MSc Minor Thesis Report

Relationship between genotype variation of Arabidopsis with different flower traits and pollinators

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Li Gu

MSc Plant Science-Plant Breeding and Genetic Resources

Wageningen UR

Thesis Supervisor: dr.ir. Iris Kappers

Register number: 911115287030

e-mail: li.gu@wur.nl

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1. Introduction

1.1 Mutualism and Pollination

Mutualism is the beneficial interaction between different species, which guarantees life reproduction and survival. Over 80% of flowering plants have beneficial mycorrhizal fungi living on and in their roots (Simon et al., 2009). In the history of evolution, almost all the milestone events are linked to mutualism, such as the origin of the eukaryotic cell and the invasion of the land by plants. Therefore, mutualism plays a central role of species diversity and biological evolution. By studying plant-insect mutualism, it indicates the function and evolution of different plant traits. The plant-insect mutualism provides three remarkable evolutionary features: pollination, protection and seed dispersal (Bernstein et al., 2006). First, the reproduction of many vascular plants is associated with attracting insect mutualists. Also, the mutualism between insects and plants is the key of conservation. Once the mutualisms are threatened, the plants that depend on them will also potentially in danger. In this minor thesis, we focus on the pollination part.

Pollination is defined as transferring pollen from an anther to the stigma of a carpel and fertilizing ovules at the end. The pollination of gymnosperms appeared a little bit earlier in time than angiosperms, which was in Permian and in the late Jurassic to early Cretaceous respectively (Crane et al., 1995; Grimaldi, 1999). First reward for early insect mutualism was excess of pollen demonstrated to be present in the Late Paleozoic (Labandeira, 1998; Grimaldi, 1999). Then nectar appeared as the reward for pollinators before the Late Jurassic (Labandeira, 1997; Ren, 1998). Angiosperms, as well as processing flower-like structure plants, relied on generalized pollinator insects, such as wasps, moths, thrips, beetles and flies, which lack specific adaptions for flower feeding. More plant-dependent insects, such as bees and butterflies, became involved in pollination from the Mid-Cretaceous and into the Tertiary (Crane et al., 1995; Crepet, 1996; Grimaldi, 1999; Bernhardt, 2000; Thien et al., 2000; Grimaldi and Engel, 2005). In addition to insects, there are two other biotic pollination forms: plants pollination by wind or water and self-pollination. However, loss of animal pollination often occurred when insect pollination is not achievable or it cannot bring plants ideal results (Bernstein et al., 2006).

1.2 Pollinators

A pollinator is an animal that transfers pollen from a male anther to the female stigma of a carpel. Insect pollinators includes bees, pollen wasps, ants, flies including bee flies and hoverflies, butterflies and moths and flower beetles (Matt et al., 2017). Also, some birds like hummingbirds, honeyeaters and sunbirds with long beaks are pollinators which can pollinate a number of deep-throated flowers (Carol, 2011). Wild and managed bees are well recognized as effective pollinators all around the world. However, the contribution by pollinators other than bees have been little focused despite their potential to contribute to crop production and stability during environmental changes. Although on average the amount of pollen deposited per visit to crop flowers is lower for non-bees than for bees, the high visitation

frequency of non-bees to crop flowers compensates for the deficit in per-visit effectiveness and results in high pollination services overall. Honey bees are good at depositing pollen in many crops, but increased honey bee visitation did not increase fruit set. In contrast, increased visits from other bees, as well as from non-bees, were associated with increased fruit set in 41 crop systems world-wide (Agustín, 2014). As argued by Garibaldi (2013), these patterns suggest that the effect of other bees and non-bees is additive to the effect of honey bees in the datasets examined. A final benefit of non-bees documented here is that they respond less negatively than bees to changes in land use (Romia et al., 2015).

Two examples of non-bee pollinators are hoverflies, which is also an important natural enemy of several aphid species and thrips which acts also asan herbivore. Hence, both species have interesting features in both pollination and herbivory.

As the name suggests, hoverflies are often seen hovering at flowers. The adults feed mainly on nectar and pollen, while the larvae eat a wild range of foods including aphids. Hoverflies are important pollinators of flowering plants in a variety of ecosystems worldwide. They are frequent flower visitors for numerous wild plants, as well as agricultural crops, and often considered as the second-most important group of pollinators after wild bees (Jamie et al., 1999).

Figure 1.2.1 Hoverfly

Thrips are small, 1mm long or less, slender insects with fringed wings and unique asymmetrical mouthparts (Bettiga, 2013). They feed mostly on plants by puncturing and sucking up the contents. They fly weakly, while their wings are not suitable for conventional flight. Some of the thrips species like *Franklienella occidentalis* are beneficial as pollinators (Kirk, 1996), although this most often does not outweigh the negative impact they have as disastrous herbivores.

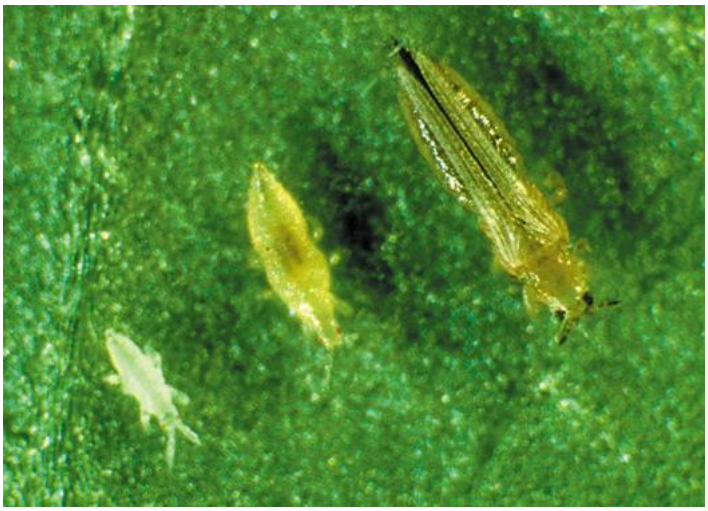


Figure 1.2.2 Thrips of different stages (first and second larval instars plus adult)

1.3 Arabidopsis

Arabidopsis thaliana is an annual winter plant in the Brassicaceae family. It is the first plant of which the genome has been completely sequenced (the Arabidopsis Genome Initiative, 2000). With a short life cycle (around six weeks), small size, small genome (135 Mb) (TAIR, 2016), and high number of offspring, *Arabidopsis* has been an ideal model for molecular analysis in plant biology for decades (the Arabidopsis Genome Initiative, 2000). As an ideal organism, it underwent abundant artificial genetic mutagenesis, including disruption of gene expression and activation tagging mutants (Radhamony *et al.*, 2005). It also allows relatively easy localization of biosynthesis expression genes and although its flowers do not excessively smell, comparison of floral volatile emission from different Arabidopsis accessions revealed variation. This implies that Arabidopsis could be used as a model species to study and discover regulation and volatile biosynthesis processes (Tholl et al., 2005).

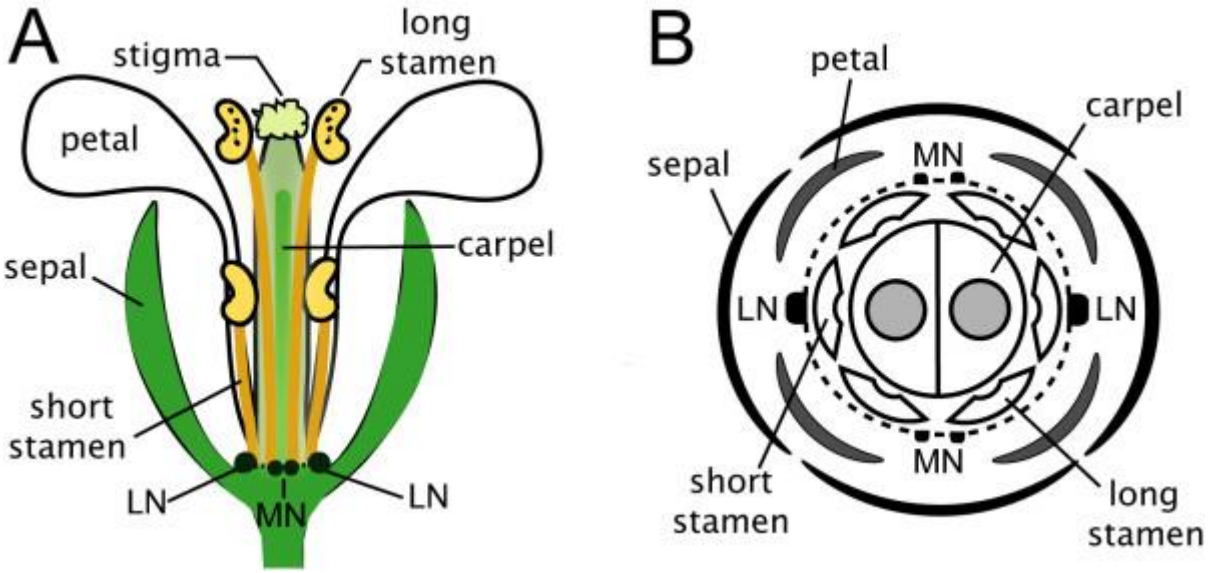


Figure 1.3.1 Flower structure of Arabidopsis

Although *Arabidopsis thaliana* is considered to be mainly self-pollinating, according to Hoffmann's study (2005), it is observed that Arabidopsis populations are visited by pollinators including bees, flies and beetles in nature and during the research period of the study of Hoffmann approximately 1% cross-pollination rate was estimated.

1.4 Plant Traits

Around two-thirds of angiosperm plants need insects to help them for pollination. To attract as many pollinators as possible, plants involved various strategies which are essential for reproduction, including visual, odour and rewards (nectar and pollen). Flower colours and odours are the simplest method for plants to attract pollinators. To lure pollinators, plants display a variety of colours of flowers and emit attractive odour complex (Raguso, 2008). Furthermore, flowering plants even can change their colours and odours of flowers to guide the pollinators to flowers that have not been pollinated yet (Weiss, 1991; Rodriguez-Saona et al., 2011). Some specific floral traits combinations appear to be visited by particularly pollinator taxa (Bernstein et al., 2006). Pollinators are also attracted by rewards, such as nectar and pollen. Pollinators even evolved characteristics that allow them to extract rewards from tough reachable flowers. Besides pollinators, herbivores can also influence flower traits (Kessler and Halitschke, 2009; Lucas-Barbosa et al., 2011). However, herbivores often negatively influence pollination by directly consuming flowers or indirectly alter the biosynthetic pathways and affect plant mutualists. Because of herbivores, plants produce defence mechanisms to reduce or avoid damage, thus change the quality of nectar and pollen, and even influence pollinators' visitation. Until now, the details about whether and how herbivores influence pollination or pollinator behaviour is still scare because plants are exposed to them at the same time (Lucas-Barbosa et al., 2016).

1.5 Objectives

In this project, we aimed to find if genotype variation existing among a selection of natural accessions of Arabidopsis (ecotypes) has consequences for the traits that plants use to attract and reward pollinators. To select genotypes, we focused on four aspects with different genes that encode for these traits: (information of genes was from ARAPORT- araport.org)

Odour (terpenoids)

The volatile blend from Arabidopsis flowers consists of compounds from several biochemical classes of plant metabolites, including terpenes, phenylpropanoids, fatty acid derivatives, and nitrogen- or sulfur-containing compounds. In this study, I focus on the terpenoids. Next to monoterpenes, Arabidopsis flowers emit over 20 sesquiterpenes (Raguso, 2008; Lucas-Barbosa et a., 2011). It not only helps plants attracting pollinators, but in a broader aspect also protecting the cell from oxidative stress, acting as mediators of thermotolerance. Even in vitro, terpenes have antibiological function (Huang et al., 2012).

We selected 3 genes AT5G44630 /AT5G23960 /AT3G53300 to conduct our experiment on odour. AT5G44630 (*AtTPS11*) is a gene which encodes a sesquiterpene synthase which

generate group B sesquiterpenes found in the Arabidopsis floral volatile blend (Tholl et a., 2005). It is strongly expressed in intrafloral nectaries (https:\\araport.org). AT5G23960 (*AtTPS21*) is a gene which encodes sesquiterpene synthase generating all of group A sesquiterpenes found in the Arabidopsis floral volatile blend. It is strongly expressed in stigma. AT3G53300 (*AtCYP71B31*) is a gene involved in oxidation-reduction process and secondary metabolite biosynthetic process. It is expressed during flowering stage, petal differentiation and expansion stage, and mainly in carpel, pollen, sepal, and stamen.

Nectar

Nectar is a sweet aqueous secretion that contains water, sugars, and amino acids. The function of nectar is attracting pollinators and defenders, and it is protected from nectar robbers and microorganisms by various secondary compounds and antimicrobial proteins (Bernstein et al., 2006). Next to flowers, nectar can be secreted from all plant organs except roots. According to different producing organs, nectar has different functions.

We chose AT2G39060 (*AtSWEET9*) to study during our experiment. AT2G39060 is a gene that encodes a sucrose transporter that is expressed in nectaries involved in nectar secretion. (Lin et al., 2014)

Colour

Plants display a variety of colours to lure pollinators and they also change flower colours in order to guide pollinators to unpollinated flowers (Weiss, 1991). AT5G13930 (*AtTT4*) is a gene that encodes chalcone synthases, a key enzyme involved in the biosynthesis of flavonoids. It is essential for the accumulation of purple anthocyanins in leaves and stems. It also involves in the regulation of auxin transport and modulation of root gravitropism. Its mRNA is cell-to-cell mobile.

Pollen

Pollen serves as a reward towards pollinators, can be used as a guide for pollinators (Crepet, 1996). AT4G13270 encodes a protein that belongs to the late embryogenesis abundant hydroxyproline-rich glycoprotein family. It contributes to pollen and tube germination which result in more pollen produced by flowers.

To address whether the above described genes play a role in the attractiveness towards pollinators, we aimed to

- select Arabidopsis accessions that differ in their basal expression of the beforehand mentioned genes

- sowing in such a way that flowering of different accessions is synchronized

- analysing secondary metabolites including total flavonoids and anthocyanins in the flower petals by (UV-Vis) spectrophotometric measurements

- analysing volatiles emitted from flowers via head space collection and GC-MS

- analysing sugar composition and amount present in the nectar by isolation of nectar from nectaries and HPLC

- introducing hoverflies or thrips to flowering plants and observe pollinator behaviour

2. Materials and Methods

2.1 Materials

A. Arabidopsis

According to the different flowers traits we want, we selected 8 Arabidopsis accessions with different genes expression level (Table 2.1) and accessions 165 (Col0) was used as genotype reference. No available accession was found for AT3G53300, that differed in its expression relative to Col0, so we abandoned it. The selected Arabidopsis plants were sown and grown in both the greenhouse (June – August, compartment 7.4) and climate chamber (12hrs light:12hrs dark, compartment B10). In total, we grow 3 batches of 8 accessions, each accession had 15 plants per batch.

Table 2.1 Arabidopsis selection

	Odour		Nectar	Colour	Pollen
Genes / Acessions	AT5G44630	AT5G23960	AT2G39060	AT5G13930	AT4G13270
Col-0/165 (Control)	L	H	L	H	H
Est-0/39	H				
Est-1/179	H				
Sha/279					L
Bay-0/146		L			
Nd-1/249			H		
Kin-0/205					L
Cen-0/162				L	

*L means low gene expression level, H means high gene expression level

B. Pollinator

Since increasing honey bee visitation do not increase fruit set, on the other hand, non-bee pollinators can help increasing fruit set while increasing visits, so we decided to use non-bees. Also, we considered about thrips are not only pollinators, but also herbivores. Hence, it can be expected that plant characters will change upon herbivory and the final experimental results of thrips will be very complicated to analyse. From the above, hoverflies are ideal pollinators for our experiment. Hoverfly (*Episyrphus balteatus*) pupates were obtained from "NATURAL biopol" that supplies beneficial insects. Every week we got 50 pupates and kept them in lab. We put pupates in the tent under light, feed them with bee pollen from Starkich and water. At the end, these pupates took around 4 days becoming adults in the lab.

2.2 Methods

We planted 8 Arabidopsis accessions of which 1 accession did not germinated (162), so in total we obtained plants from 7 accessions that could be used for the experiments.

A. Headspace

The plants were taken from greenhouse to the lab in the morning. Inflorescences were enclosed in a PE plastic food container (see figure/photo) and the opening was closed with cotton-wool. At the inversed bottom of the container (and hence now at the upperside) a SWAGELOCK connector was constructed so that a stainless-steel liner containing 200 mg of TENAX could be connected. Air was pulled through the TENAX liner using a PAS pump at a flow of 100 ml/min for 4 hours and volatiles emitted by the inflorescences were trapped on the TENAX (Figure 2.2.1). Each batch had 7 accessions with two repeats, and we did each sample with two plants. In total, there were three batches. After 4 hours pumping, the Tenax liners were dried by tube conditioner TC-20 for 15 mins and after that metal tubes were sent to do the GC-MS analyse.

