hierarchical cluster analysis was performed. In the resulting image, four different clusters are observable. Similar to the first data point all control samples create a group that is different from the other treatments. In comparison to the first data point OS samples do not group alone, instead there is a second large cluster consisting of OS, FAC, and boiled OS treatments. One OS sample creates a cluster for itself. Although not all boil samples are included into this group, it seems to include all treatments containing FACs.

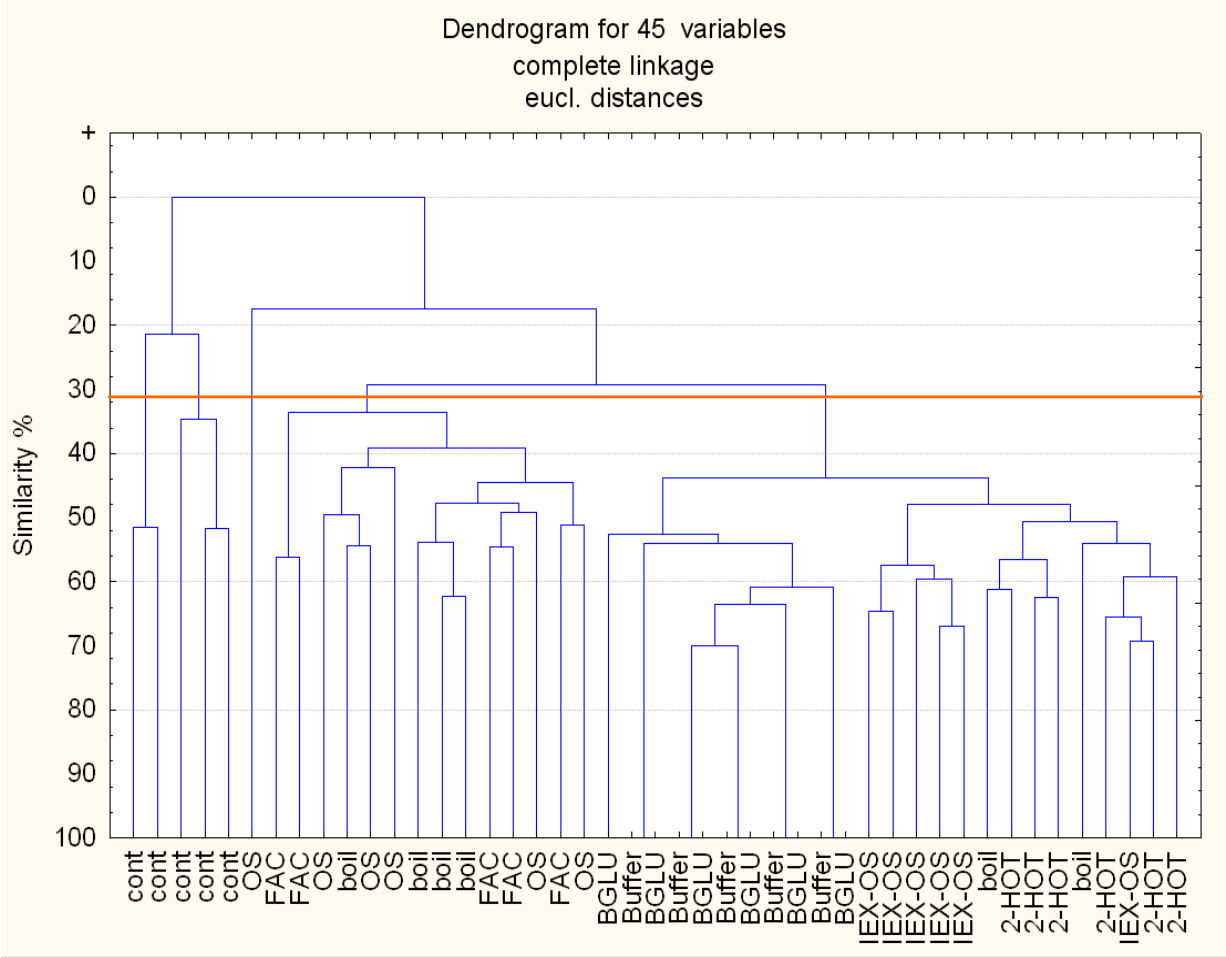

Figure 21: cluster analysis showing the similarity

By separating clusters at a similarity close to 35 % (figure up) in the obtained dendrogram, a slight difference can be seen. As before, FAC containing samples cluster together except one OS sample. The control samples are also divided into two clusters. These great similarities between all the samples are again due to biological differences of used plant individuals. However the achieved clustering is explainable by the composition of the used treatments.

