Herbivory induced aggregation of tortoise beetles on morning glory plants

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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July, 2021

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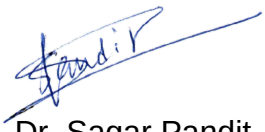
Certificate

This is to certify that this dissertation entitled “Herbivory induced aggregation of tortoise beetles on morning glory plants” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Sujay Manoj Paranjape at Indian Institute of Science Education and Research under the supervision of Dr. Sagar Pandit, Assistant Professor, Department of Biology, during the academic year 2020-2021.

Sujay Manoj Paranjape



Dr. Sagar Pandit

Committee:

Dr. Sagar Pandit

Dr. Nishad Matange

Declaration

I hereby declare that the matter embodied in the report entitled “Herbivory induced aggregation of tortoise beetles on morning glory plants” are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Sagar Pandit and the same has not been submitted elsewhere for any other degree.

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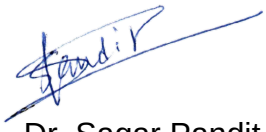

Dr. Sagar Pandit

This thesis is dedicated to Aai and Baba, for their sacrifices towards my education.

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Abstract

Tortoise beetles (*Chiridopsis spp.*) are herbivores of morning glory plants (*Ipomoea spp.*) and they exhibit high level of host specificity. In nature, we have observed these beetles aggregating only on host plants with a founder population present on them. Through this study, we tried to understand the role of founder beetles, origin, and the composition of the aggregation signal by conducting behavioural assays, GCMS analysis, and SPME-HS analysis followed by an attempt at reverse genetics. Using *Chiridopsis nigropunctata* and *Ipomoea elliptica* as our model system, we report that signal for *C. nigropunctata* to aggregate originates in the plant itself and a founder population of beetles is not necessary to bring about aggregation on *I. elliptica*. These beetles aggregate similarly on a plant with just mechanical damage and no founder beetle, as they do on plants having herbivory and a founder beetle. It means beetles do not differentiate between these two treatments. GC-MS and SPME-HS data suggest that the said aggregation signal may be composed of plant VOCs induced in response to beetle herbivory as well as mechanical damage. α-copaene, β-copaene and δ-cadinene were the three compounds that show significant induction in concentrations 12 hours post herbivory and mechanical damage alike. Hence, they are the top candidates to be (or constitute) the said aggregation signal. We also attempt to PCR amplify and identify the genes responsible for producing these sesquiterpenes in *I. elliptica* and ultimately silence them using the VIGS technique and observe the effect on aggregation in silenced plants.

Acknowledgements

I would like to sincerely thank my guide and supervisor, Dr. Sagar Pandit for providing me with this opportunity to work with his lab group. I am thankful to him for providing all the support I needed to conduct this study and for being available to help at any hour. His excitement and motivating attitude have helped under challenging times.

I want to thank my mentor, Gauri Binayak, for helping me with field collection, writing, experiments and brainstorming ideas. Her mentoring and notions have kept me motivated. I also extend my gratitude to Rituparna Ghosh, who has critically taught me methods of GC-MS, including machine handling. She has been a source of information and has motivated me multiple times.

I would like to thank Rutwik Bardapurkar, Surhud Sant, Ganesh Pawar and Maroof Shaikh for their help in collecting insects from the field multiple times. I express my gratitude to Sahil Pawar and Manish Kuman for their assistance in molecular biology experiments.

I appreciate and thank Gauri Binayak and Surhud Sant for their valuable and critical comments on the earlier drafts of this thesis.

I thank all the concerned IISER Pune authorities for their help and guidance during the time I was infected with COVID-19 and had to be isolated from 23rd March to 15th April 2021.

I am thankful to my parents for their unconditional support throughout my education. I also thank my local guardians and friends who have been supportive and helpful all these years al IISER Pune.

Chapter 1 **Introduction**

For any two or more organisms to communicate with each other, there must be a signal emitted by one organism. It should be able to travel through a suitable medium, and the receiver must have appropriate machinery to interpret the signal and ultimately respond to it (Shorey 1973). Plants and insects have coexisted for millions of years and have evolved a plethora of ways to interact with each other and with themselves. Many insects consume plants as their food. Plants have evolved various ways to protect themselves from these insect herbivores. One of the defence strategies includes producing chemicals that either deter the herbivore or render it dead (BENNETT and WALLSGROVE 1994). Most of these plant defence chemicals are secondary metabolites like terpenoids, phenolics, flavonoids and alkaloids (Boncan et al. 2020; Böttger et al. 2018).

Plants respond to insect herbivory by increased emission of volatile organic compounds (VOCs) which help mount a direct defence against herbivores by deterring them (Unsicker, Kunert, and Gershenzon 2009) and also an indirect defence by attracting predators and parasitoids of the herbivore (Arimura, Kost, and Boland 2005). Some VOCs also help plants cope with abiotic stresses such as heat stress, exposure to ozone or photodamage (Holopainen 2004). Terpenes and their derivatives are a part of these VOCs, and they also constitute the largest section of plant natural products (Theis 2011); they are formed from repeating multiple isoprene (5 carbon) units- Hemiterpenes (5C), monoterpenes (10C), sesquiterpenes (15C), diterpenes (20C), sesterterpenes (25C), triterpenes (30C), tetraterpenes (40C) and polyterpenes (>40C). Compared to other plant secondary metabolites, little is known about the biosynthesis and functions of thousands of reported terpenes and their derivatives in plants (Gershenzon and Dudareva 2007).

Along with plants, insects have also been reported to used terpene-based signals. Defence secretions of some leaf beetles contain iridoid monoterpenes which help them repel potential predators (Laurent et al. 2003). Defensive secretions from individuals of soldier castes in seven European termites are known to contain mono-, sesqui- and diterpenes which help in defending the colony against predators and competitors (Quintana et al. 2003). Because of the structural similarities between

insect and plant terpenes, their origin remains a debated topic (Gershenzon and Dudareva 2007).