Figure 2.2.1 Headspace in the lab

GC-MS

Gas chromatography-mass spectrometry is an analysing method which combines gas-chromatography and mass spectrometry to separate, identify and quantify different substances in a complex test sample. We used TD-100 from Thermal Desorber, 7890B GC system and 7200 Accurate-Mass from Agilent Technologies to analyse the volatile compounds emitted by the flowers.

The sample solution from metal tubes was injected into the GC inlet where it was vaporized and swept onto a chromatographic column by the carrier gas (usually helium). The sample flowed through the column and the compounds comprising the mixture of interest were separated by their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase), and this allowed the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The latter part of the column passed through a heated transfer line and ended at the entrance to ion source where compounds eluting from the column were converted to ions.

The next component is a mass analyser, which separated the positively charged ions according to their mass-to-charge ratio. Several types of analyser exist: quadrupoles, ion traps, magnetic sector, time-of-flight, radio frequency, and cyclotron resonance. 7200 Accurate-Mass we used was the time-of-flight (Q-TOF) analyser. After the ions were separated they enter a detector where the output was amplified to boost the signal. The detector sent information to a computer, converted the electrical impulses into visual displays and hard copy displays (Hites, 2016).

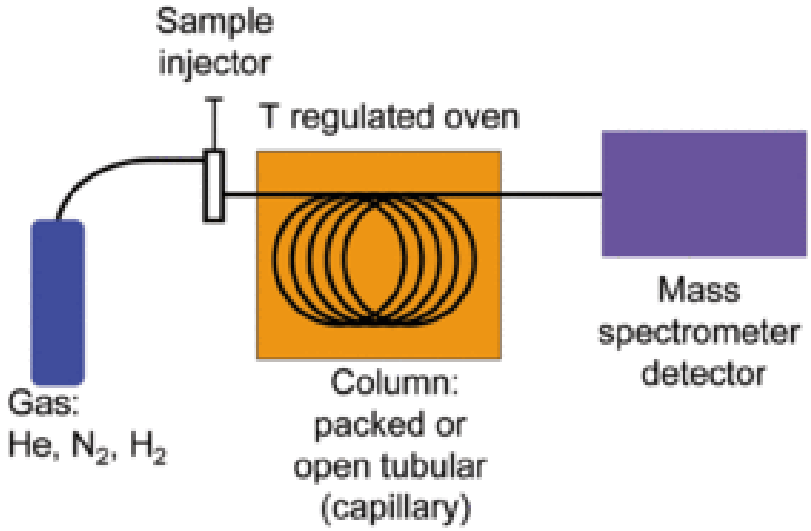


Figure 2.2.2 GC-MS schematic

MZmine 2.27 was used to align chromatogram data and the output was exported into an Excel file. The peak areas of chromatogram determined in the EIC mode at m/z=93 which helped expending region of interest, the data was exported as MZ files. Multivariate data analysis was done through Metaboanalyst (https://metaboanalyst.ca) using principal component analysis (PCA) and Partial least squares discriminant analysis (PLS-DA) to study whether genotypes differed in their volatile blend and which of the volatile components contributed to such a separation. Metaboanalyst.ca was used to do the data analyses. Next, Students t-tests was performed to analyse significances between genotype comparisons using RStudio.

B. Sugar Extraction

Because the nectar of Arabidopsis was too tiny to cut, so the small pieces from the flower bottom which contained nectars were collected (Figure 2.2.3), and these small pieces were directly frozen in liquid nitrogen. After collecting, the samples were stored at -80℃ until sugar extraction. In total there were three batches, all 7 accessions were done for the first two batches, and each batch had two repeats which contained 50 small pieces separately. However, only 3 accessions were done for the third batch (see in result) with two repeats for each accession. Each sample in batch 3 consisted of 150 small pieces.

For sugar extraction, first 500µL of 400mg/L melezitose in 80% MeOH was added to the frozen samples and extracted for 15 min at 76℃ in a water bath. After the extraction, the samples were put into the SpeedVac (Thermo SAVANT SC210A SpeedVac Concentrator) to evaporate the MeOH. After around 3 hours, the dried samples were dissolved into 500µL mQ water. Next, the samples were vortexed and centrifuged in an Eppendorf Centrifuge 5424 for 5 mins at 14000 rbp. Finally, the cleaned supernatant was diluted 10 times with XX and stored at -20℃ until HPLC analysis.

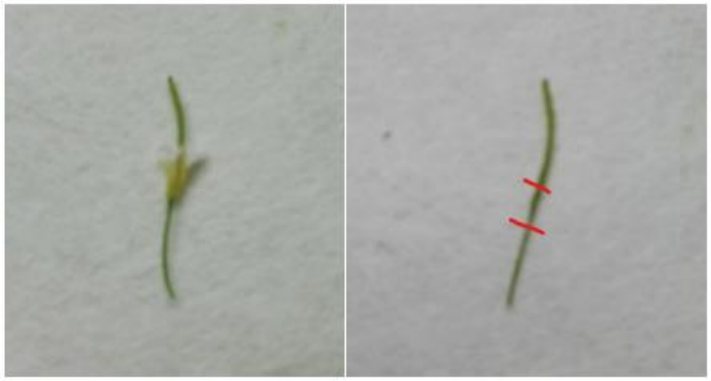


Figure 2.2.3 Arabidopsis flower and small piece of nectar cut

HPLC

High-performance liquid chromatography is an analytical chemistry technology used to separate, identify, and quantify components in a test mixture. HPLC used high pressure pumps to pass a pressurized liquid and sample mixture through a column filled with absorbent, causing the separation of the mixture due to their different interaction with the absorbent particles. These interactions are mainly physical in natural, e.g. hydrophobic, dipole-dipole and ionic. Then the components were delivered to the detector which generates a signal according to the amount of sample component emerging from the column, allowing for quantitative analysis (Karger, 1997). Dionex DX5000+ dual system and Dionex Carbopac1 250x4mm column + Carbopac1 50x4mm guard column was used as HPLC system. The detector was electrochemical ED50. And eluent gradient 20-150mM NaOH over 30 minutes plus 10 minutes isocratic with 150 mM NaOH.

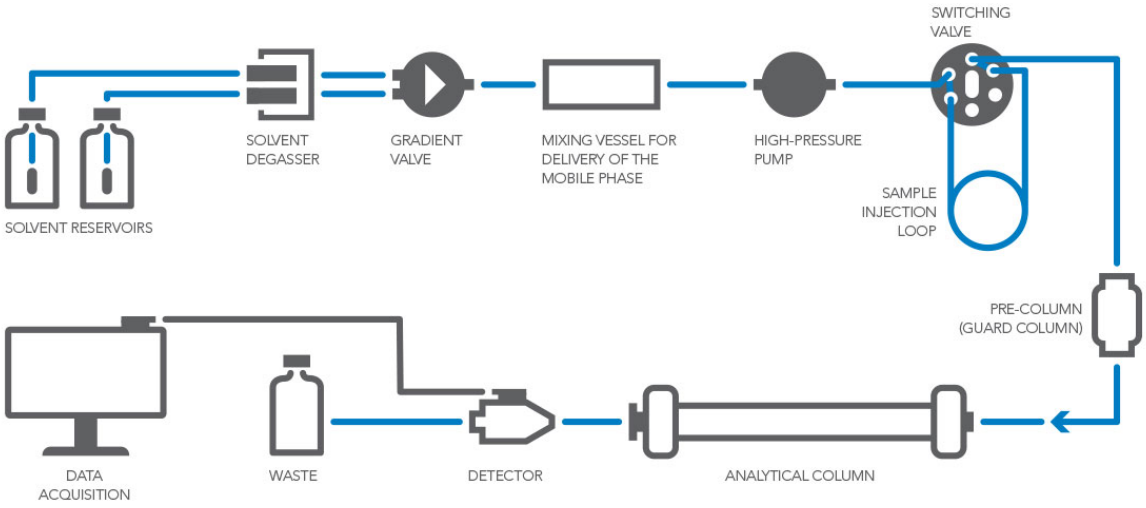


Figure 2.2.4 HPLC flow chart (from idex.com)

The data analyse of sugar also done by Metaboanalyst for PCA, PLS-DA and RStudio for T-test, same as volatile analysing.

C. Anthocyanin measurement

The flower petals of 7 Arabidopsis accessions were collected for anthocyanin measurement. Each sample had 50 petals for the first batch, 100 for the second, and 200 for the last batch. Also, same as nectary's, petals were directly put in the liquid nitrogen and stored at -80℃ before use. First, 1ml aliquot of 1% HCL (v/v) (the concentration of HCL is 37%) in 40% MeOH was added to the samples and mixed properly. The mixture was centrifuged at 16000g for 15mins at 4℃ (Thermo Centrifuge) and the upper phase was collected. 1ml chloroform was added to cleared supernatant to remove chlorophyll and the samples were centrifuged at 16000g for 15mins twice. At the end, the aqueous phase was collected and subjected to spectrophotometry.

UV-Vis

Ultraviolet-visible spectroscopy is an analysing method using absorption spectroscopy in the ultraviolet-visible spectral region. UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different components, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules. The method is most often used in a quantitative way to determine concentrations of an absorbing species in solution. UV-Vis spectrometry will be used to determine anthocyanins and flavonoids in the flower sepals. The absorption of samples was measured at 535nm and 650nm by Molecular Devices.

D. Bioassay

Bioassay is defined as estimation or determination of concentration or potency of physical, chemical or biological agents by means of measuring and comparing the relative strength of a substance by comparing its effect on a test organism with that of a standard preparation. Bioassay is a successful tool in estimation and discovery of biologically active substances (e.g. drugs, toxin) and important application in sensitivity and specificity of pharmacological applications.

In this experiment, hoverflies were used to compare the influence of different Arabidopsis flower traits. There were 4 couples of comparisons, in which the control genotypes were compared with those different in their volatile blend (165&39,179,146), control with nectar (165&205), control with pollen (165&249,279), and volatile with nectar (39,179,146&205). The bioassay had two repeats in total, 3 plants were used for each accession per repeat. For each comparison, at least 8 hoverflies were released in the experiment tent (depended on activities of hoverflies). To keep the same sunlight, plants were put in a row in the middle of the tent (Figure 2.2.5). And each round took 40mins to observe.

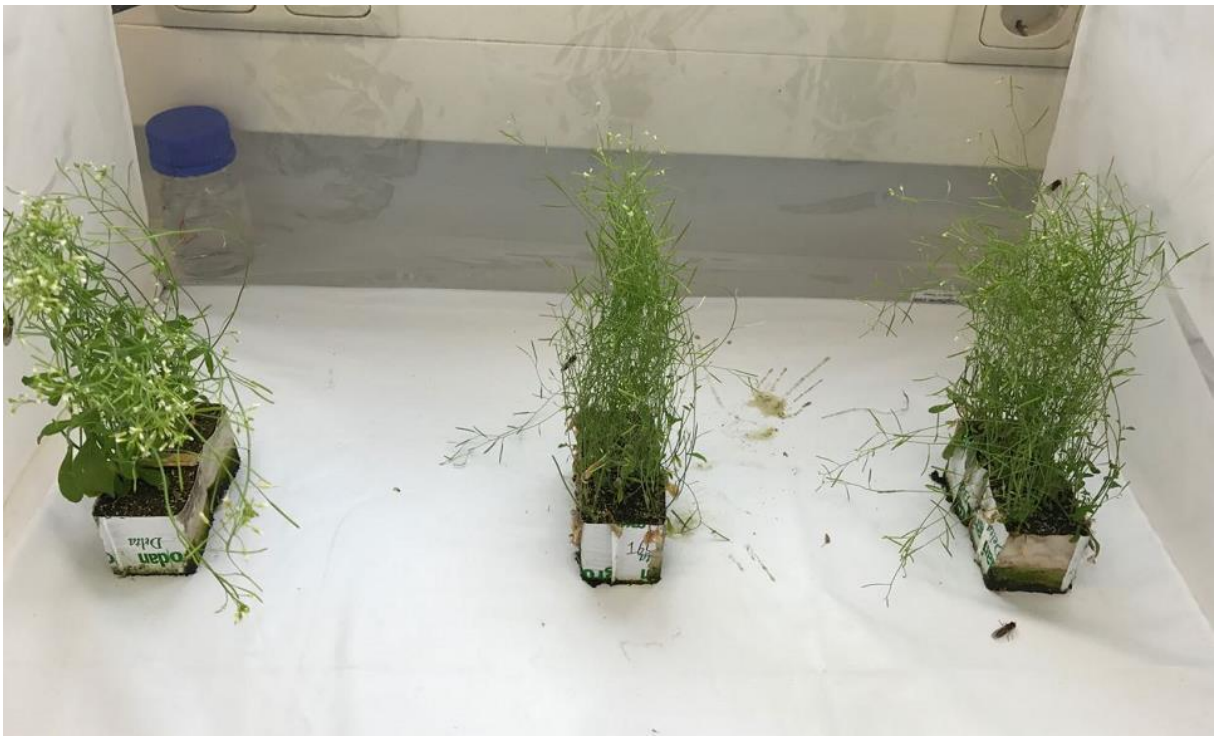


Figure 2.2.5 Bioassay of hoverfly behaviour observation

3. Results

3.1 Volatiles Analysis

For headspace, in total 42 (7accessions*2repeats*3batches) samples, 6 controls and 6 empty clean tubes were tested. After GC-MS analyse, it gave a chromatogram (Figure 3.1.1). The x-axis shows the retention time, and the y-axis shows the intensity (abundance) of the signal. As it can be seen from Figure 3.1.1, there were several peaks detected with their retention time and each peak representing a different compound which was present in the volatile mixture.

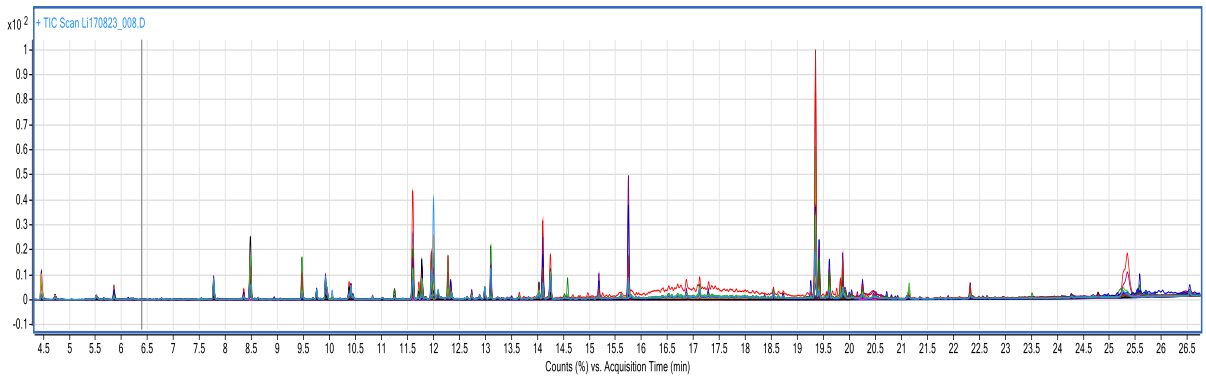


Figure 3.1.1 Chromatogram of batch 1

However, as there were many samples with complex blends containing many compounds, it was hard to compare chromatograms directly. So, data were normalized before analysing. Samples were normalized by sum to correct for differences introduced e.g. by different biomasses. Data were log transformed, mean-centred and divided by the by the square root of standard deviation of each variable (pareto scaling) (Figure 3.1.2). As a start point for data analysis, an unsupervised PCA was performed (Figure 3.1.3) which shows that empty liners and background odour deviated from the volatile blends of the tested accessions among the first PC. The second PC shows that volatile blends of genotypes clustered in 3 groups, most likely representing different days of analysis (batches). A discriminant analysis with a categorical response (PLS-DA) (Figure3.1.4) confirmed that the empty and controls had significant different volatile blend from plant samples.