5.3.2 Principal Component Analysis (PCA)

According to the scree plot (figure 22) only the first three principal components were investigated. Examination of PC 1 and PC 2 in a 2D plot confirms the grouping of FAC-containing samples and of control samples is confirmed. A third group differing from other treatments can be observed. This third group consists of β-glucosidase-treated plants (BGlu) and is mostly influenced by PC 2. Control plants are mainly influenced by PC 2. FAC-treated plants are negative affected by PC 2.

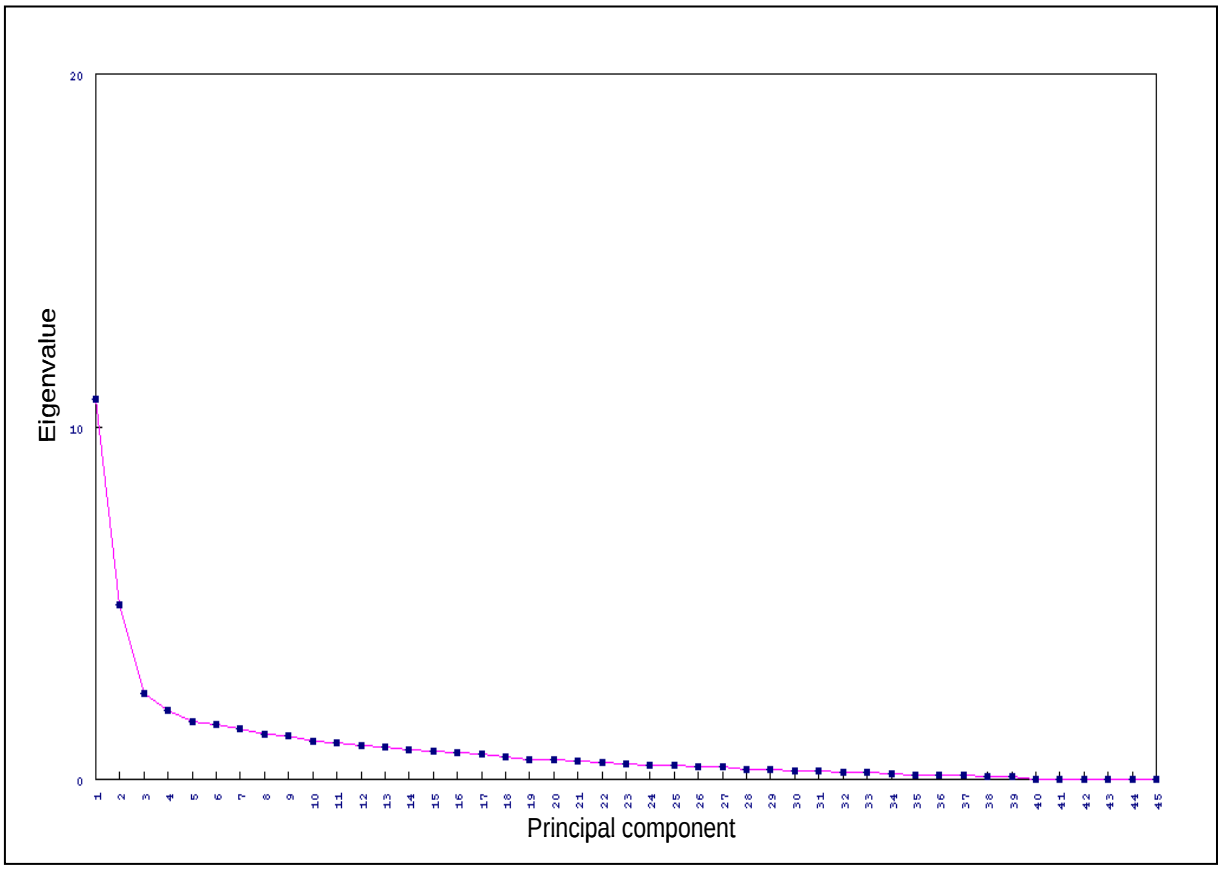

Figure 22: screeplot of principal components

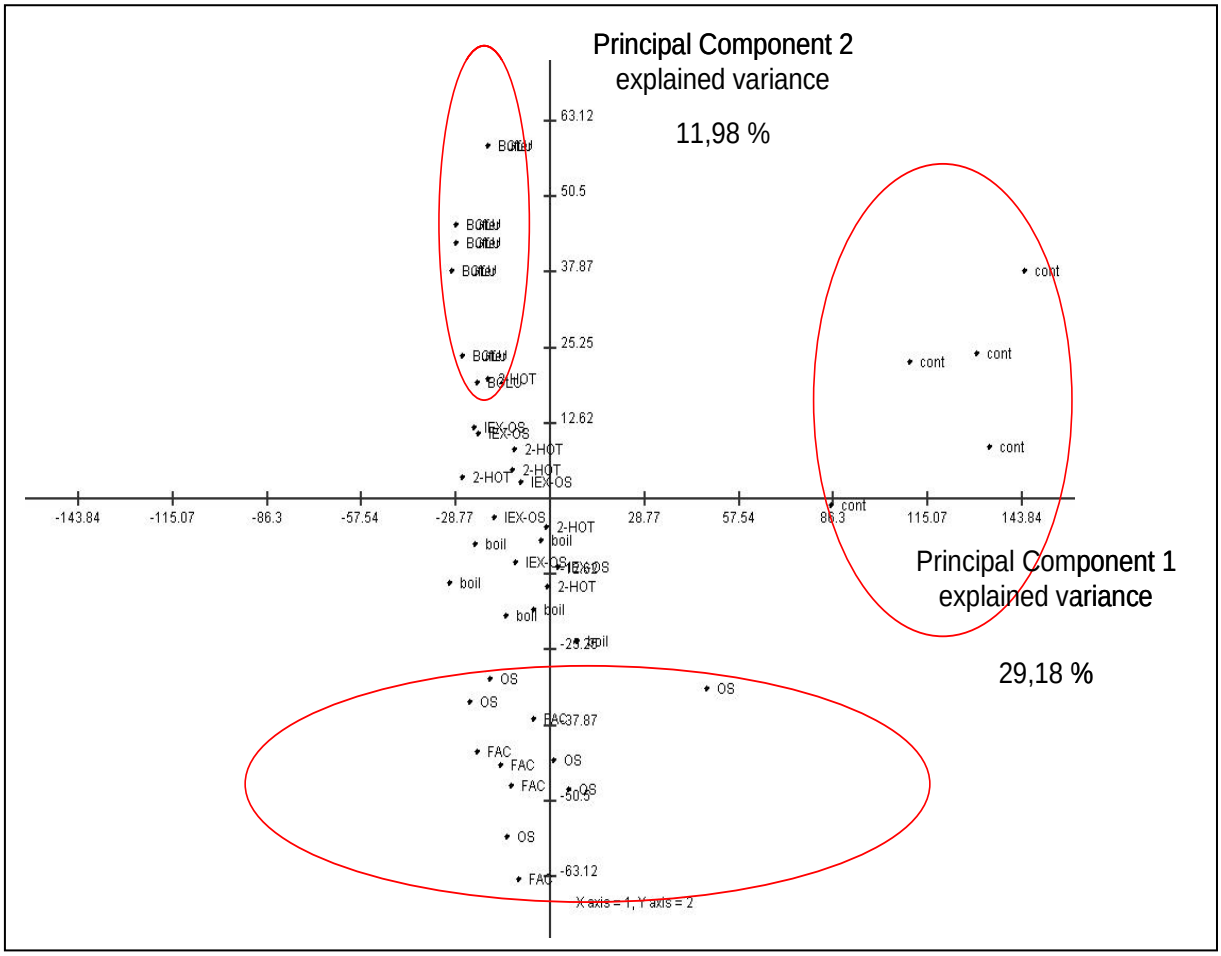

Figure 23: 2D plot of PC 1 against PC 2

Some of the substances belonging to the single principal components are presented in figure 24. The mostly control plants influencing PC 1 is represented by a mix of substances. Two of these compounds, naphthalene and limonene, containing a positive eigenvector and seem to be responsible for the separation of control plants from other treatments. Compounds with a negative eigenvector, e.g. *trans*-α-bergamotene, slightly influence the other treatments. Therefore PC 1 can be explained by the wound induced volatile emission. In contrast to this result PC 2 divides the wounded plants in two groups. A first group influenced by terpenoid emission, and a second group influenced by emission of C6-esters. PC 3 does not provide any explainable information.