The tortoise beetles (*Chiridopsis spp*., family- Chrysomelidae) feed exclusively on morning glory plants (*Ipomoea spp*., family- Convolvulaceae). From our lab, we have reported a high level of host specificity (figure 1) in this plant-herbivore interaction, which could possibly be attributed to the metabolomic profiles of the individual host species. This detailed study on host plant preferences was previously carried out on five Ipomoea plant species and their corresponding four herbivore *Chiridopsis* beetle species, as shown in the figure.

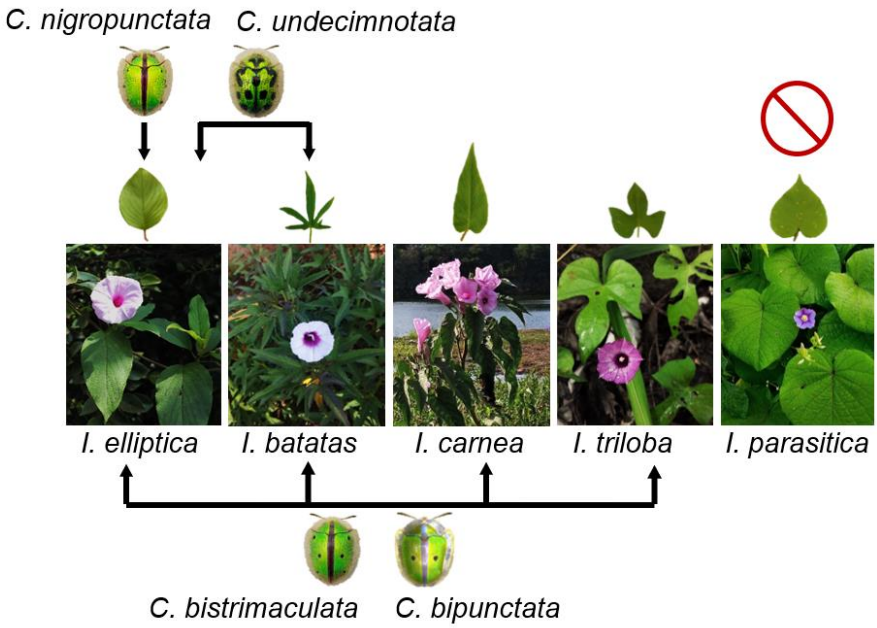


Figure 1- Host specificity in the plant-herbivore system of Ipomoea spp. and Chiridopsis spp. beetles. *Arrows indicate that C. nigropunctata is monophagous, C. undecimnotata is biphagous, and C. bistrimaculata and C. bipunctata are oligophagous.*

In nature, we observed all the of mentioned *Chiridopsis spp.* beetles aggregating in large numbers on their host plants in monsoon season in the forests of Western Ghats. This aggregation is specifically only on host plants and only upon initiation of herbivory by the first visitors. Further, even if the two beetle species having a common host coexist in a particular area, they did not co-aggregate on the same host plant. In another study carried out in the lab, I have reported significant

induction in the concentrations of sesquiterpenes present in *Ipomoea carnea* 12 hours post herbivory by *Chiridopsis bistrimaculata,* which served as a pilot assay to suggest that terpenes could play a role in this plant-herbivore interaction.

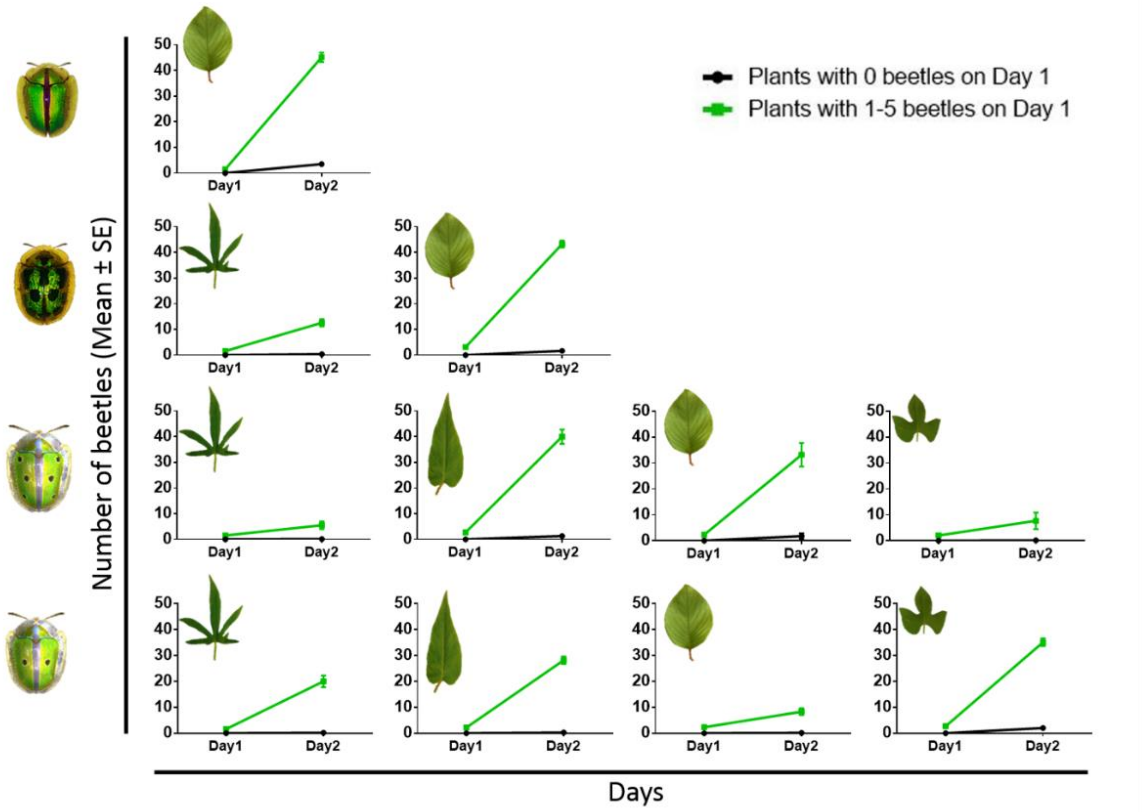


Figure 2- Observations on Chiridopsis aggregation in nature. *All Chiridopsis spp. aggregated on their hostplants. Hostplants which had no beetles on day 1 of observation had none or few beetles on day 2. On the contrary hostplants which had 1-5 beetles on day 1 had a large number of conspecifics on day 2. The trend was consistent across all species of beetles and their respective hostplants. Figure designed by Gauri.*