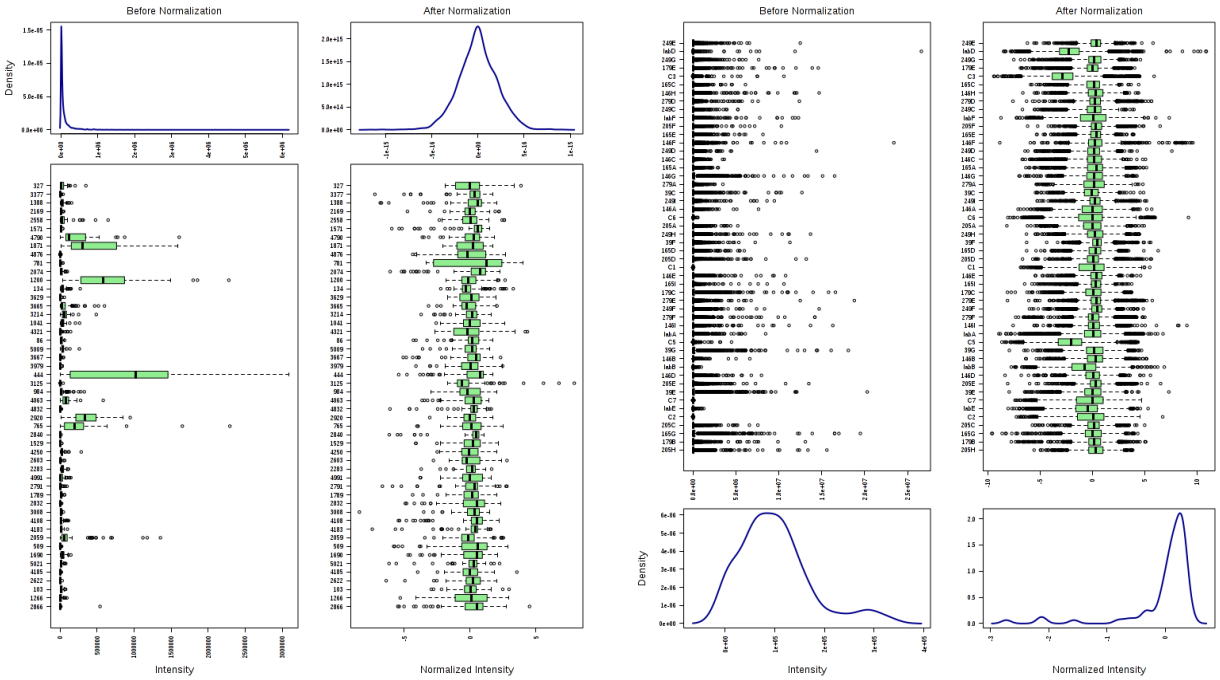


Figure 3.1.2 Normalization of all samples after sum normalized, log transformation and pareto scaling

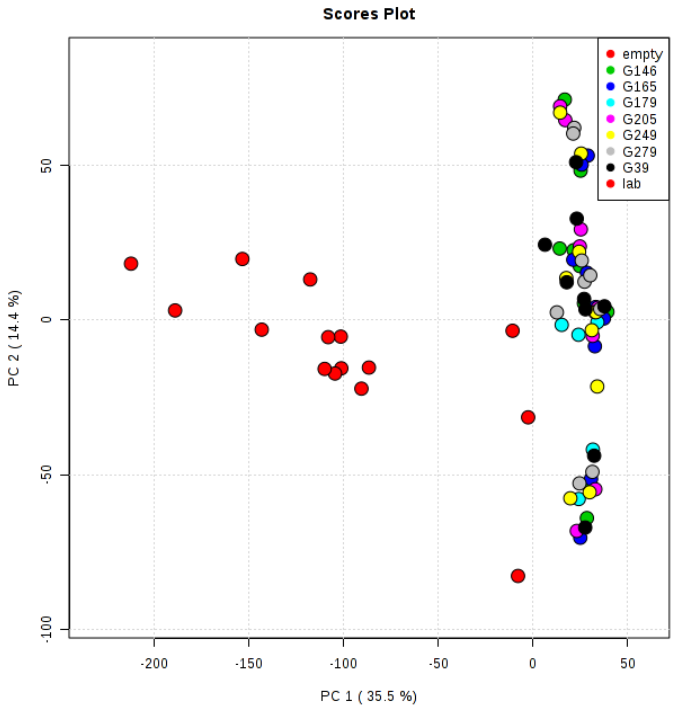


Figure 3.1.3 PCA of all samples, showing that most of the experimental variation (PC1, 35.5% of total variation) deviated empty liners and lab background odour from volatile blends emitted by the genotypes.

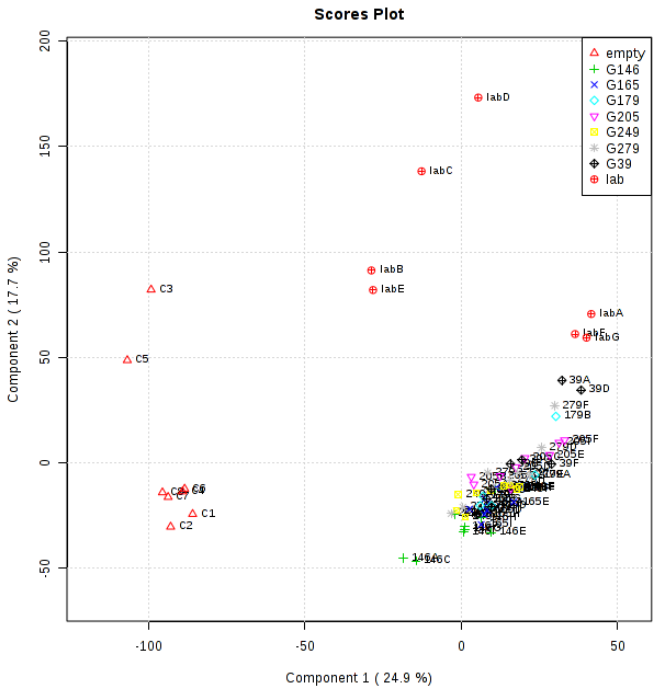


Figure 3.1.4 PLS-DA of all samples, showing that most of the experimental variation (Component 1, 24.9% of total variation) deviated empty liners and lab background odour from volatile blends emitted by the genotypes.

To study differences between volatile blends of the genotypes, empty liners and controls were excluded from the data analysis. The result of genotype data is shown as Figure 3.1.5, 3.1.6 and 3.1.7. PCA mainly divided measurement into three groups using 43.4% (PC1 29.5% + PC2 13.9%) of variation. It can be seen that measurements of batch A and B clustered together and were significant different from other batches. Also, the last batches, G and H grouped together and deviated. As the batches were collected from June until September and from plants grown in the greenhouse, it can be expected that seasonal and daily weather (sunny/rainy days) could have an effect. Using the genotype as categorical identified, PLS-DA showed that genotype contributed 7.2% of the variation found, Green part represented the genotype control (165). Based on PLSDA, the 95%-confidence intervals showed that genotype 146 (red) has a significantly different volatile blend than other genotypes, while other genotypes (green part representing the genotype control (165), grey (39), blue part (205), and yellow part (279) did not significantly differ from 165.

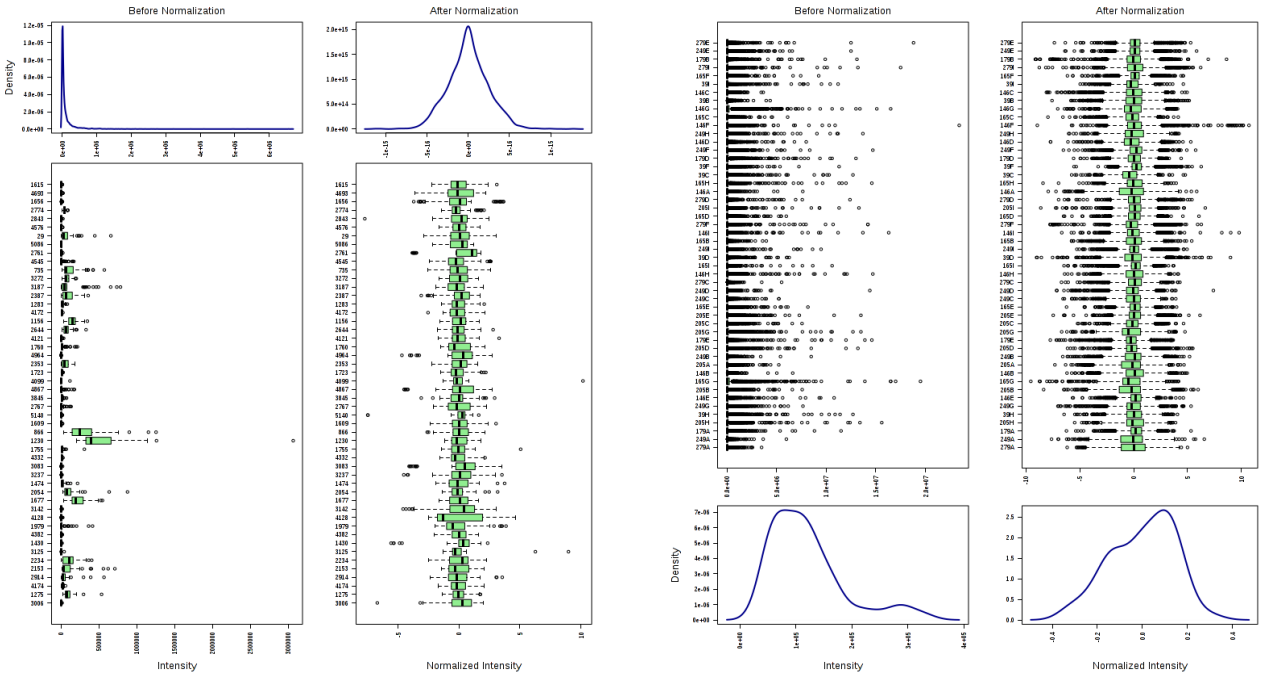


Figure 3.1.5 Normalization of genotype samples after sum normalized, log transformation and pareto scaling

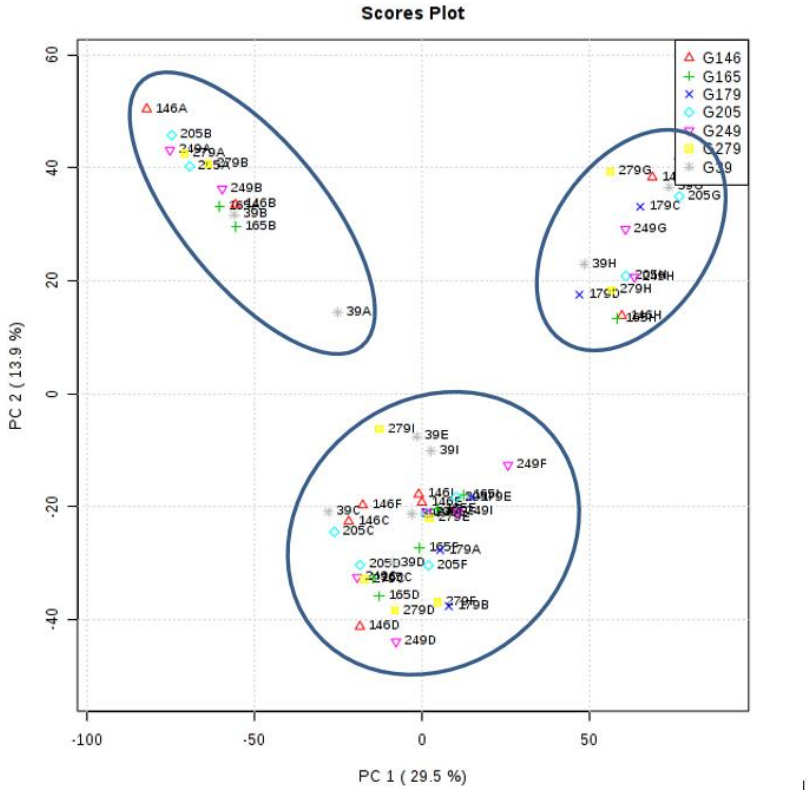


Figure 3.1.6 PCA of genotype samples, showing that most of the batch variation (PC1, 29.5% of total variation) deviated batch A and B, G and H from other batches of volatile blends emitted by the genotypes separately.

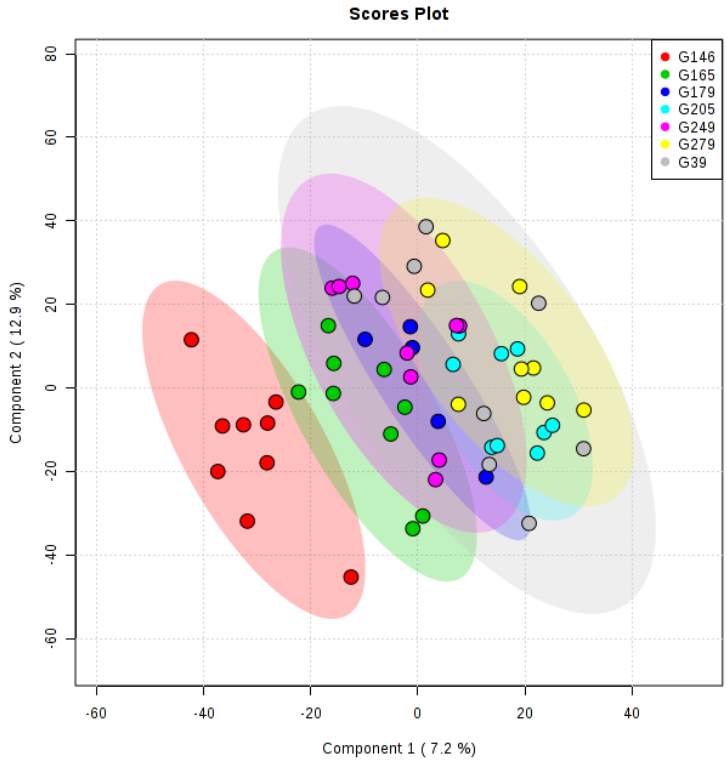


Figure 3.1.7 PLS-DA of genotype samples, showing that most of the genotype variation (Component 1, 7.2% of total variation) deviated accession 146 significantly from volatile emitted of 165, while 39, 205 and 279 were not significant.

However, there were still too many peaks, as the aforementioned analysis was based on all mass fragments obtained from the mzmine output. MS CLUST was used to reduce the number of peaks from 5184 [m/z@retention time] fragments to 604 centrotypes. The data analyse was done for MS CLUST output same as before. The final results are shown as Figure 3.1.8-3.1.10. The PCA and PLS-DA of the cluster data were similar with genotype result. There were three groups when 51.2% (PC1 37.6% + PC2 13.6%) of variation can be explained by the first two PC of PCA. The data of batches A and B had similar results but significant differed from other batches, as well as batches G and H. Based on PLS-DA, the 95% confidence intervals showed that genotype 146 (red) has a significantly different volatile blend than other genotypes, while other genotypes (green part representing the genotype control (165), grey (39), blue part (205), and yellow part (279) did not significantly differ from 165.

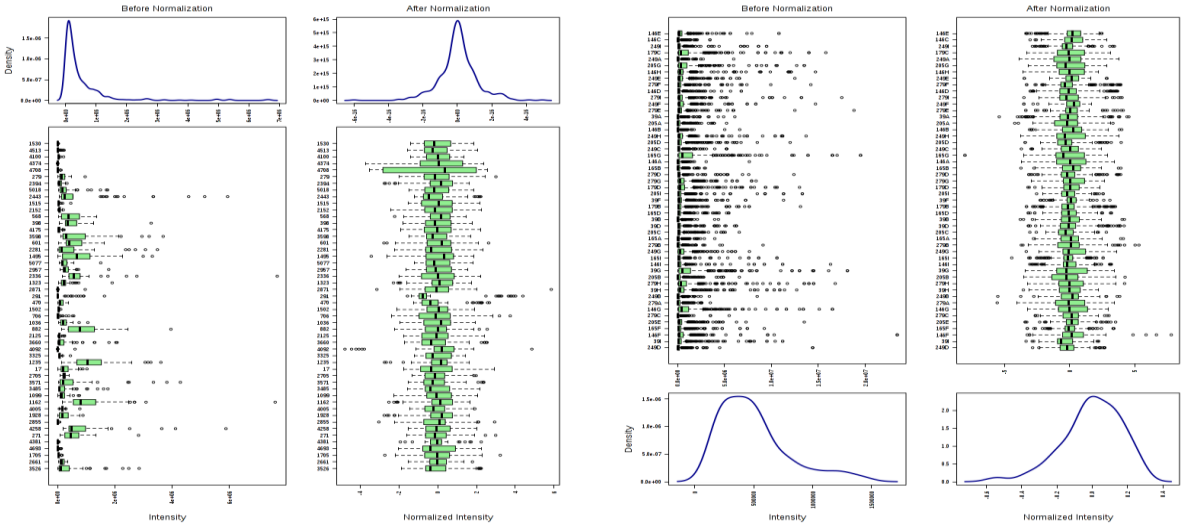


Figure 3.1.8 Normalization of cluster data after sum normalized, log transformation and pareto scaling

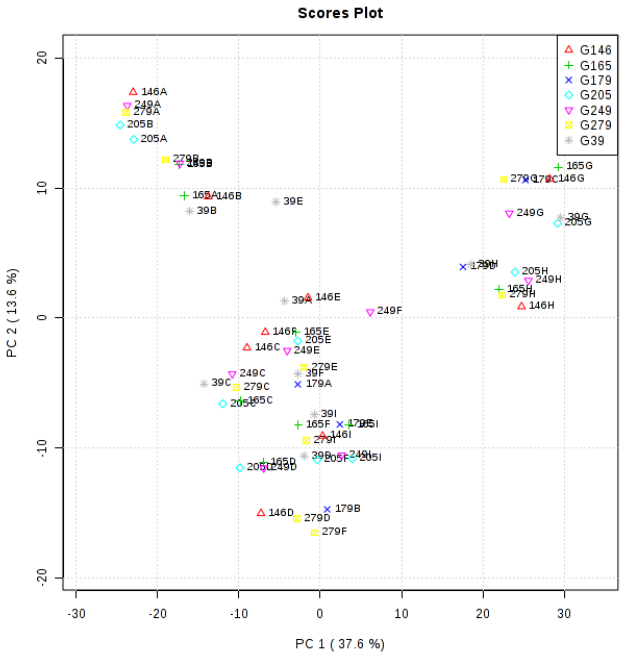


Figure 3.1.9 PCA of cluster data, showing that most of the cluster variation (PC1, 37.6% of total variation) deviated batch A and B, G and H from other batches of volatile blends emitted by the genotypes separately.