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	RT 1D	RT 2D	eigenvector	compound
	888	4.33	1.95	Naphtalene
	600	2.33	1.40	limonene
PC1	1302	2.33	-1.01	*trans*-α–bergamotene
26.18%	834	2.56	-1.07	hexenyl ester 1
	558	2.77	-1.12	octanal/ hexenyl acetate
	558	2.29	-1.93	monoterpene 1

	RT 1D	RT 2D	eigenvector	Compound
	606	2.64	1.94	eucalyptol
	990	2.52	1.46	*cis*-3-hexenyl-methylbutyrate
PC 2	912	2.64	1.30	*cis*-3-hexenyl-butyrate
11.98%	1302	2.33	-1.10	*trans*-α–bergamotene
	738	2.84	-1.33	monoterpene 3
	1176	3.32	-1.54	sesquiterpene 1

	RT 1D	RT 2D	eigenvector	compound
PC 3	738	2.84	1.66	monoterpene 3
5.93%	1236	2.39	-1.86	sesquiterpene 2

Figure 24: principal components and their corresponding compounds

Eukalyptol and *cis*-3-hexenyl-methylbutyrate belong to PC 2. Both compounds own a high positive eigenvector for this component, indicating a strong influence on β-glucosidase-treated plants. Figure (below) shows the normalized peak areas for these compounds, which are indeed significantly higher for β-glucosidase-treated plants compared to plants treated by OS/FAC.