Much like these tortoise beetles, many non-social insects such as desert locusts, monarch butterflies, Japanese beetles, etc. are known to aggregate in large numbers. Aggregation provides advantages like increased chances of mate finding, facilitation in feeding, protection from predators and modification of micro-habitat (Wertheim et al. 2005; Wertheim, Dicke, and Vet 2002). For these herbivorous insects to aggregate, the signal could be a single compound or a cocktail of semiochemicals. They may be of several possible origins-

1. Synthesized de-novo by the insect herbivore (Chiu, Keeling, and Bohlmann 2018)
2. Derivative(s) of plant compound modified by the herbivore insect (Dickens 1989)
3. A compound or cocktail of compounds from the plant's herbivory-induced volatile blend has been selected by the insect herbivore as a signal to aggregate (Loughrin et al. 1996)

Males of the pine bark beetles (*ips spp*.) secrete an aggregation pheromone cocktail. It contains oxygenation products of monoterpene olefins derived from their diet of pine and spruce barks. But recently two more monoterpene constituents of the aggregation pheromone were discovered which are produced de-novo by these beetles. The monoterpene synthase responsible for this is the first of its kind reported from metazoa (Fernandez-Cornejo et al. 2014). After knowing that some beetles use terpene-based signals for aggregation, we ask a question regarding the role of induced sesquiterpenes in *Ipomoea spp.* 12 hours post herbivory and aggregation of *Chiridopsis spp.* on their host plants by the end of 48 hours (figure 2).

This study tries to uncover the origin and components of the aggregation signal by performing dual choice and no choice assays, GC-MS-based volatile profiling, headspace analysis, and an attempt at reverse genetics towards the end. We selected the *Ipomoea elliptica* (IE) and *Chiridopsis nigropunctata* because this pair exhibits the most stringent host specificity among other *Chiridopsis spp* and their hostplants.

Figure 3- Aggregation of C. nigropunctata on Ipomoea elliptica.

Chapter 2 **Materials and Methods**

Plants-

Ipomoea elliptica is a climber found in tropical rainforests of the Indian subcontinent, often found growing in dense thickets where shade and humidity are plenty. Since these conditions are substantially different from those prevalent in Pune, it is quite challenging to maintain and propagate these plants in Pune. During this study, I was able to standardize a protocol to vegetatively propagate them in laboratory conditions. A woody cutting of an *I. elliptica* branch approximately 15 cm long was brought from the IISER Pune field, and the proximal end of the stem was given a slant transverse cut in order to maximize the contact with soil. The cutting was inserted in a soil-filled grow bag and watered thoroughly. Multiple such bags were kept together in bunches in a tray containing ~2- 3 cm water. The bags were then covered with a large autoclave bag to trap and maintain high humidity. The cuttings were provided with Keradix rooting solution the day after planting, following which they were regularly watered every two days. Micro- and macro-nutrients were sprayed once every week. Temperature and light conditions were maintained at 27 ˚C and 8 h light/ 16 h dark.

Insects-

Chiridopsis nigropunctata adults were collected from the forests of Mahabaleshwar and Panchgani near Pune, Maharashtra. These insects are usually collected from the wild in the monsoon (June to September), but due to the COVID-19 pandemic these collections were not possible, and existing cultures from the lab had perished during the nationwide lockdown. Hence all the collections for the project were done around the sparse rainfall that occurred in the months of November and December 2020.

Collected insects were reared in ventilated jars kept in an insect rearing chamber at 27 ˚C and 75% humidity. They were fed *I. elliptica* twigs, and jars were cleaned on alternate days.

Experiment 1- Understanding the nature of aggregation signal

From the field data, we know that *C. nigropunctata* aggregate on herbivory damaged leaves having a founder population of the same species of beetles. To understand the nature of the signal that calls for this aggregation, we conducted the following assay.

Since the beetles can identify the leaves from a distance, the aggregation signal could be either visual or olfactory. The majority of the reported aggregation signals in insects, especially beetles, are olfactory (Leal 1998)(Seybold 1993), i.e., the insects are attracted to the source by volatile chemical cues released by the source. To understand the nature of *C. nigropunctata* aggregation signal, we conducted dual choice assays where visual cues to the beetles were blocked. Five beetles were released in a Y-tube, and treated *Ipomoea elliptica* leaves in both arms were visually occluded by opaque filter paper sheets, as shown in the figure. The treatments on *Ipomoea elliptica* leaves (figure- 4) were 1. Undamaged (control), 2. Mechanical damage, 3. Herbivory. This assay was performed between every pair of treatments as performing three dual-choice assays gives us more information than a single triple-choice assay. The number of beetles on the leaves inside both arms after one hour were counted (n= 10).

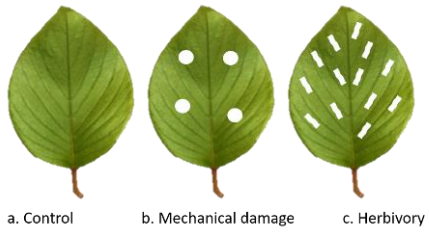


Figure 4- Representation of treatments

Experiment 2- Preferences of *C. nigropunctata* towards leaves of *I. elliptica* with various treatments

Once we knew the nature of the aggregation signal, we tried to understand what individual beetles prefer when given choices of *Ipomoea elliptica* leaves bearing various treatments as they would encounter in nature, i.e., leaves with herbivory (henceforth called herbivory leaves), mechanically damaged leaves and intact control leaves (figure 4).