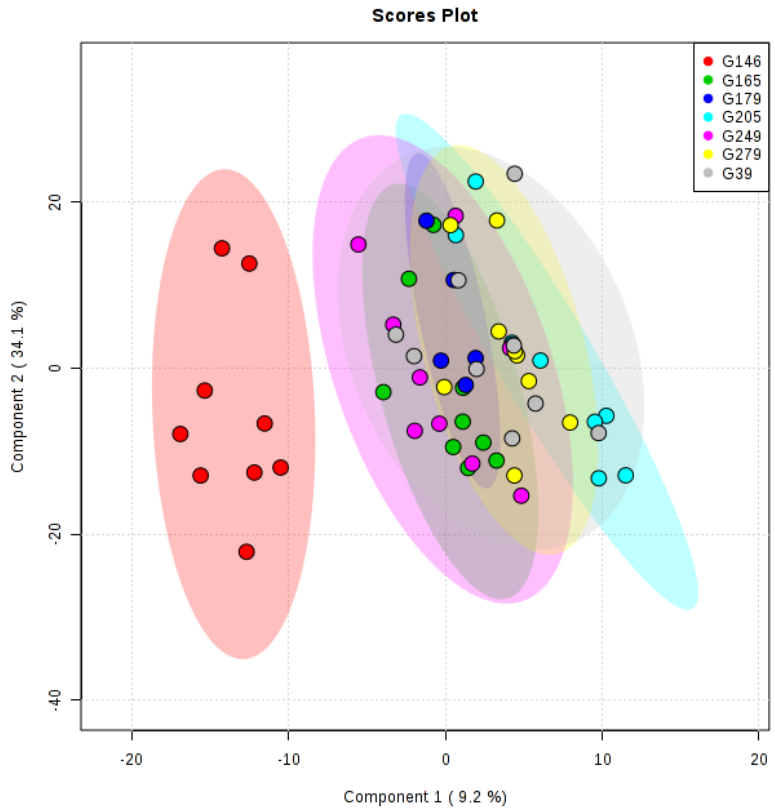


Figure 3.1.10 PLS-DA of cluster data, showing that most of the genotype variation (Component 1, 9.2% of total variation) deviated accession 146 significantly from volatile emitted of 165, while 39, 205 and 279 were not significant.

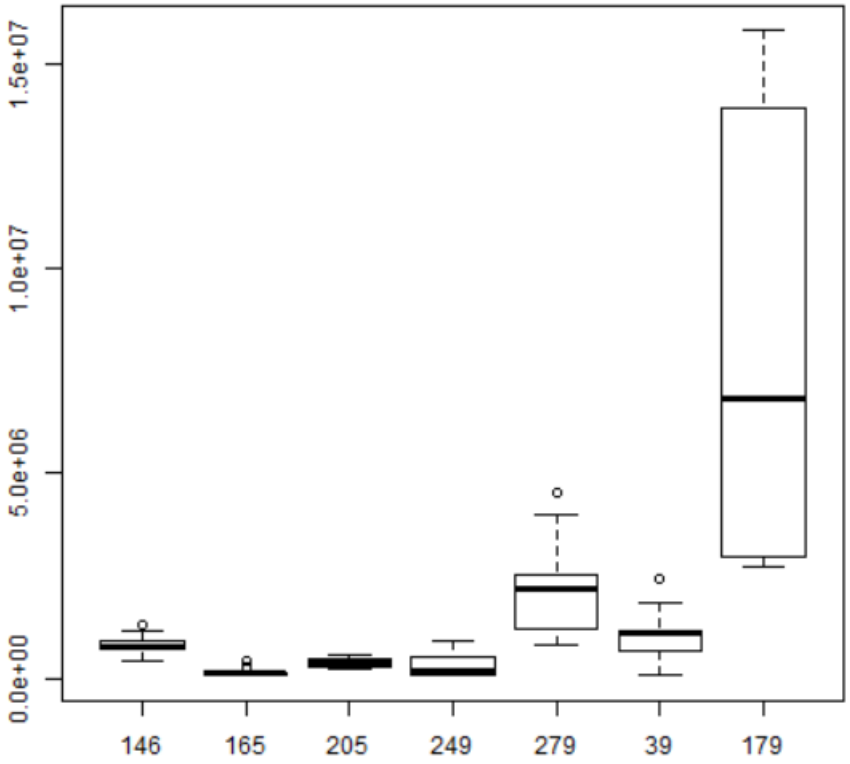
To test, which of the individual compounds were responsible for the separation of the ecotypes, Student T-test was performed using Rstudio, comparing each of the accessions with the reference genotype, 165 respectively. Comparing the T-test result and original chromatogram data, the significant different accessions under several terpenes were listed in Table 3.1.1. In the table, (E)-β-ocimene is monoterpene, all other terpenes are sesquiterpenes. Accessions 39 and 146 were significant different from 165 for the different sesquiterpenes, while accessions 179 was significantly different from 165 for the monoterpene.

Table 3.1.1 T-test result of cluster data, showing that what accessions at what Retention Time (RT/min) had significant different from genotype control 165 of which terpene. i.e. at 19.29min, genotype 146 had significant different from 165 of sesquiterpene geranylacetone.

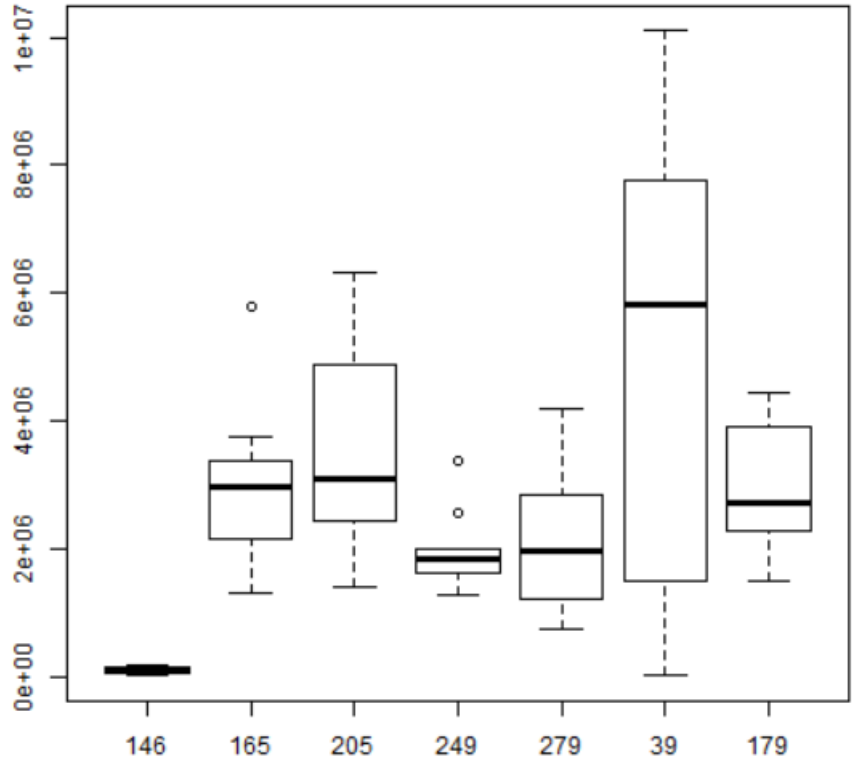
Terpene	(E)-β-ocimene	geranylacetone	(E)-β-caryophyllene	α-barbatene	thujopsene	humulene	chamigrene	cuparene
RT	13.08764865	19.29472973	19.33662162	19.41256944	19.598324	19.84982	20.2504	20.5102222
Accessions	179	146, 39	146, 39	146, 39	146, 39	146, 39	146, 39	146, 279, 39

Boxplots (Figure 3.1.11) showed the concentration for relevant compounds between different accessions. Except for (E)-β-ocimene and cuparene, the concentrations of the relevant compounds of genotype 146 were much lower than those of genotype 165 while the compound concentrations of genotypes 39 were much higher than those of 165. For

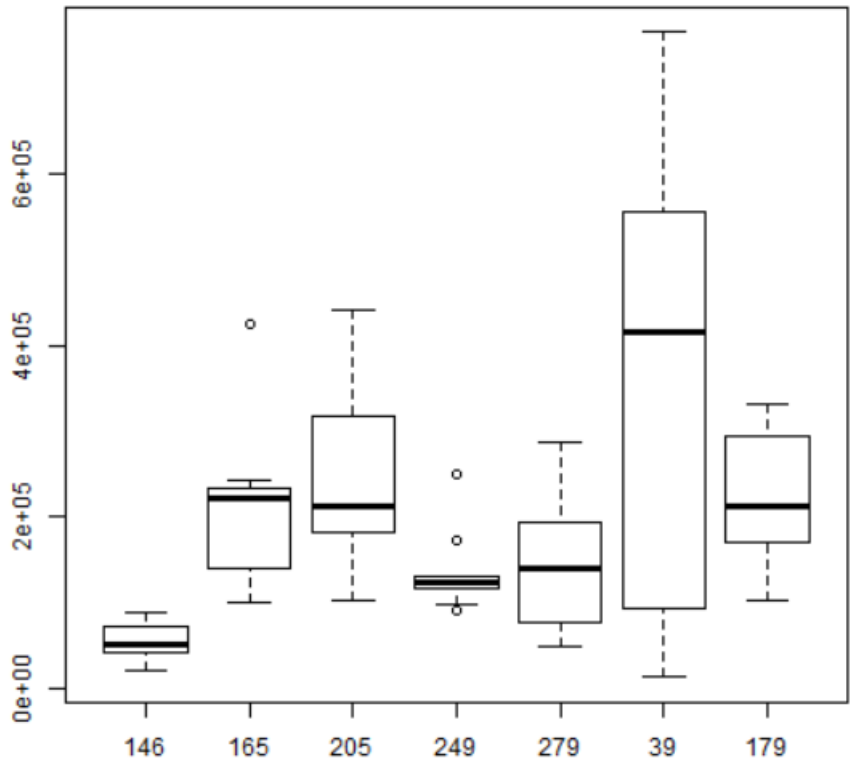
monoterpene (E)-β-ocimene, accession 179 had the highest concentration level while the concentrations of other accessions were more or less similar. As for the sesquiterpene cuparene, the concentrations of accession 39, 146 and 279 were all lower than that of 165.