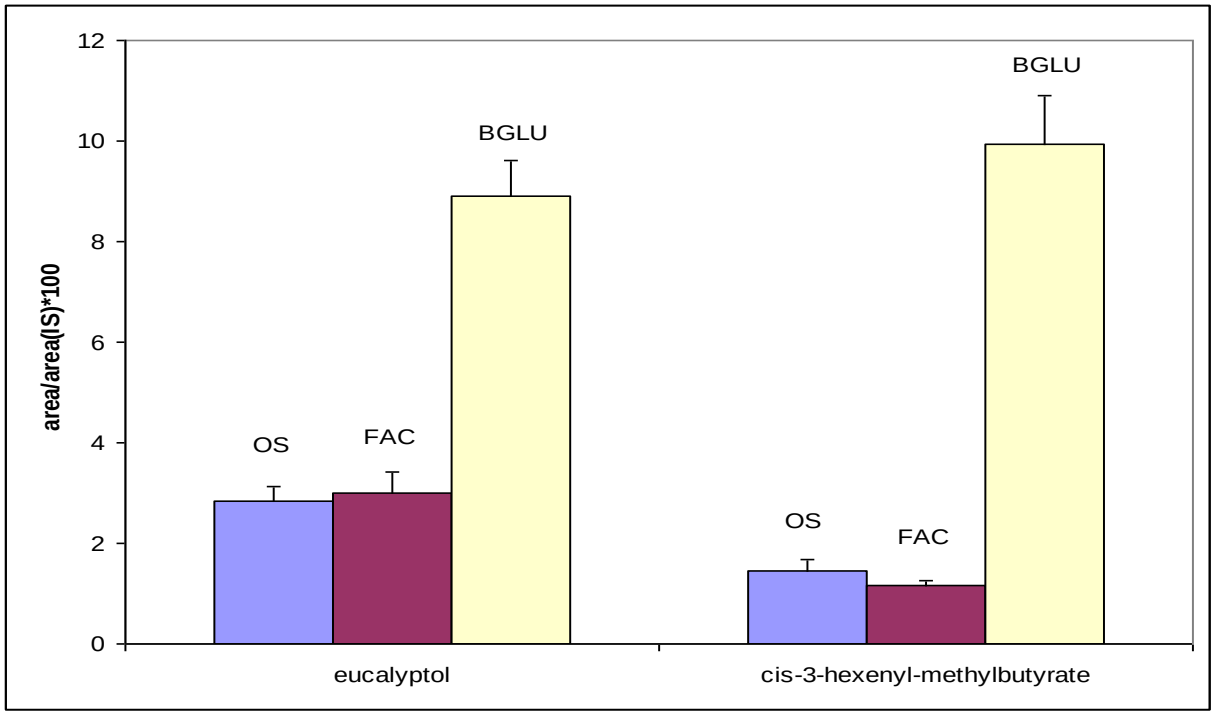

Figure 25: comparison of two compounds responsible for BGLU clustering

5.4 General Discussion

Statistical analysis showed that different induced volatile emission could be successfully classified, according to the used treatments of wounded plants and even unwounded control-plants. Separation of control plants showed the well described effect of wounding and the resulting release of volatiles. The great amount of raw data could be reduced by ANOVA and introduced into multivariate statistical methods such as cluster analysis and principal component analysis.

Cluster analysis provides a well explainable clustering for the first two trapping periods. Four different clusters are created for the first trapping period. These clusters represent control plants, OS-induced plants, FAC-induced plants, and in a fourth cluster all other treatments. The single grouping of unwounded plants is due to the wounding and the following release of volatiles. Separate grouping of FAC- and OS-treatment indicates different plant response, related to the components in the treatment solutions. Although both treatments (FAC, OS) contain a similar elicitor of defense response, FACs, they differ in the inducing of volatile emission from treated plants. This might be due to the effect of other elicitors present in the oral secretion.

However, the other treatments may not contain these unknown elicitors, because they group different from the OS treatments. It is also possible that only combination of different elicitors as existing in oral secretions lead to this different volatile emission. Because the IEX-OS samples do not behave like OS samples, an elicitor can be assumed that is removed by ion-exchange chromatography, e.g. FACs or 2-HOT. As already mentioned FAC and 2-HOT treatments do also not behave like the OS sample, differences are possibly due to interactions between different elicitors.