Beetles were subjected to dual choice assays between- 1. mechanically damaged and herbivory leaves, 2. Herbivory leaves and control leaves and, 3. Mechanically damaged leaves and control leaves, where the choice was considered to be the leaf on which the beetle settled. These assays test whether the beetle can differentiate between each mentioned pair of leaves. Five such assays were conducted (n= 5, each consisting of 25 different beetles).

Experiment 3- Determining if the aggregation signal is of plant or insect origin

After knowing the preference of individual beetles, we studied the aggregation behaviour of *C. nigropunctata* on *I. elliptica* leaves under controlled conditions. To determine whether the aggregation signal is of plant/ beetle/ mixed origin, we performed a series of dual choice assays. In a glass Y-tube, five experimental beetles were allowed to choose between two types of *I. elliptica* leaves, ones with a beetle and the other without any beetle. These experiments were done using the following leaf treatments: 1. Undamaged (control), 2. Mechanically damaged, 3. Herbivory. Beetles' choice was estimated as the leaf on which it settled (n= 10).

In addition to these, we also performed a series of no-choice assays to know how the beetles behave in presence of only one of the treatments from last choice assays. In each assay, a clean, odourless box was taken, and five beetles were placed in it along with a single one of the above treatments at a time. The treatment beetle was not allowed to feed during the assay. In each assay, the number of test beetles found on the leaf at the end of one hour were noted (n= 10).

Experiment 4- Final aggregation assay

After knowing the preference of single beetle from experiment 2, we also needed to understand the collective choice of a group of beetles towards the same treatments. This experiment was a controlled replication of aggregation happening in nature and was performed after knowing the volatile nature of the signal and its origin.

In a clean glass Y-tube, five beetles were released at the mouth while the arms contained *Ipomoea elliptica* leaves having one of these treatments- 1. Control, 2. Mechanical damage, 3. Herbivory. All possible combinations of these treatments

were experimented with and at the end of one hour, the number of beetles on leaves inside each arm was counted (n= 5).

Experiment 5- Analysis of damage induced volatiles

We studied the volatile blend induced by beetle herbivory on the *I. elliptica* to determine whether it is any one compound or the complete blend that attracts the conspecifics. A fresh *I. elliptica* leaf was placed in a 40 ml glass vial and subjected to herbivory by a *C. nigropunctata* beetle starved for 12 hours. The setup was sealed using polytetrafluoroethylene (PTFE) tape to ensure no gaseous exchange with the surrounding. The vial was incubated at 27℃ in a water bath for 0, 12, and 24 hours (n= 6). As controls, two similar setups were used, one containing an intact leaf and another containing a mechanically damaged leaf, both without any beetles. At each time point, the beetle was removed from the vial, and the leaf was weighed. 10 ml of dichloromethane (DCM) (spiked with internal standards- valencene, hexanal and para-tolualdehyde) was then added, and volatile organic compounds were extracted over a period of 6 hours. After this, the leaf was removed, and anhydrous sodium sulfate was added in order to remove aqueous components. This DCM extract was concentrated in a vacuum concentrator to approximately 250 µl and filled in autosampler vials. Extracts were run in an Agilent 7000D gas chromatography-triple quadrupole (GC-QQQ) MS with 2 µl injection volume in a splitless method. Compounds were separated in a DB-5MS capillary column using helium as carrier gas at a flow rate of 2 ml/ min. Peaks obtained in the chromatogram were identified using mass spectral libraries NIST11 and Wiley 8th edition. Detected compounds were quantified with respect to internal standards. Valencene was used to quantify terpenoids, hexanal for aldehydes, alcohols and alkanes and para-tolualdehyde for phenolics.

Experiment 6- Standardization of headspace analysis by manual air injection

Volatile chemicals released by a source into its immediate surroundings are called headspace volatiles. These need to be analyzed because the aggregation signal must be air-borne if it must reach conspecifics at a distance. To analyze headspace volatiles, the same experimental setups described above were arranged. After the

incubation period, 1 ml of air was collected into a clean air-tight glass syringe by piercing the PTFE tape. This air was immediately injected into the gas chromatography sample inlet by simultaneously pressing the manual override button on the Agilent 7000D GC-QQQ machine. Compounds were separated in a DB-5MS capillary column using helium as carrier gas at a flow rate of 2 ml/ min. Peaks obtained in the chromatogram were identified using mass spectral libraries NIST11 and Wiley 8th edition. Incubation times of 0, 1, 2, 3, 6 and 12 hours and was tried multiple times in order to saturate the glass vial with the volatiles, but the concentration still remained under the detectable levels, and we had to try out other methods of headspace volatile analysis.

Experiment 7- Standardization of headspace analysis by HS-SPME fibre adsorption

Headspace solid-phase microextraction (HS-SPME) is a technique working on the principle of adsorption and desorption. An adsorptive as well as absorptive fibre is coated on a needle, and when this structure is exposed to a sample, molecules from the sample enter the stationary phase till an equilibrium is reached. After an equilibrium is reached, the fibre is plunged into a chromatographic system, gas chromatography (GC) in this case where the sample molecules are desorbed at high temperature.

Two twigs (3- 4 leaves on each) of *Ipomoea elliptica*, whose basal ends were covered with a moist tissue paper in a 2 ml tube were placed in a 1000 ml conical flask. The mouth of the conical flask was sealed with a tight cotton plug (to reduce excess moisture) and through this plug the SPME fibre in its holder was inserted. The setup was kept at 24℃ for 6 hours in a well-lit room for plant-released volatiles to be adsorbed onto the SPME fibre. Desorption of sample molecules was done thermally in the inlet of the GC at 240˚C of an Agilent 7000D gas chromatography-triple quadrupole (GC-QQQ) MS. Compounds were separated in a DB-5MS capillary column using helium as carrier gas at a flow rate of 2 ml/min. Peaks obtained in the chromatogram were identified using mass spectral libraries NIST11 and Wiley 8th edition.