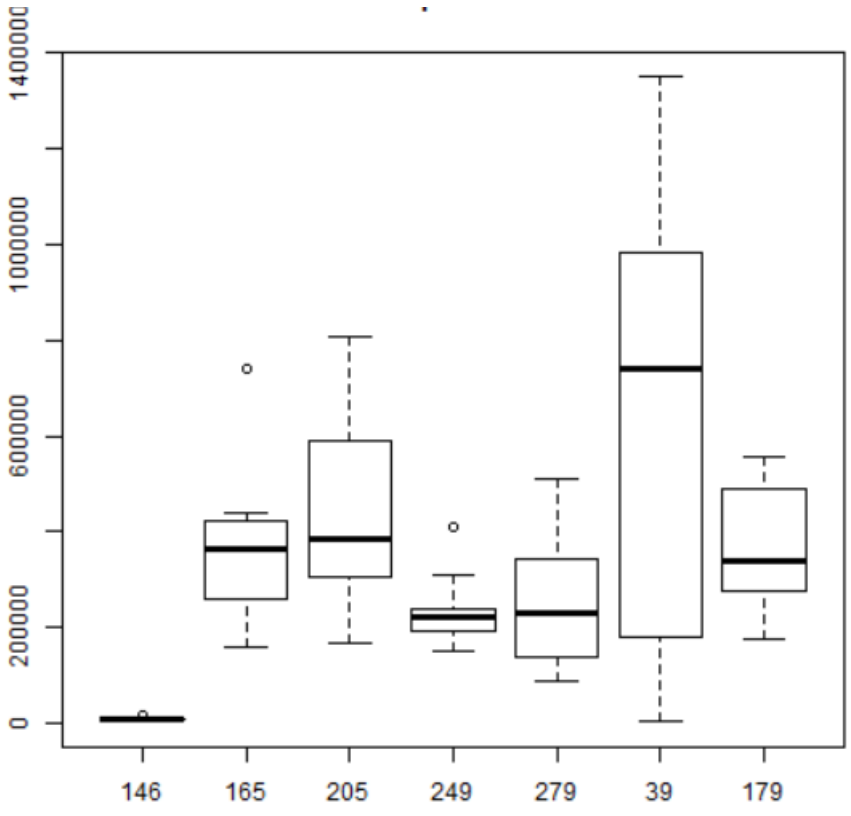
(E)-β-ocimene



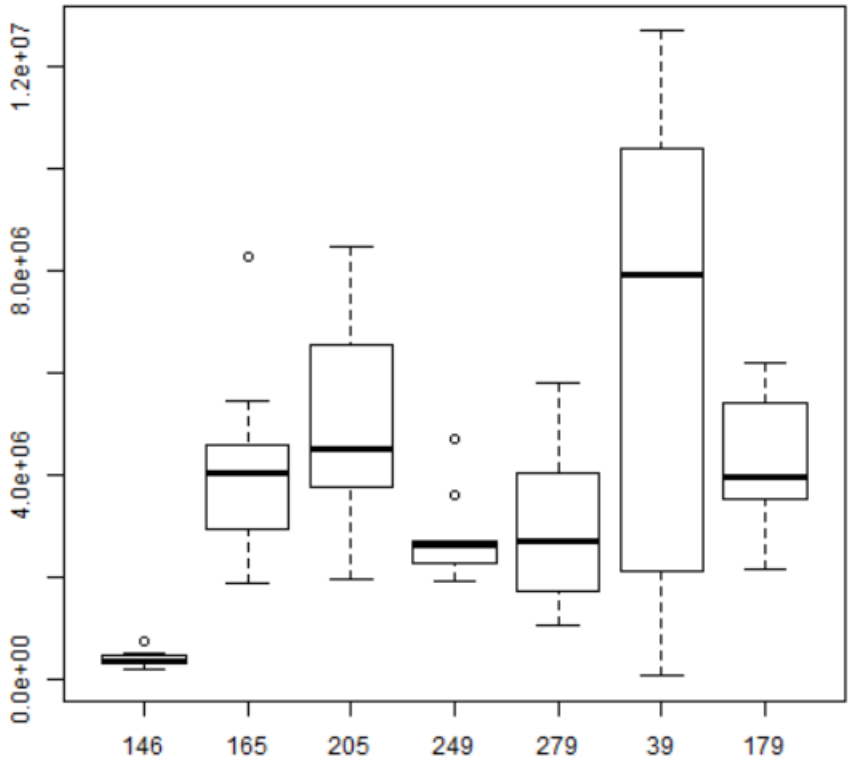
geranylacetone



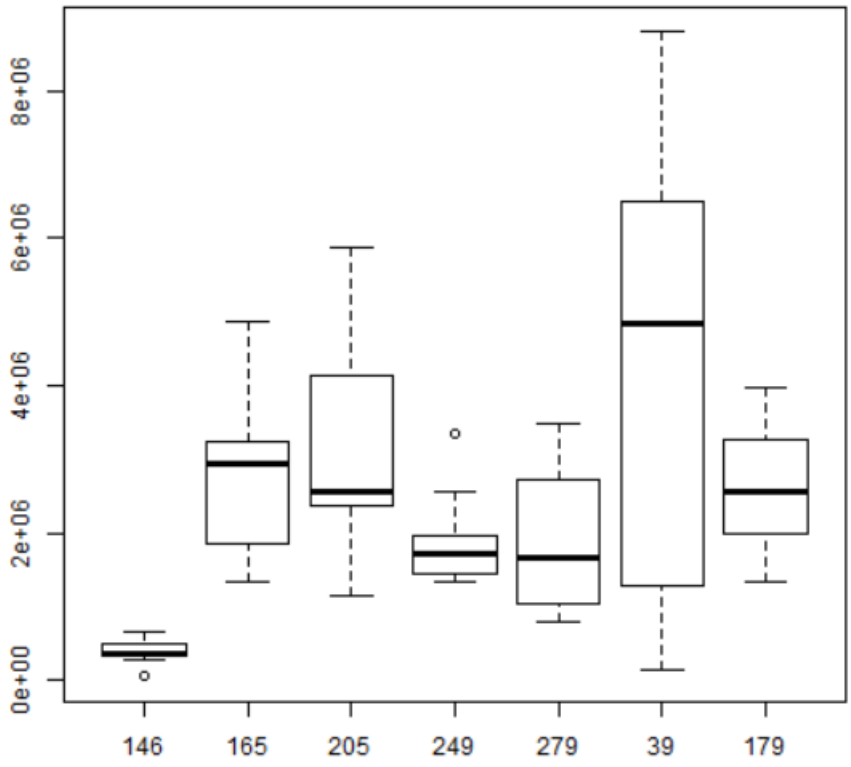
(E)-β-caryophyllene



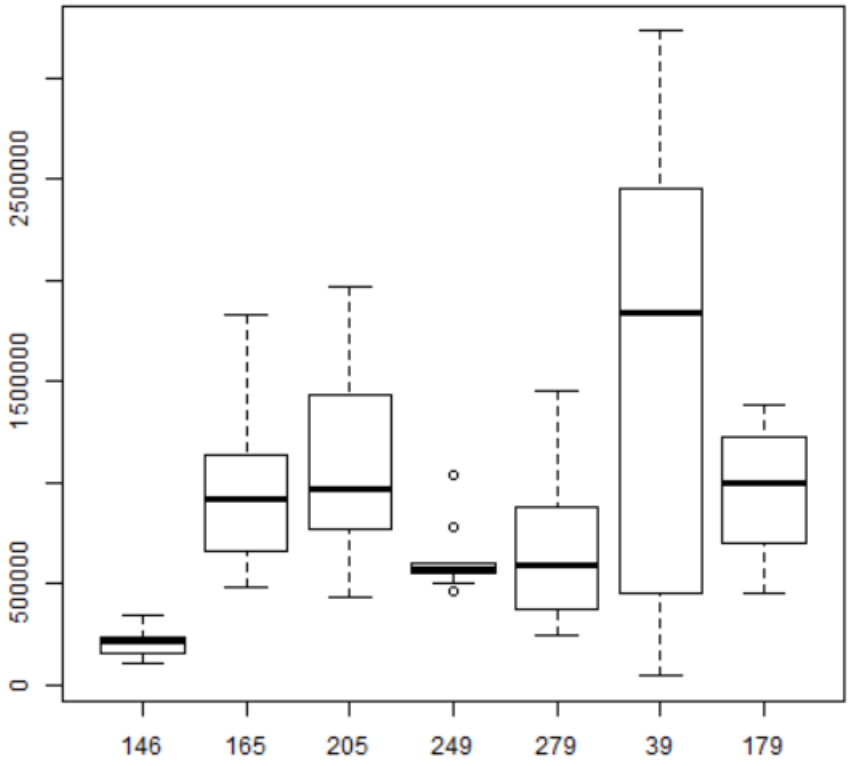
α-barbatene



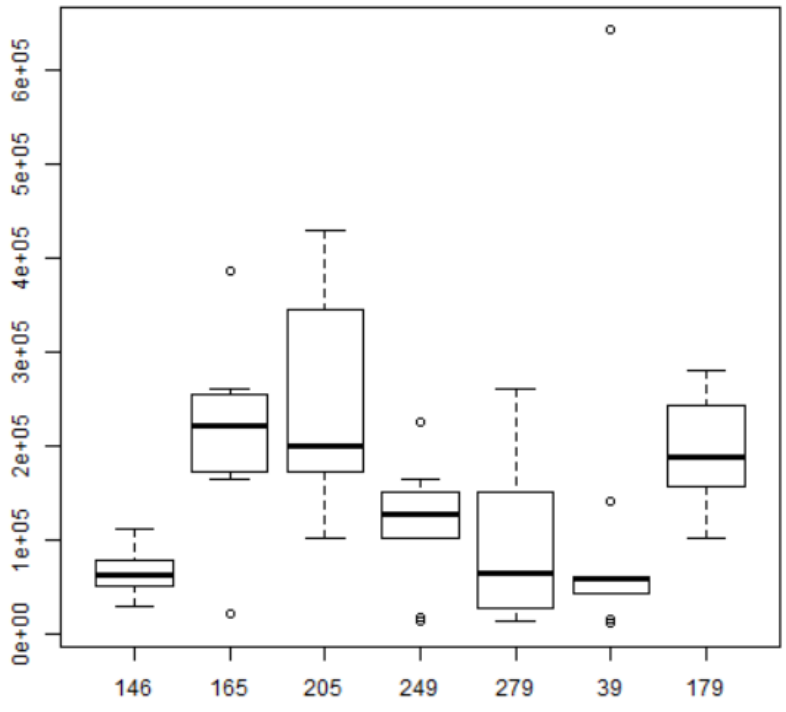
thujopsene



humulene



chamigrene



cuparene

Figure 3.1.11 Box-plots of important terpenes showing that the concentrations of the relevant compounds of genotype 146 were much lower than those of other genotypes after normalized by to the sum of volatiles to correct for differences in biomass while the compound concentrations of genotypes 39 were much higher. So, genotype 146 should have relatively

high abundance of other type of compounds than other genotypes which 39 should have relatively low concentration.

3.2 Sugar Extraction

At first, the first two batches of sugar extraction were done for all accessions. The same data processing as volatiles had been done for sugar. Before the data analyse, standardization had been applied to the data for different batches of different sugars. Samples were normalized by sum to correct for differences introduced. Data were log transformed, mean-centred and divided by the by the square root of standard deviation of each variable (pareto scaling). In the PCA (Figure 3.2.1), green part represented the genotype control (165). Accession 205 (purple part) had significantly different from 165 (green part) based on the 95% confidence intervals under 76.4% (PC1 47.8%+PC2 28.6%) of variation. To test, which of the individual compounds were responsible for the separation of the ecotypes, Student T-test was performed using Rstudio, comparing each of the accessions with the reference genotype, 165 respectively. The T-test results shown as Table3.2.1. It showed that accession 205 was significantly different from 165 for glucose, inositol, fructose, stachyose and sorbitol, while the concentration of accession 279 had significant difference with 165.

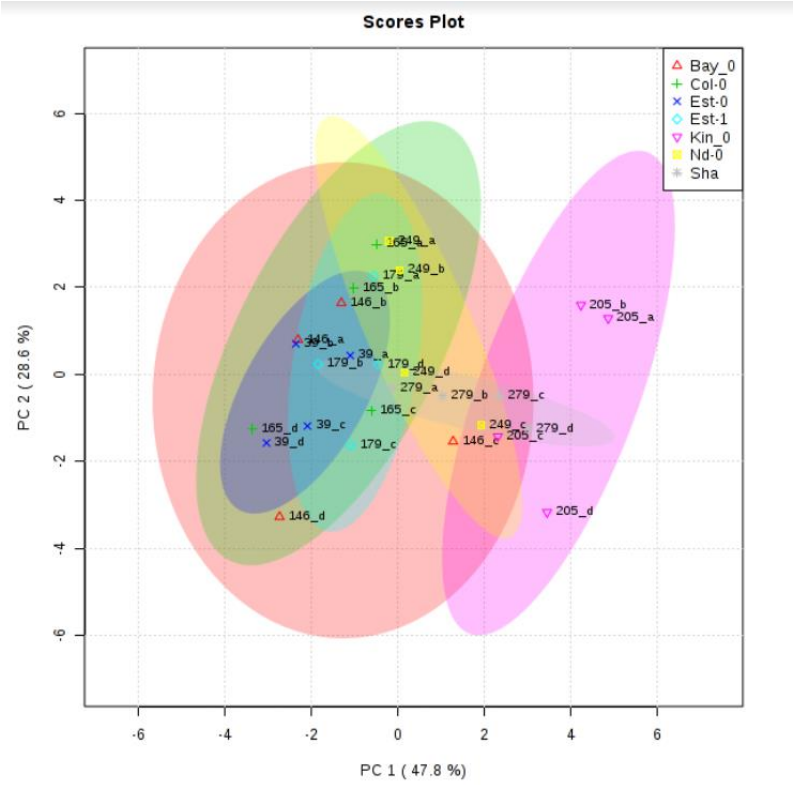


Figure 3.2.1 PCA of sugar extraction (1-2 batches) of all samples, showing that most of the experimental variation (PC1, 47.8% of total variation) deviated genotype 205 from components by the left genotypes.

Table 3.2.1 T-test of sugar extraction (1-2 batches) of all sample, showing that which genotypes had significant difference (P<0.05) from genotype control 165 of which components. i.e. Genotype 205 significantly different from 165 of the concentration of glucose.

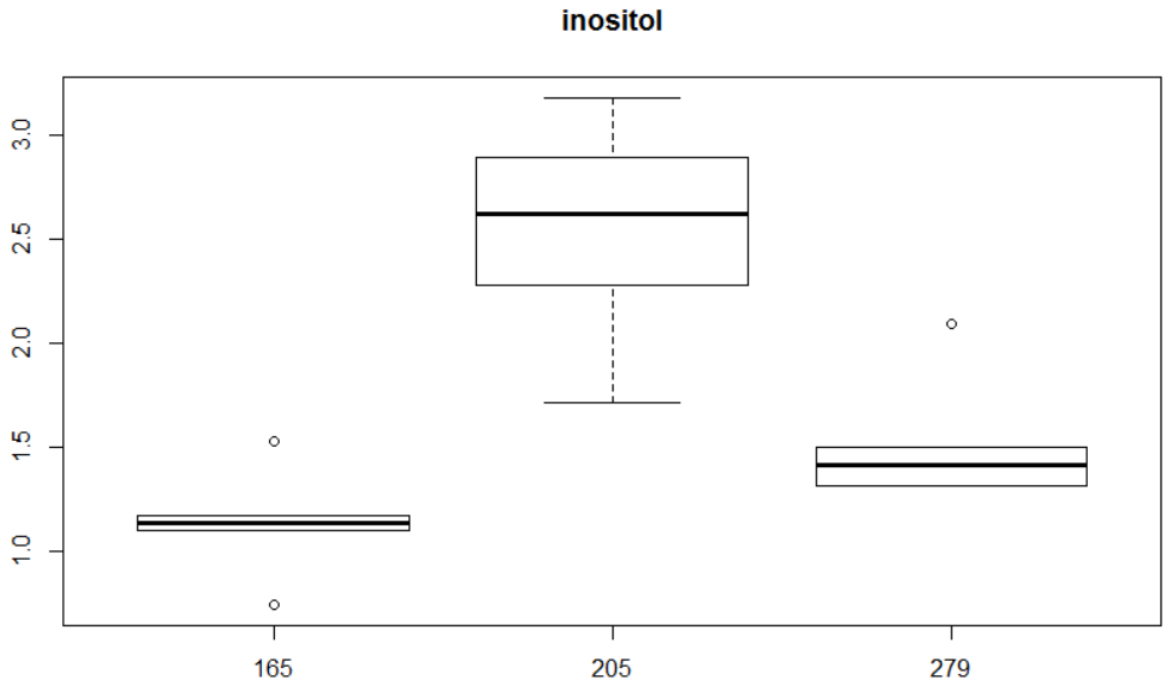
Sugar	glucose	inositol	fructose	glucose	stachyose	sorbitol
Accessions	205	205	205	279	205	205
P value	0.000461	0.000922	0.002537	0.006516	0.011078	0.033038

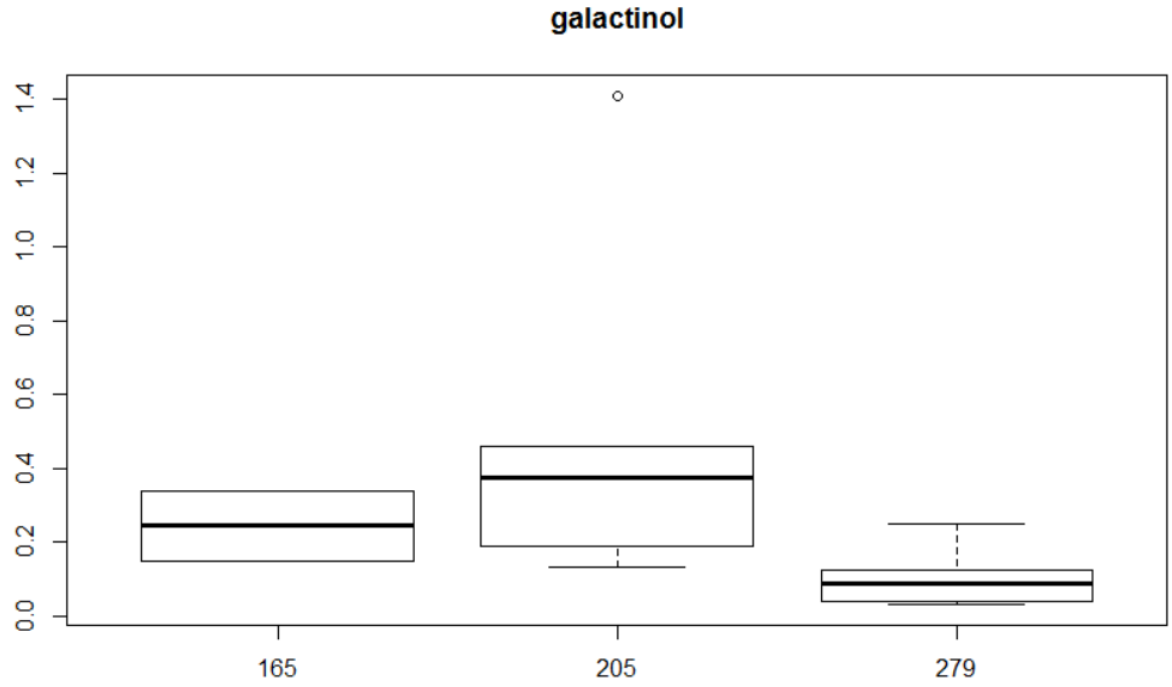
However, several sugars could not be detected because there was not enough amount of nectars. To study differences between sugar of the genotypes and save time, other accessions were excluded from the third batch except for 165, 205 and 279. And the amount of nectar pieces in the third batch was increased from 50 to 150. Then the same data processing had been applied, i.e. normalization, MetaboAnalyst and Student T-test. Comparing the T-test results of 3 accessions (Table 3.2.2) and the T-test results of all accessions (Table 3.2.1), there were some slight differences. As the batches were collected from June until September and from plants grown in the greenhouse, it can be expected that seasonal and daily weather (sunny/rainy days) could have an effect. It showed 205 was significantly different from 165 for inositol, fructose, glucose, stachyose and melezitose, while 279 had significant differences of galactinol and inositol with 165.

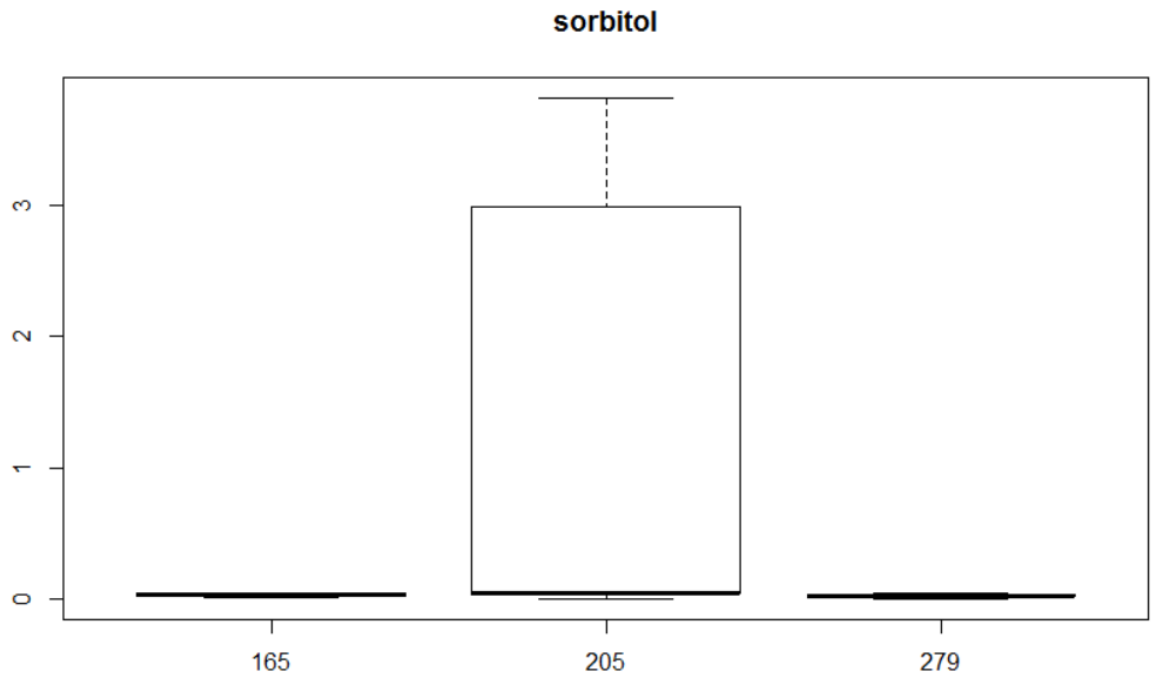
Table 3.2.2 T-test result of sugar extraction (1-3 batches) of genotypes 165, 205 and 279, showing that which genotypes had significant difference (P<0.05) from genotype control 165 of which components.