PCA analysis confirms the results achieved by cluster analysis. There were 3 principal components examined, explaining at least 31 % of the total variance. Because control samples are less affected of PC 1 and negative of PC 2, these components reflect the wounding effect and the release of volatiles after wounding. Therefore PC 1 could be explained by OS induced terpene emission (figure 17) and PC 2 by OS induced hexenyl ester emission. In PC 3 terpenes as well as hexenyl esters are present and the FAC treatments are separated from OS and Control.

Thus PC 3 represents the FAC induced volatile emission. Separation of control plants is obviously due to the mechanical wounding and the following release of volatiles in the other treatments.

Non-grouping of FAC and OS (figure 16) shows how different plants react to single elicitors (FACs) and mix of elicitors (OS), if treated with. The comparison between the two identified compounds hexenyl ester 1 and trans-α-bergamotene shows significant differences in the induction of these compounds. While hexenyl ester 1 seems to be up regulated by OS, trans-α-bergamotene is up regulated by FACs. This indicates an inhibiting effect of other ingredients of oral secretion towards the release of this sequiterpene. Increasing emission of trans-α-bergamotene, caused by FAC treatment, was shown by Halitschke et al. [14]

Compared to the first trapping period, analysis of the second trapping period by cluster analysis gives slightly different results for treatment grouping. Control samples are again separated in a single cluster, while all FAC containing treatments, including boiled OS, are combined in a second cluster and all other treatments in a third one. These results show that the effect of other specific OS elicitors, except FAC, decreases after 24 h because FAC containing treatments behaving more closely. FAC may have, compared to the other investigated elicitors, the greatest effect on long term volatile emission.