Experiment 8- Degenerate primer designing

Since α- copaene, β- copaene and δ- cadinene are significantly induced in damaged leaves, and these are also present in the headspace of *Ipomoea elliptica*, we hypothesized that these three sesquiterpenes are candidate aggregation signals. To confirm that they constitute the aggregation signal, we tried to silence the genes responsible for their production and observe the effects on aggregation.

Copaene and cadinene are sesquiterpenes (a type of terpene made of three isoprene units) produced by higher plants using enzymes copaene synthase and cadinene synthase, respectively. These enzymes belong to a class of enzymes known as sesquiterpene synthases.

Ipomoea elliptica is not a model organism for scientific study and its genome is not sequenced. The only plant from our system of *Ipomoea* spp. to be sequenced is *I. triloba* but its sesquiterpene synthase genes are not annotated. In order to clone a sesquiterpene synthase from *Ipomoea elliptica*, we had to rely on *Ipomoea triloba* and other plants whose sesquiterpene synthases are annotated or at least have predicted sequences. Phylogenetically closest plant to *Ipomoea* of all the plants with annotated copaene synthase is *Ricinus communis* (castor bean plant, family Euphorbiaceae). We used the mRNA sequences of this copaene synthase to find matching sequences in family Convolvulaceae. Two sequences from *Ricinus communis*- JN315864.1 and NM_001323756.1 were first BLASTed onto family Convolvulaceae and top hits from this were chosen to BLAST specifically onto the genus *Ipomoea*. Because of very high number of hits in both the stages of nucleotide BLASTs (nBLAST), we also performed protein BLASTs (pBLAST) and the common top hits (>95% query coverage and >85% identity) from both nBLAST and pBLAST were chosen for primer design.

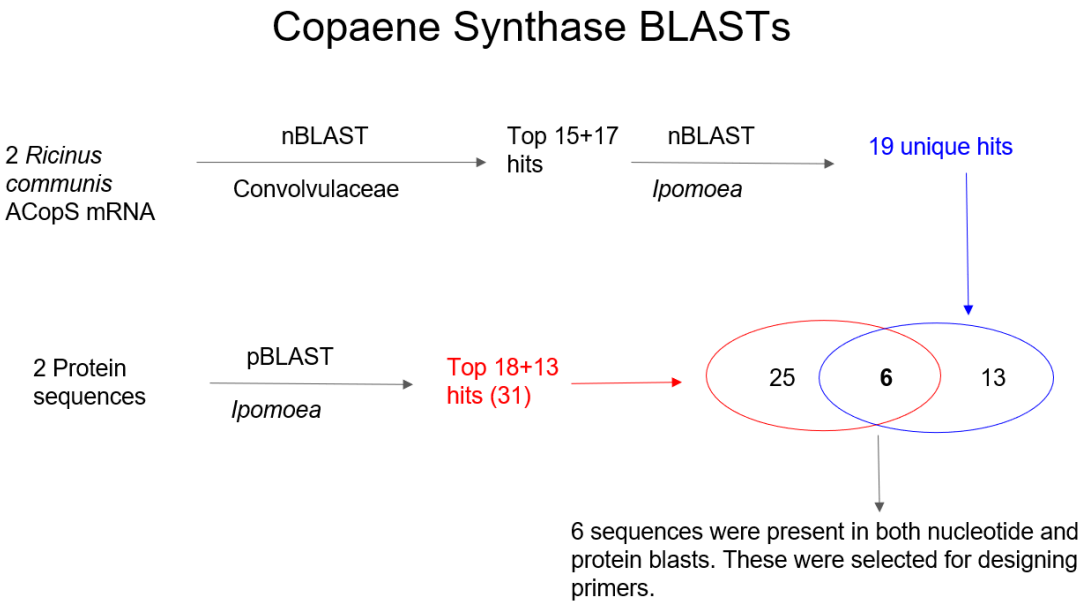


Figure 5- Steps followed to design degenerate primers for amplification of copaene synthase gene.

Similarly, cadinene synthase has been sequenced and annotated from two plants, namely, *Gossypium arboreum*- NM_001330005.1 and Y16432.1 (tree cotton, family Malvaceae) and *Cucumis melo*- NM_001297453.1 (muskmelon, family Cucurbitaceae). Similar algorithm of nBLAST and pBLAST was used to obtain putative sequences for designing primers.