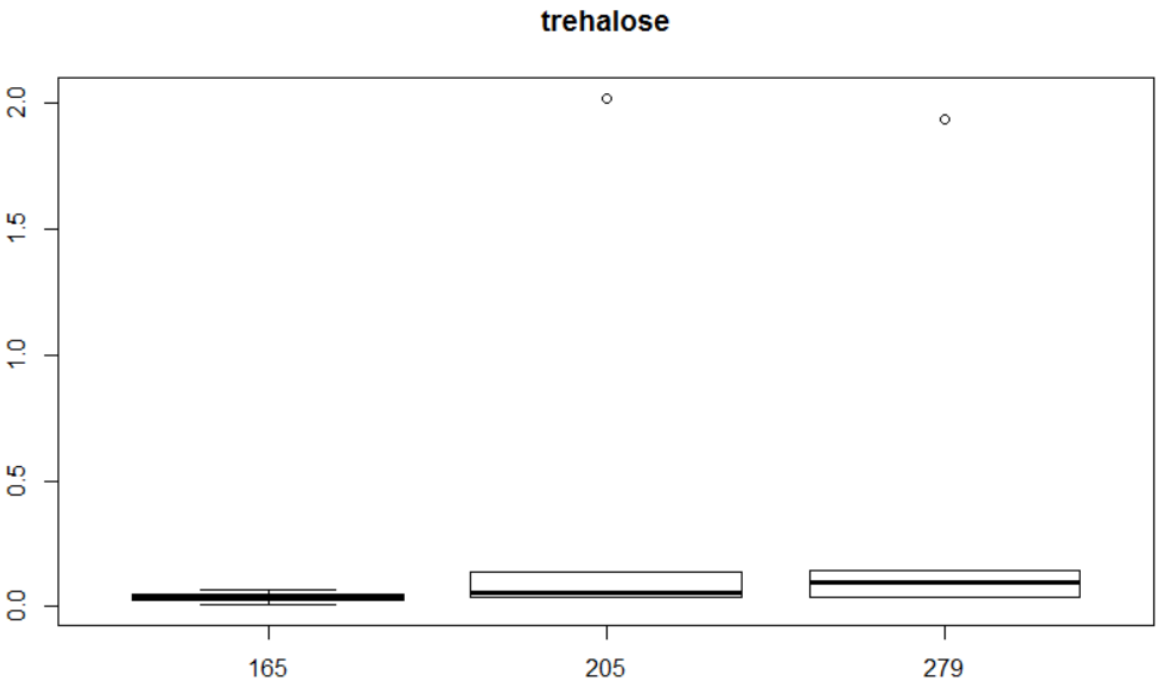
Sugar	inositol	fructose	glucose	galactinol	inositol	stachyose	melezitose
Accession	205	205	205	279	279	205	205
P value	0.000497	0.002721	0.003864	0.018729	0.040598	0.040669	0.0447803

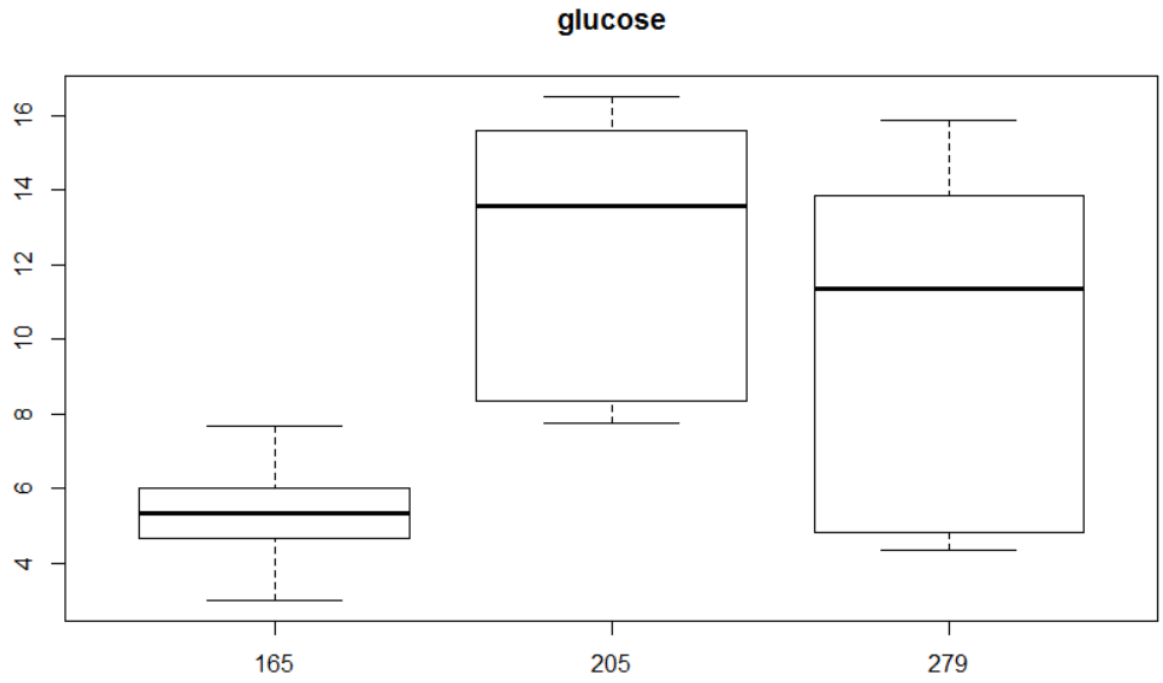
Besides the T-test, box-plot (Figure 3.2.2) run by RStudio showed the concentration for relevant components between different accessions. Except for amino acid serine and sugar melezitose, the concentrations of the relevant sugars of genotype 205 were higher than those of genotype 165 and 279. For genotype 279, the concentration of glucose and fructose were much higher than those of 165. For amino acid serine and sugar melezitose, genotype 165 had higher concentration than 205 and 279.