The obtained clustering is confirmed by the PCA analysis as control and FAC-containing samples are separated in individual groups. Nevertheless, also a third group of samples is shown. Representing all β-glucosidase treated samples, this group did not occur in the analysis of the first trapping period. This result indicates that a longer period of time is needed to evolve the effect of this elicitor on volatile emission. This group seems to be mostly influenced by PC 2. A closer examination of the compounds involved in this principal component reveals two compounds that could be identified, eukalyptol and *cis*-3-hexenyl-methylbutyrate. They have a strong positive effect on PC 2 and comparison of normalized peak areas shows a significant increase for these compounds, if plants are treated only with β-glucosidase. It has been shown that this enzyme triggers volatile emission in cabbage plants ([1] here it could be demonstrated in *N. attenuata*. The β-glucosidase induced release of volatiles occurs during the second trapping, indicating a longtime response of the plant to this single elicitors.

6. Summary

The aim of this work was to decipher the different effects of elicitors present in the oral secretion of *M. sexta* to volatile emission released by wounded leaves of *N. attenuata.* Differences in the bouquet of volatile emission should be examined for different elicitors.

Therefore *N. attenuata* wild type seeds were germinated and plants were grown under stable conditions in the greenhouse.

Plants were wounded, to mimic herbivore attack, and then 8 solutions containing different elicitors were applied to the wounded leaves, while one group of plants was not wounded as a control. Plants were treated with oral secretion collected from *M. sexta* larvae, as well as with solution containing FACs, a well known elicitor of volatile emission, 2-HOT and β-glucosidase, which is found in oral secretion of *M. sexta* and known to trigger volatile emission in cabbage plant [1]. For investigating the effect of FAC within the oral secretion IEX-OS was used. Boiled OS was applied for examining the enzymatic effect, while a pH 9 (pH of *M. sexta* digestive tract) buffer solution was applied to examine volatile emission by mechanical wounding.

Volatiles were trapped over 36 hrs after wounding. Traps were changed every 12 h, resulting in three subsequent trapping periods. After collecting the traps were eluted and introduced into chemical analysis.

Collected samples were analyzed by GCxGC-ToF, which is a powerful and versatile tool for the analysis of complex volatile mixtures. This analytic power is due to the two orthogonal separations, which take place on the both columns used in this chromatographic technique. A modulator preserves the achieved separation of the first column. ToFMS provides a fast enough detection and adequate resolution of analytes.

Obtained data were analyzed by the ChromaTOF software, using the comparison feature of this software. One OS-induced sample was set as a reference and compared to other samples.

Statistical analysis was performed with TIGR MeV and STATISTICA software. ANOVA provided significant different peaks, which were analyzed by agglomerative hierarchical cluster analysis and principal component analysis. It was possible to achieve a classification of samples collected in the first trapping period. Because of the large release of volatiles after mechanical wounding, that do not appear in unwounded control plants, they could be successfully separated.

The effect of FACs treatment could be successfully demonstrated on two identified compounds. Treatment of wounded plants with a solution containing only FACs leads to an increase of trans-α-bergamotene emission, while hexenyl ester 1 emission decrease compared to OS treatment.

It has been showed that β-glucosidase has a positive effect on the release of two volatiles eucalyptol and *cis*-3-hexenyl-methylbutyrate. Comparable results were published by Mattiacci et al [1], here we demonstrate this effect in *n. attenuata.* The effect of β-glucosidase appears after 24 hrs, indicating a long time response.

For the third trapping period no explainable grouping was observed. Assuming that volatile emission decrease to unwounded levels, single treatments could not be separated by statistical analysis.

A data processing method, using the ChromaTOF software, was created which allows analyzing the complex composition of volatile blends. Further work should focus on the creation of a more stable reference, because artifacts in the used reference caused some problems when applying data to statistical analysis.
Nevertheless, this approach to data processing combined with the great separation power provided by the GCxGC-ToF, could contribute for deciphering the effect of special plant elicitors to volatile emission of plant and therefore to understand their role in direct and indirect defense.

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Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung das angegebenen Hilfsmittel und Literatur angefertigt habe.

Jena, 18. März 2009

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