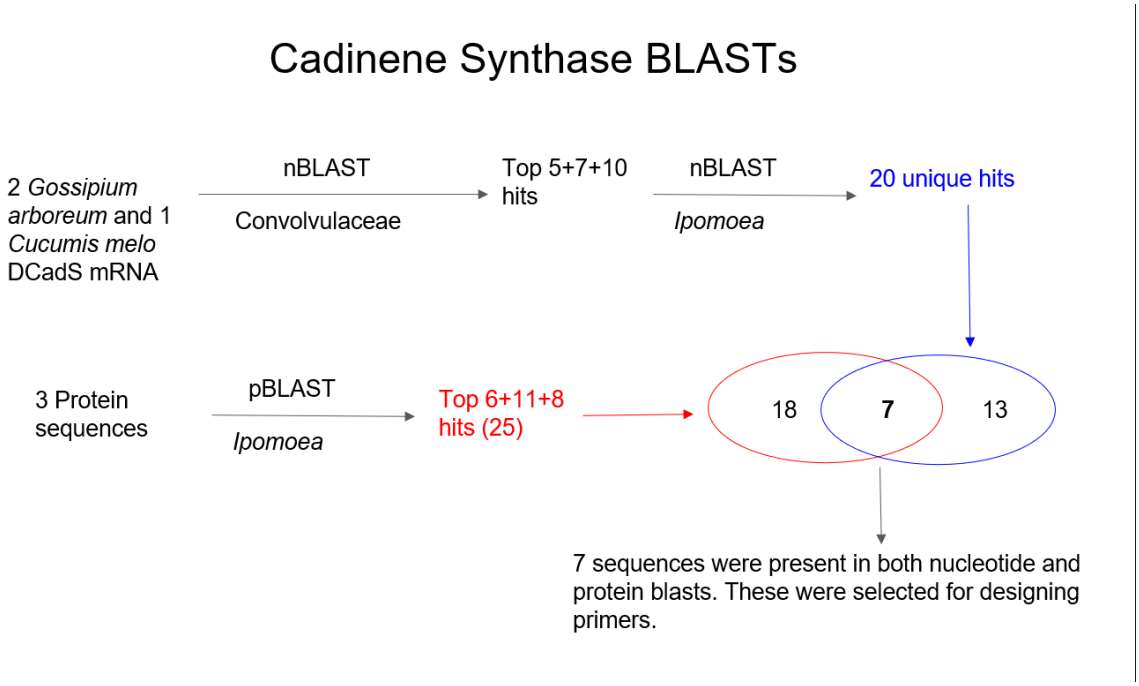


Figure 6- Steps followed to design degenerate primers for amplification of cadinene synthase gene.

When we compared the accession numbers of the six putative copaene synthase top hits and seven putative cadinene synthase top hits, four accession numbers were common to both lists (figure 7). This tells us about the highly conserved nature of sesquiterpene synthases- one enzyme can produce more than one sesquiterpene and it is also possible that one sesquiterpene can be produced by more than one enzyme, depending on the conditions and amount of structural similarity between the products.

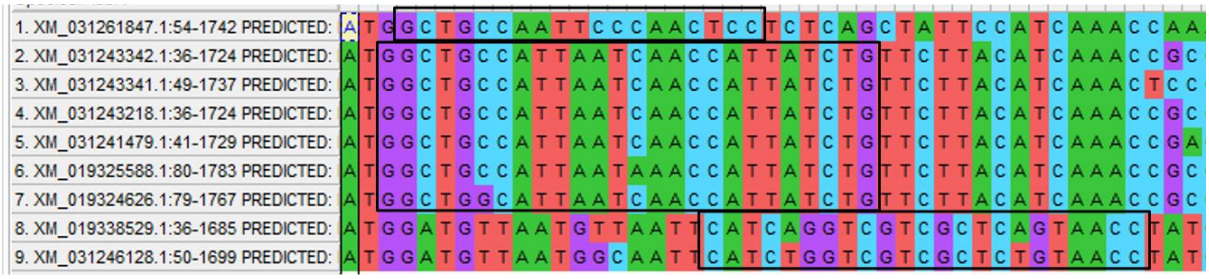
Copaene Synthase like	Cadinene Synthase-like
XM_031243341.1	XM_031243341.1
XM_019325588.1	XM_019325588.1
XM_031261847.1	XM_031261847.1
XM_031241479.1	XM_031241479.1
XM_031246128.1	XM_019324626.1
XM_019338529.1	XM_031243342.1
	XM_031243218.1

***Figure 7- List of common accession numbers**. Common hits are indicated by same text colour*

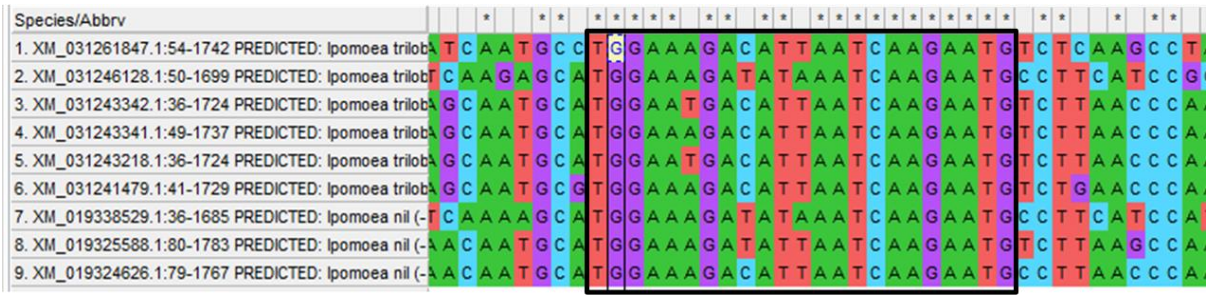
The nine obtained sequences were aligned to find conserved regions at the beginning and the tail of the gene. It was challenging to find a stretch long and conserved enough such that a single primer could apply to all of them. Hence, we

designed four forward degenerate primers and two reverse degenerate primers that would cover all the sequences. There is one degenerate primer to each of the blocks outlined in black.

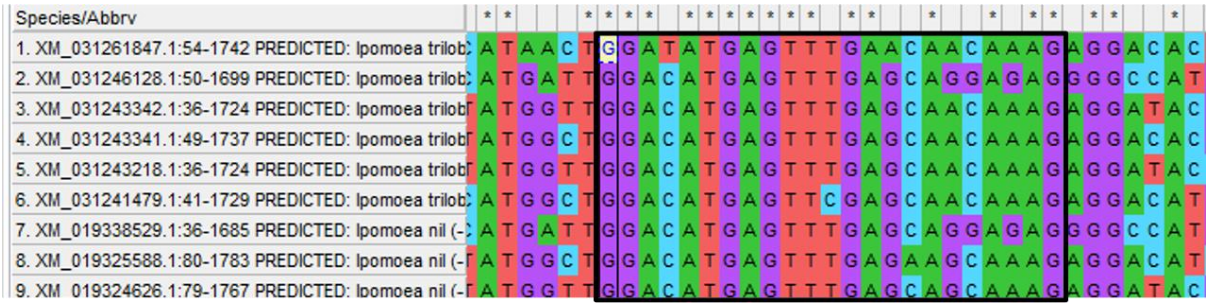
Forward primer blocks-



Reverse primer block 1-



Reverse primer block 2-



Sequences of the degenerate of the primers are as follows-

Forward-

1847- 5’-GCTGCCAATTCCCAACTCC-3’