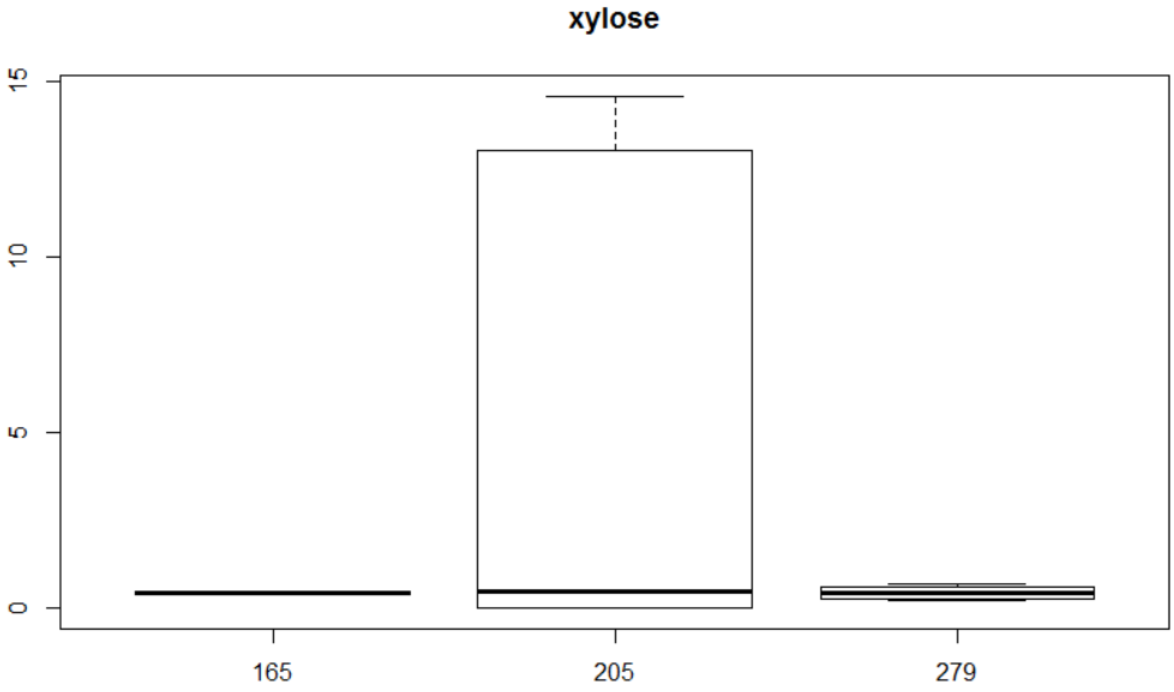

inositol
3.0
2.5
2.0
1.5
1.0
165
205
279

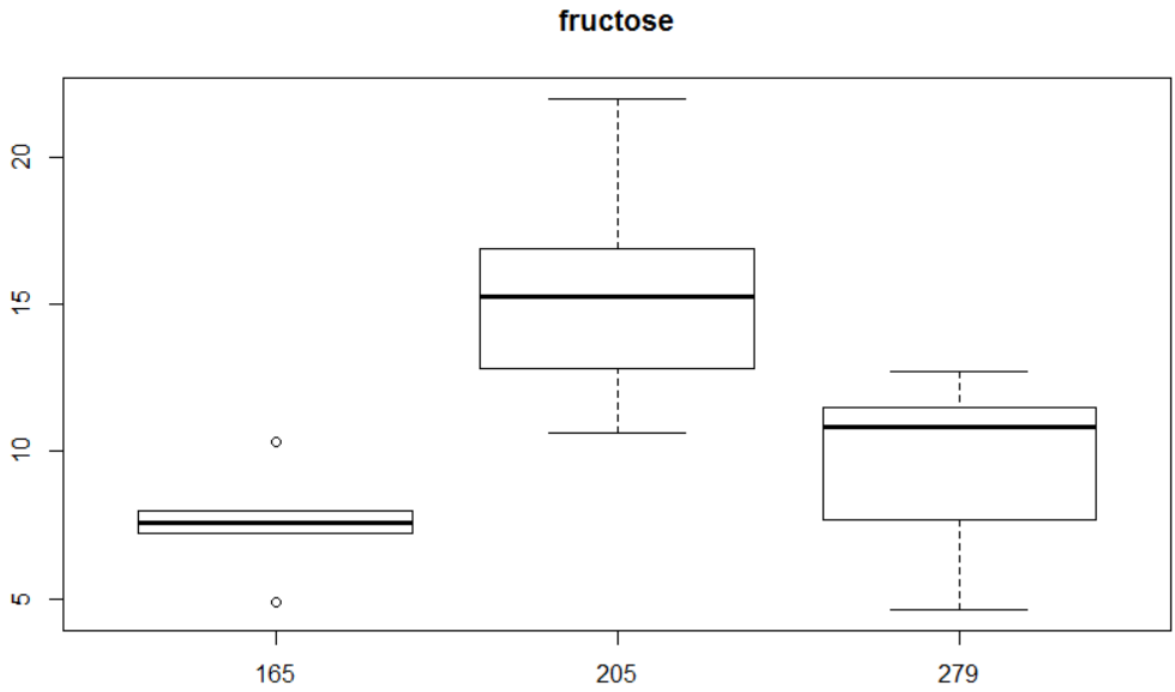

galactinol
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
165
205
279

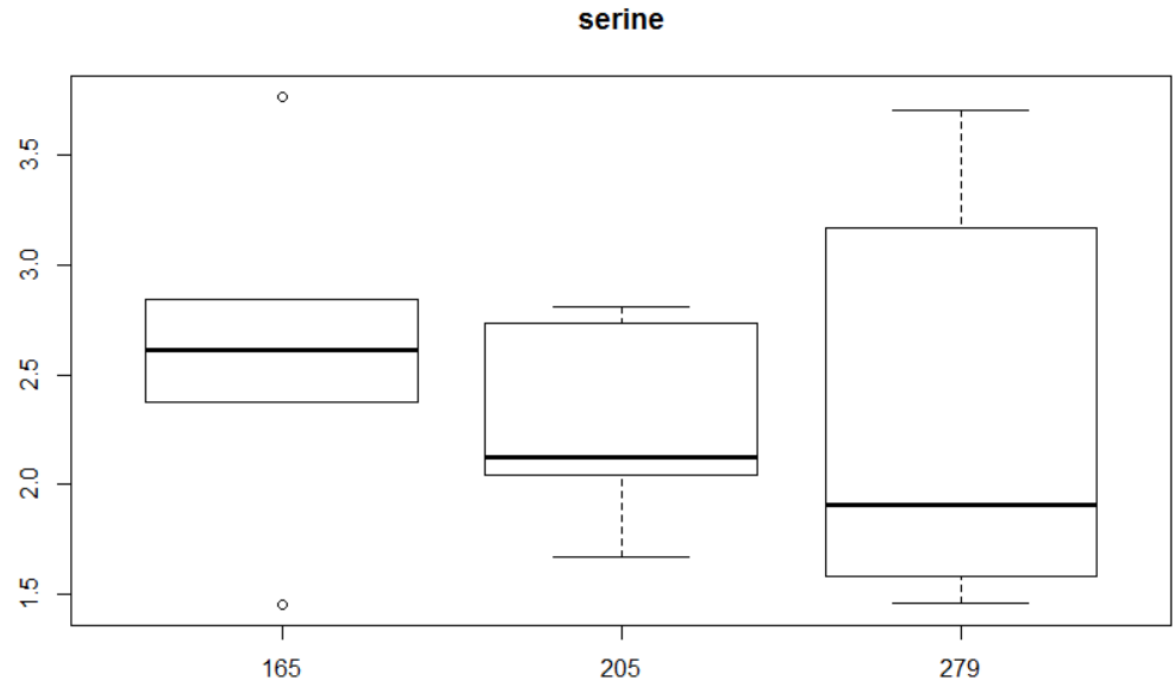

sorbitol
0
1
2
3
165
205
279

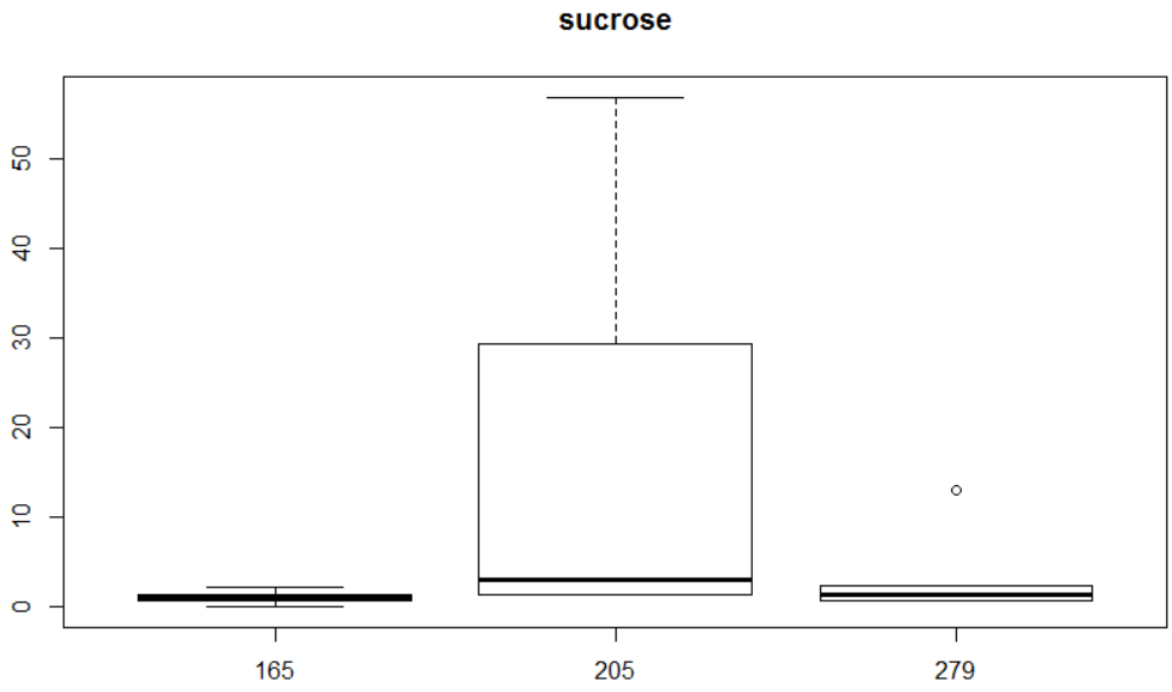

trehalose
0.0
0.5
1.0
1.5
2.0
165
205
279

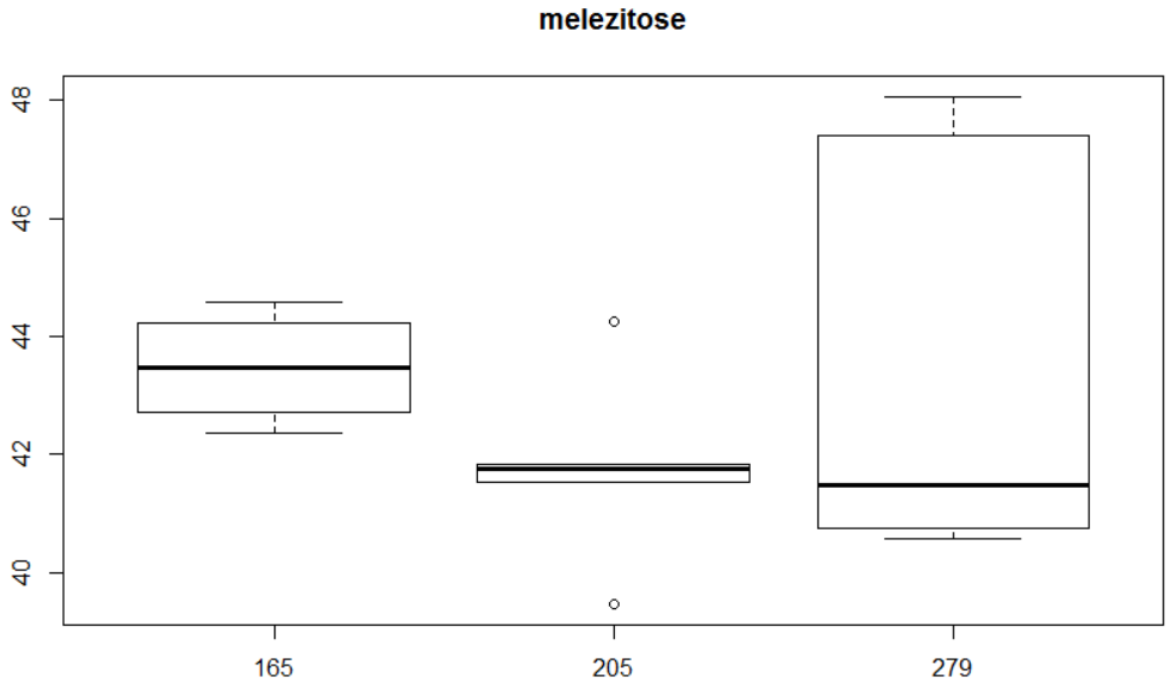

glucose
4
6
8
10
12
14
16
165
205
279

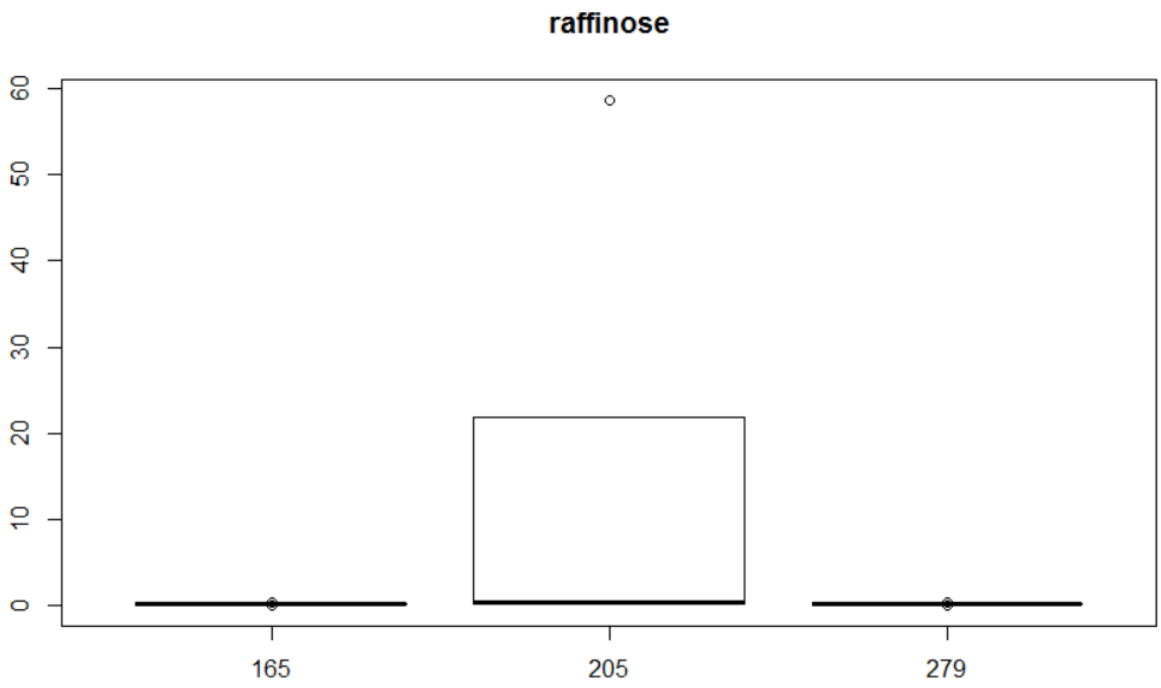

xylose
0
5
10
15
165
205
279


fructose
20
15
10
5
165
205
279


serine
3.5
3.0
2.5
2.0
1.5
165
205
279


sucrose
0
10
20
30
40
50
165
205
279


melezitose
40
42
44
46
48
165
205
279


raffinose
0
10
20
30
40
50
60
165
205
279

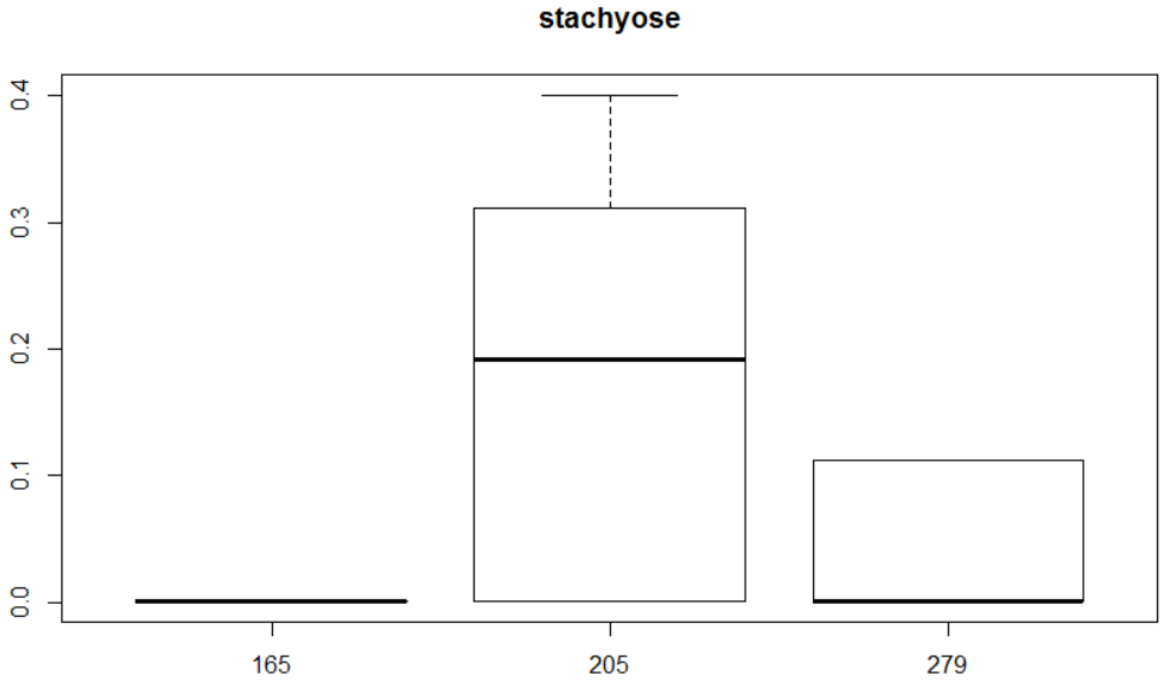

stachyose
0.0
0.1
0.2
0.3
0.4
165
205
279

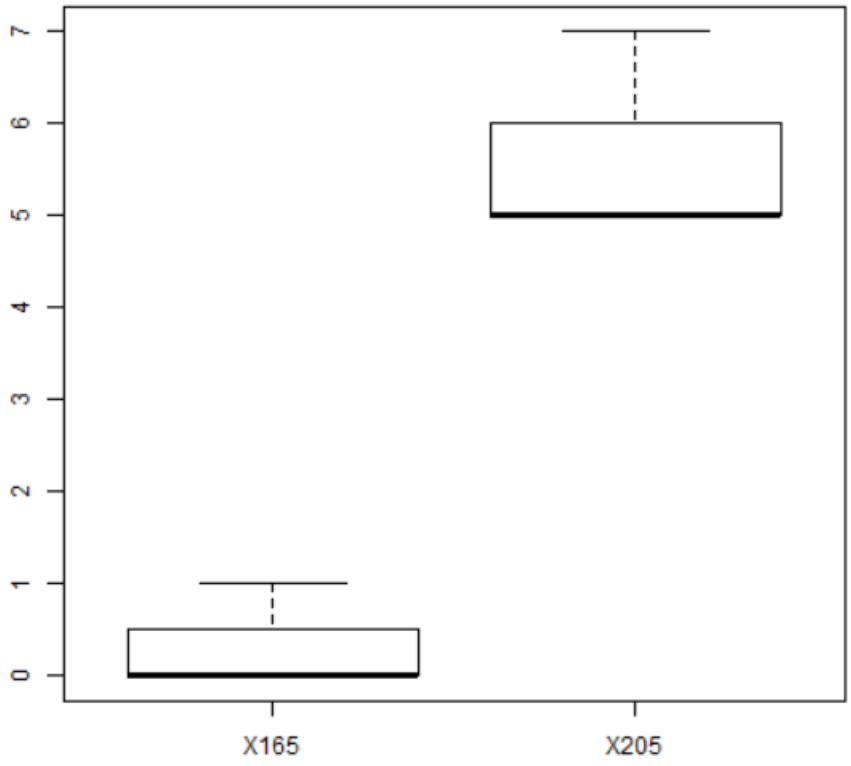
Figure 3.2.2 Box-plots of sugar extraction (1-3 batches) showing that 205 had more sugar than other accessions

3.3 Anthocyanin

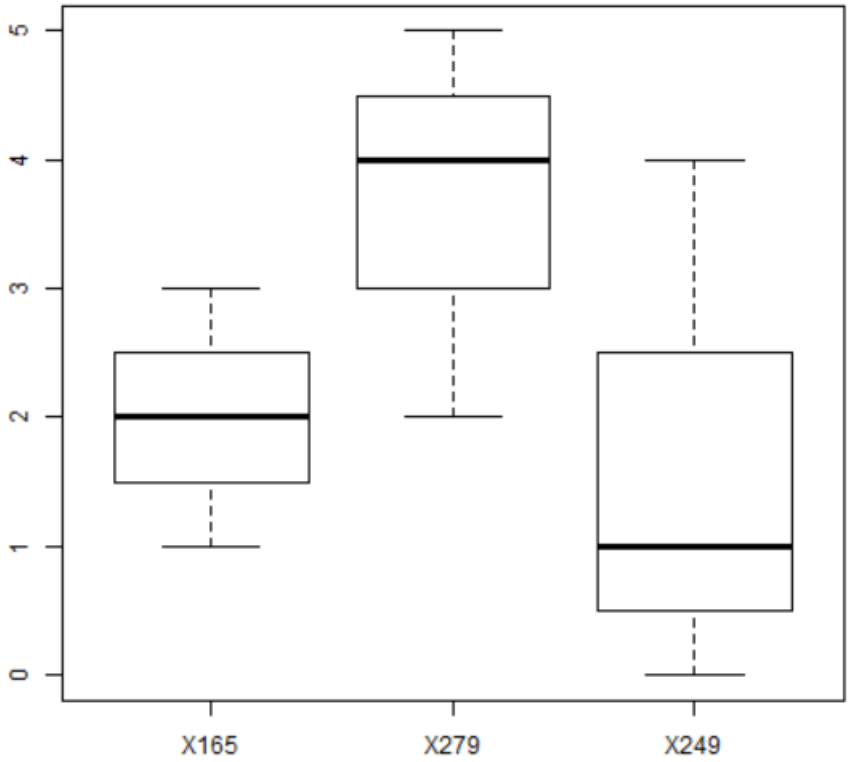
To test the anthocyanin, the flower petals from all accessions were used. Because it was not sure about how many petals were needed, so the amount of 50 petals, 100 petals and 300 petals had been tested. Since the flower petal of all accessions were the same white for human eye to distinguish, so it was almost impossible for SpectraMax from Molecular Device to detect with too less amount of anthocyanin. All the results were nearly 0, as same as blank control.

3.4 Bioassay

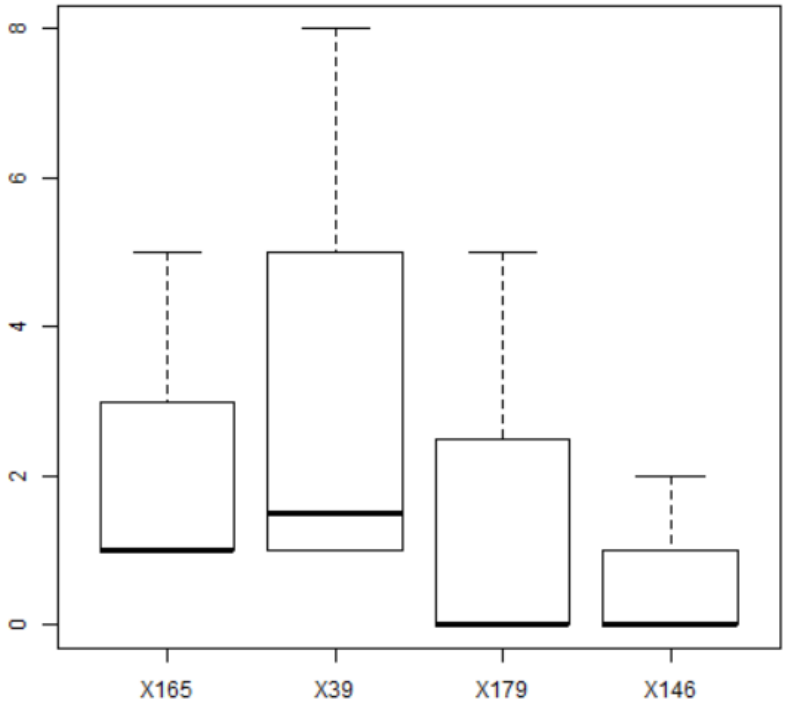
To test whether differences in various flowers traits affect the preferences of hoverflies, contrasting genotypes were tested in a two-choice assay. Each time, two plants of each genotype were enclosed in an insect tent and 8 hoverflies were released. The number of visits to flowers, and also the duration of the visit was recorded for 30 minutes. The records of visited times were shown in Figure3.4.1 and stay duration in Figure 3.4.2.