6.1- 5’-GGCTGSCATTAATMAACCATTATCTG-3

6.2- 5’-ATGGCTGSCATTAATMAACC-3’

2.1- 5’-ATCAGGTCGTCGCTCWGTAACC-3’

Reverse-

9.1- 5’-CATTCTTGATTWATRTCWTTCC-3’

9.2- 5’-CTRTSRTMRTCYAACTCATYTCCA-3’

Experiment 7- Sesquiterpene synthase cloning

Once the degenerate primers were obtained, workflow was decided as follows-

a. Extract RNA from *Ipomoea elliptica* leaves
b. Construct cDNA based on this RNA
c. Try all the combinations of degenerate primers (a to h) on the cDNA template to PCR amplify a sesquiterpene synthase gene. Expected amplicon size- 1.4kb
d. Sequence this amplification product
e. Construct two pairs of specific primers based on the new sequencing data
f. Use one pair of these primers to clone this gene into VIGS vector for virus induced gene silencing in the plant
g. Use the other primer pair to clone the gene into an expression vector for in-vitro demonstration of enzyme function which will serve as a proof that the silenced enzyme is indeed a sesquiterpene synthase

Twigs of *Ipomoea elliptica* were allowed to be fed upon by *C. nigropunctata* for 8- 10 hours (estimated time for the required genes to be transcribed in higher amount because compounds show induction at 12 hours). After which, the beetles were removed, and the leaves were flash-frozen in liquid nitrogen.

Step a- Total RNA extraction

Harvested leaf tissue was homogenized in liquid nitrogen, and about 150 mg of the tissue weighed and added to a 2 ml tube containing 1.5 ml TRIzol solution. It was vortexed well and incubated at room temperature for 15 minutes, followed by centrifugation at 10000 rpm to remove tissue debris. The supernatant was transferred to a fresh 1.5 ml tube without taking any debris. 300 µl chloroform was added to it and mixed well for 2 min by gentle inversion till an emulsion was formed. Tubes were then centrifuged at 10000 rpm for 10 min at 4˚C. Using a cut tip, the

upper aqueous phase was carefully transferred to a 1.5 ml tube while making sure that it is pipetted smoothly, and the two phases do not bump into each other. Aqueous phase from two tubes of same biological replicate were mixed at this step to increase the yield. Equal volume chloroform: isoamyl alcohol (24:1) was added and mixed for 2 min by gently inverting to form an emulsion, followed by centrifugation at 10000 rpm for 10 min at 4˚C. Using a cut tip, the aqueous phase was carefully transferred to a fresh 1.5 ml tube. 0.1 volume sodium acetate (3M, pH 5.2) was added and mixed well by gentle inversion, and then 0.8 volume pre-chilled isopropanol was added. Tubes were incubated at -20˚C overnight, after which they were centrifuged at 10000 rpm for 30 min at 4˚C. The supernatant was discarded and the pellet was resuspended in 200 µL pre-chilled (~4˚C) 70% ethanol very gently, followed by a spin at 10000 rpm for 30 min at 4 ˚C. The ethanol was decanted, and the supernatant was entirely dried by keeping tubes open for few minutes in a laminar air flow hood. The dry pellet was dissolved in 15 µl diethylpyrocarbonate (DEPC) water. DEPC inactivates RNase enzymes that may have remained in the sample during the RNA extraction procedure.

Step b- cDNA preparation was done as per the instructions on the Takara kit.

Step c- PCR amplification using degenerate primers

The possible primer combinations from the four forward and two reverse primers are mentioned in table 1-

Table 1- Primer combination codes

Primer combination name	Forward primer code	Reverse primer code	Amplicon length (bp)
a	1847	9.1	1529
b	1847	9.2	1409
c	6.1	9.1	1530
d	6.1	9.2	1410
e	6.2	9.1	1533
f	6.2	9.2	1413
g	2.1	9.1	1512
h	2.1	9.2	1392

PCR amplification of the putative sesquiterpene synthase genes was tried on the *I. elliptica* and *I. triloba* cDNA template under various conditions. This includes changing annealing temperatures (56˚C, 60˚C, and 52˚C), concentrations of primers (0.25µl and 0.5 µl of 10mM stock in a 10 µl reaction) and template (50ng in case of genomic DNA), type of template (cDNA and genomic DNA) and changing the polymerase enzymes (R-Taq and Ex-Taq) for all the possible primer combinations on both *Ipomoea triloba* and *Ipomoea elliptica* templates.

Chapter 3 **Results**

Through a series of choice assays, GC-MS analysis and headspace analysis we tried to understand the nature and composition of the aggregation signal and tried to replicate the said aggregation phenomena in lab environment.

Results of experiment 1- Aggregation signal is olfactory

The preliminary field data from the lab suggests that beetles aggregate on plants where herbivory has already been initiated by some founder beetles. For such gregarious behaviour to occur, there must be a communication signal associated with it.

Since beetles can locate these herbivory damaged plants from a distance, the signal that helps the beetles locate these plants must be visual or olfactory. In order to understand the exact nature of the said aggregation signal, we conducted a special aggregation choice assay (figure 8A) where the choices were visually occluded from the five beetles released in a Y-tube.