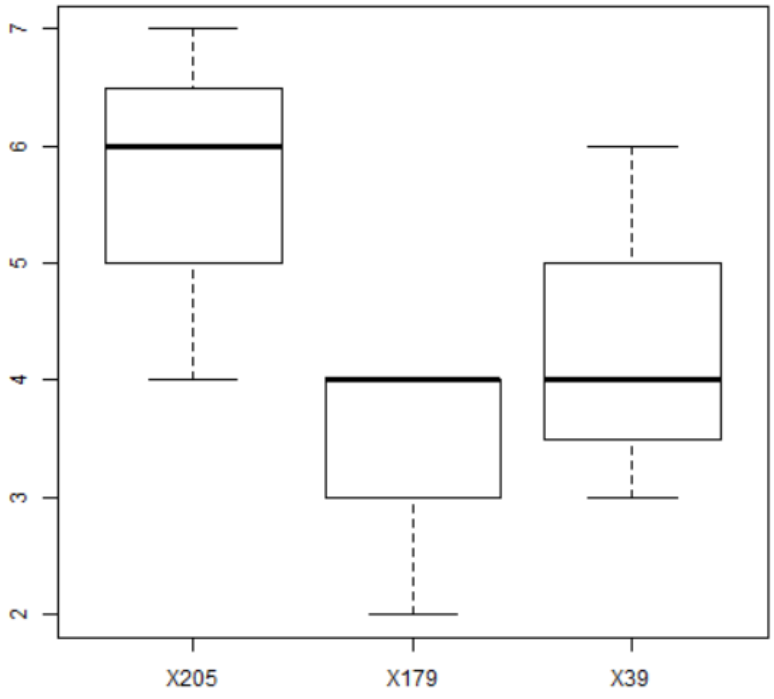
Control&Nectar



Control&Pollen



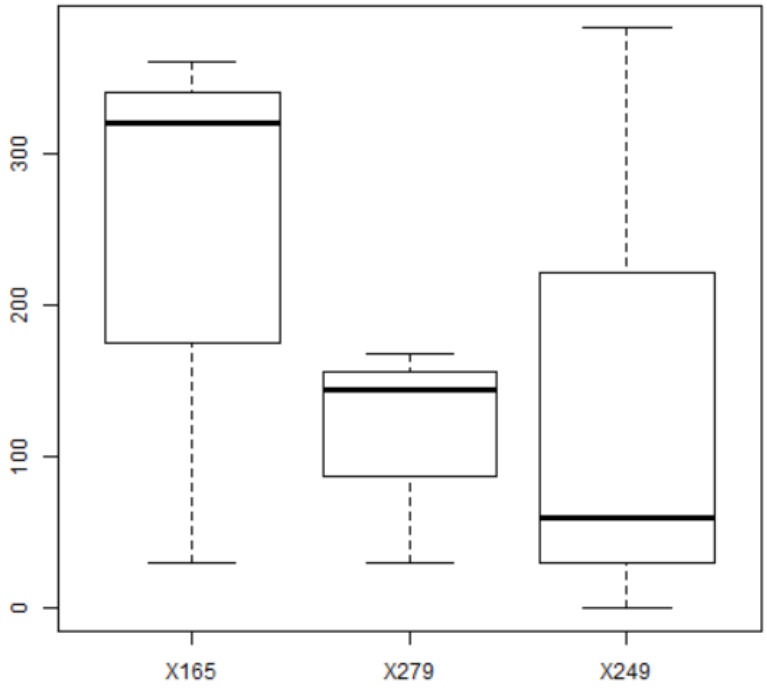
Control&Volatile



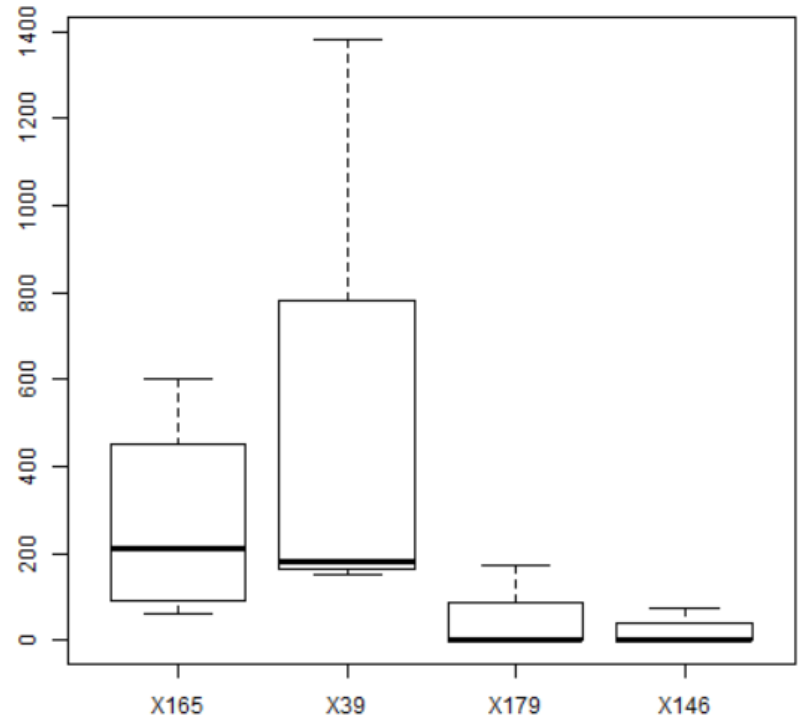
Nectar&Volatile

Figure 3.4.1 Visiting times of hoverflies by different comparisons (Genotype control & Volatile/Pollen/ Nectar, Nectar & Volatile). According to different comparisons, it showed which genotype had more visited times, which had less.

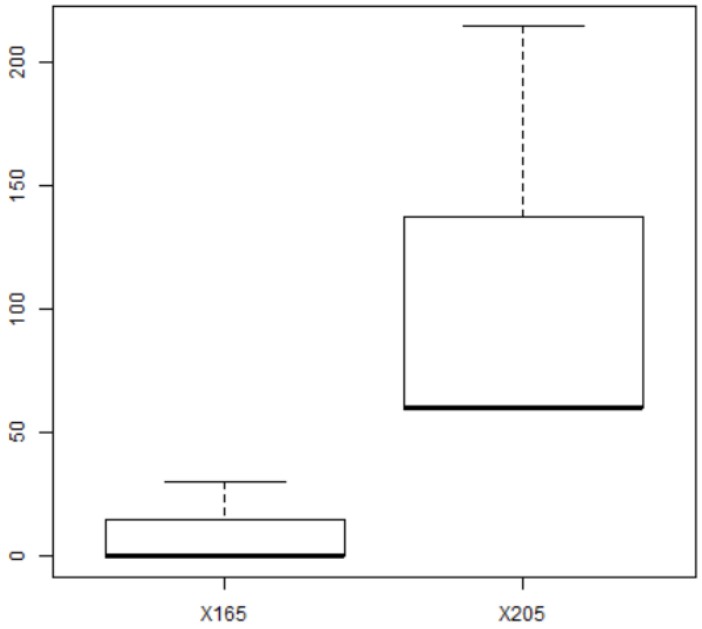
Based on the number of visits compared to the genotype control (165), hoverflies preferred visiting nectar genotype (205) and volatile (39) accessions. In contrast, low expression level genotype 146 was the least attracted one of the volatile accessions. By comparing volatile accessions with the nectar accession of hoverflies interested, it showed that the nectar accession (205) was the most popular for hoverflies.



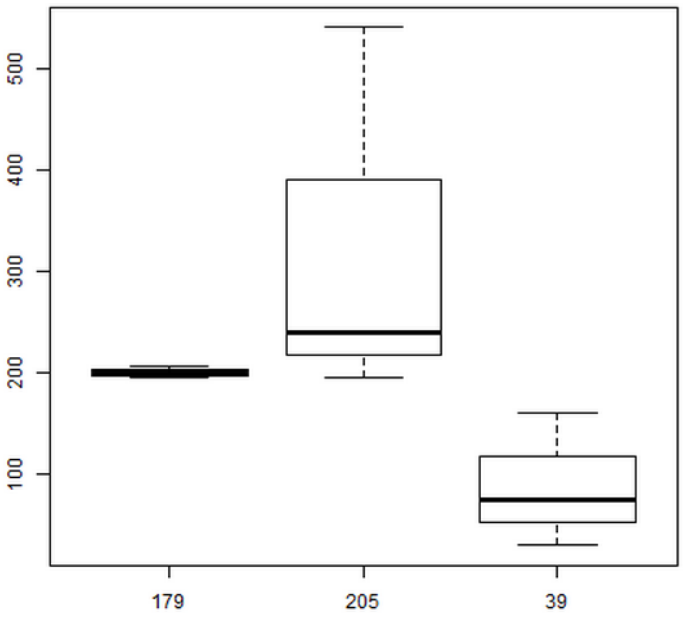
Control&Pollen



Control&Volatile



Control&Nectar



Nectar&Volatile

Figure 3.4.2 Average stay duration of hoverflies by different comparisons (Genotype control & Volatile/Pollen/ Nectar, Nectar & Volatile). According to different comparisons, it showed which genotype had longer stay duration, which had less.

The average stay duration (Figure 3.4.2) showed compared to genotype control 165, hoverflies more liked stay at nectar (205) and volatile (39 and 179) accessions. Compared to volatile accessions, the nectar genotype took advantage by seducing hoverflies to stay for longer time. The volatile genotype 179 attracted less number of hoverflies but longer stay duration.

4. Discussion

The genotypes of Arabidopsis we selected are natural ecotypes. These natural ecotypes are known clearly about the expression level of specific gene. Gene AT5G44630 (*AtTPS11*) and AT5G23960 (*AtTPS21*) related with volatiles (Tholl et a., 2005), while AT2G39060 (*AtSWEET9*) involved in nectar secretion. AT5G13930 (*AtTT4*) helps the biosynthesis of flavonoids and AT4G13270 contributes to pollen and tube germination. These genes are expressed in Arabidopsis flowers for different flower traits. To selected relevant ecotypes for putatively differed in these traits, we chose 8 accessions in total based on the reported expression levels of genes in seedlings as reported in the Araport database, including Col-0 (165) as genotype control. And according to the results from the experiments of different flower traits, it showed that some of the selected ecotypes indeed had different flower characteristics as we expected. However, there is no one literature mentioned our selected accessions but only chosen genes with specific flower traits until now. So, we still need gene sequencing to prove our guess for the next step.

4.1 Volatile

We selected accessions 39 (Est-0) and 179 (Est-1) for higher AT5G44630 gene expression level relative to that of accession 165 (Col-0). Furthermore, accession 146 had lower AT5G23960 gene expression level compared with 165. Sesquiterpene (E)-β-caryophyllene and humulene generating encodes by AT5G23960, while sesquiterpenes geranylacetone, α-barbatene, thujopsene, chamigrene and cuparene are encodes by AT5G23960 (Throll et al., 2005). As expected, accession 39 had higher concentrations of (E)-β-caryophyllene and humulene, but also that of other sesquiterpenes, including geranylacetone, α-barbatene, thujopsene, chamigrene and cuparene. Accession 146, which was expected to have lower concentrations of the group B sesquiterpenes as described in Chen et al (2013), indeed has relative low geranylacetone, α-barbatene, thujopsene, chamigrene and cuparene, but it also had lower concentrations of (E)-β-caryophyllene and humulene, when compared to 165. In contrast, accession 179 was also expected to have a lower emission of group B sesquiterpenes, but was found only to have higher concentration of (E)-β-ocimene, which is a monoterpene. Furthermore, accession 279, which was selected for a pollen-related trait also had higher concentrations of cuparene.

As a result, accessions 39 and 146 significant differed from accession 165 for sesquiterpenes (E)-β-caryophyllene, humulene, geranylacetone, α-barbatene, thujopsene, chamigrene and cuparene. Even though accession 179 was selected on basis of higher AT5G44630 gene expression level in seedlings, it had higher concentration of a monoterpene. So maybe there were differences of gene expression level between seedlings and adult plants, otherwise the gene expression level of selected volatile accessions was not exactly the same as we expected may due to abiotic or biotic stress. So, for further study, RNA transcript analysis (RT-PCR) and headspace of different plant growing stages are necessary for further study.

Besides, the differences of comparisons, there was also a very interesting result to study. The order of retention time was different from the literature (Chen et al., 2003) for (E)-β-caryophyllene and α-barbatene. The reason may cause by different GC column we used and this still needs to be find out.

4.2 Sugar

As the result from sugar extraction showed, accession 205 with higher expression levels of AT2G39060 had significant higher concentration of inositol, fructose, glucose, stachyose and melezitose compared to Col0 (165). In addition, accession 279, selected for a pollen-related gene had significant higher concentrations of galactinol and inositol compared with 165. According to Lin et al. (2014), AT2G39060 encodes a nectary-specific sugar transporter SWEET9. High expression level of SWEET9 helps plants generating more sucrose, which becomes available for CWINV4, leading to more fructose and glucose. But besides those sugars, 205 also had higher concentration of stachyose and melezitose which was unexpected. So, the transcript level of SWEET9 and CWINV4 should be check by using q-PCR in the future.

However, our results should be carefully being interpreted as variation was found in sugar ratios within duplicates of a similar accession. The most logical explanation is that, even though we tried to isolate nectaries from flowers of the same growing stage, they still had differences because of the daytime of isolation or because the for-weather conditions varied over the experimental period (June to September). As a result, some samples had different ratio of the same sugar from the same accession. So, we need more samples from different growing stage to study. It would be interesting to study, whether sugar composition (ratio) varies with developmental stage and over the day period.

4.3 Anthocyanin

Although this part of experiment was failed, at least we knew if we want to test anthocyanin, we need more colourful flower petals. Unfortunately, as far as known Arabidopsis petals are all white. Maybe there are some white accessions can be tested by UV-visible, even they have no visible differences for human eye. According to Sajjad and Saeed (2010), hoverflies prefer yellow and white colour of petals.

4.4 Hoverfly behaviour

Compared to control accession 165, hoverflies loved visiting 39 and 205. By comparing 39 and 205, hoverflies preferred to visit 205. The result of average stay duration was similar to visiting times, except for hoverflies also prefer stay on 179 for longer time besides 39 and 205 compared to 165. And 205 was the longest staying time accession. Multiple visiting times stands for the plants can attract hoverflies, while longer stay duration means hoverflies preferred pollinating for this plant for its reward (nectar or pollen). In conclusion, 205 was the favourite accession for hoverflies. The influence of plants for hoverflies was nectar>volatile>control=pollen. For this experiment, we only did 4 comparisons and we released 8 hoverflies each time of comparison because of their inactivity. Besides comparing

visit times or stay duration, different weather or environment or other conditions also can be tested in the future (Nalam et al., 2012). Also, releasing too much hoverflies could cause we cannot find out if the hoverfly was revisit or it was another hoverfly. If there will be camera which can help recording, then releasing one hoverfly would be better in the further study.

4.5 Overall

Combining all the experimental results together, we can get few conclusions. First, hoverflies liked accession 205 most, but in the number of visits and the duration of a visit. So, sugar had the most significant influence on hoverflies. Specifically, a high fructose, glucose, stachyose and melezitose had the most significant influence on hoverflies. Secondly, hoverflies also preferred accessions 39 while they were least interested in accesison 146. So, sesquiterpenes, (E)-β-caryophyllene and humulene, geranylacetone, α-barbatene, thujopsene, chamigrene and cuparene had significant influence on hoverflies. Besides the sesquiterpenes, monoterpene (E)-β-ocimene also attracted hoverflies for longer stay duration. Until now, none of the same study has been found. But there is a literature mentioned about the syrphid fly had response to various monoterpenes but inactive to sesquiterpenes by electroantennographic (EAG) test (Verheggen et al., 2008).

To study the function of a specific volatile or sugar, next step we need to test with the significant genes one by one, using knockout or gene over-expression.

4.6 Limits and further study

From this minor thesis, we selected several different flower traits based on the gene expression level in seedlings which could have influences on behaviour of hoverflies. And the results of the experiments showed that these ecotypes we selected indeed differ from the flower traits of reference genotype, Col-0 (165), including volatile emission and nectar composition. However, considering this was the trail of this experiment, more repetitions and sampling within a tighter experimental period (or growing of plants in a climate-controlled environment) should be performed for a substantiate evidence. For example, we were not able to sequence the gene of 7 accessions, and pollen analyses are needed to find out the function of At4G13270.

For behaviour of hoverflies, we found that hoverflies had higher preferences for genotypes with higher emission of sesquiterpenes and higher hexoses in their nectary's. Additional studies are needed to study individual hoverfly preferences as well as their choices to plants that differ in a single volatile, e.g. by using gene over-expression or knock-out plants. It would also be interesting to study to whether the change of daily weather or time of the day would influence hoverfly behaviour. Also, releasing one hoverfly each time is a better choice in case of mix up revisit or visits by different hoverflies.

5. Conclusion

In this minor thesis, 8 different accessions were sowed in the greenhouse (one not germinated) and 7 accessions were tested for volatile, sugar, anthocyanin and bioassay. The Col-0 was determined as genotype control among those 7 accessions to compare with. The T-test and box-plots were made for volatile and sugar analyse. Also, a simple record of visiting times and stay duration were made for bioassay of hoverflies. In spite of many things need to improve, we found out that hoverflies preferred sugar (reward) most, volatile second. Pollen was the least interesting objective for hoverflies. For volatile, sesquiterpenes (E)-β-caryophyllene, humulene, geranylacetone, α-barbatene, thujopsene, chamigrene, cuparene and monoterpene (E)-β-ocimene were important for attracting hoverflies. Those sesquiterpenes not only can attract hoverflies for more visiting times, but also for longer stay duration, while the monoterpene only can keep hoverflies staying for a longer time. For sugar, fructose, glucose, stachyose and melezitose helped Arabidopsis attracting hoverflies no matter on visiting times or staying time. However, there were some results not the same as our expected or different from what other people got, like different order of volatile retention time. Overall, this is a very interesting topic and it needs further study to optimize the results.

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