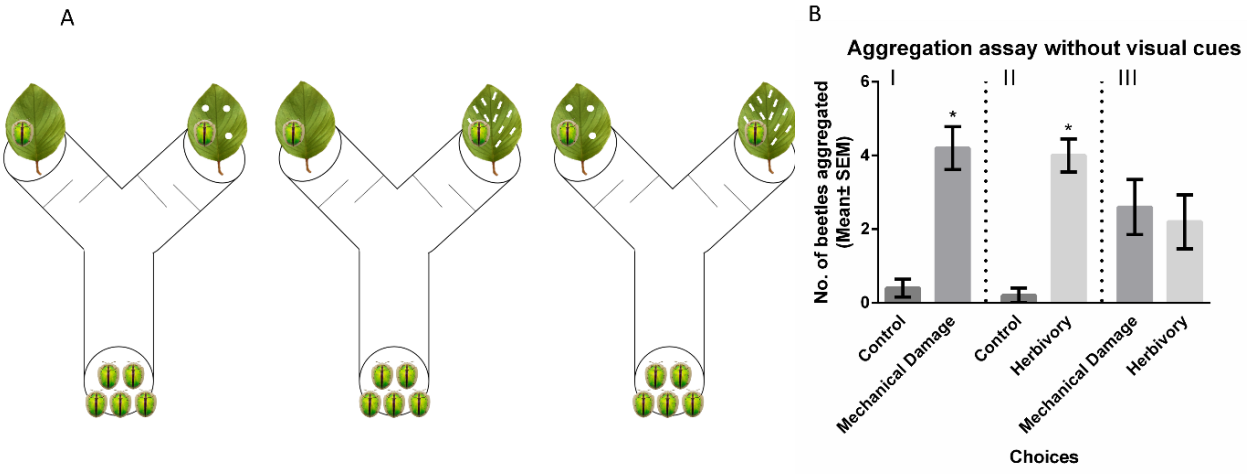


Figure 8- Aggregation assays without visual cues- *A. Schematic of the assay. Five beetles released in each Y-tube with visual barricades made choices based only on olfactory cues. Assay ended when all five of the beetles have settled on the leaves in any of the arms of the Y-tube. (n=5 for each choice assay.) B. Dotted line separates two different choice assays. I- Control vs mechanical damage, unpaired t-test, p=0.0014, significant. II- Control vs herbivory, unpaired t-test, p=0.0004,*

significant. III- Herbivory vs mechanical damage, unpaired t-test, p=0.7129, not significant. Experiment done by Gauri; data compiled and analyzed by me.

Despite all visual cues being occluded, we observed that a significantly greater number of beetles still aggregated on damaged leaves (figure 8B). These observations clearly indicate that the potential aggregation signal is not visual and is likely to be olfactory since it is functional even when visual cues are eliminated. We hypothesized that the aggregation signal involved in this *Chiridopsis-Ipomoea* interaction comprises volatile organic chemicals released from damaged hostplants.

Results of experiment 2- Individual beetles prefer mechanically damaged and herbivory leaves over undamaged control leaves

After concluding that aggregation signal is not visual but olfactory in nature, we went a step backwards and tried to understand how individual beetles behave when they are provided choices between control and mechanically damaged leaves, control and herbivory leaves, and herbivory and mechanically damaged leaves (figure 9A).

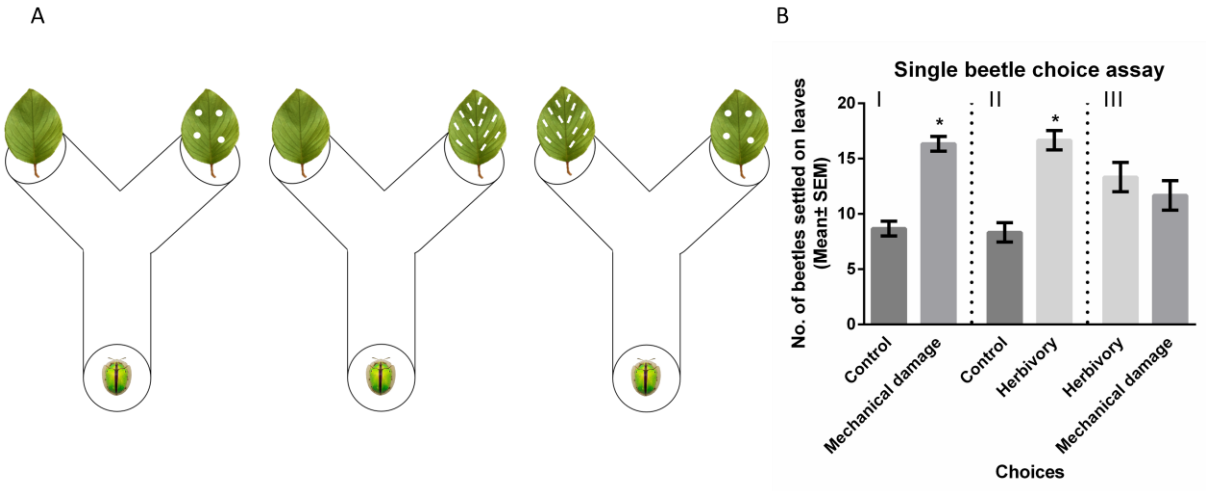


Figure 9- Single beetle choice assays. *A- Schematic of the assay where Individual beetle's choice was recorded when it settled on the leaves present in one of the arms of that Y-tube. n=5 with 25 different beetles in each replicate of the three choice assays. B. Dotted line separates two different choice assays. I- Control vs herbivory, unpaired t-test, p=0.0026, significant. II- Control vs mechanical damage,*

unpaired t-test, p=0.0012, significant. III- Herbivory vs mechanical damage, unpaired t-test, p=0.4267, not significant.

From the above data (figure 9B), we conclude that an individual beetles prefers damaged leaves (both herbivory and mechanically damaged leaves) more than intact control leaves. The data also suggests that individual beetles are not able to differentiate between herbivory and mechanical damage on the leaves. Peculiarly, experiment 1 uses founder beetles in the arms of Y-tube while experiment 2 does not, still the results of fig 8B.III and fig 9B.III are similar i.e. a group of five beetles was also not able to distinguish between the two types on damages on the leaves. This made us question the necessity of the founder beetle for aggregation.

Results of experiment 3- Founder beetle is not necessary for aggregation. Aggregation signal is of plant origin.

After noticing the peculiar similarity in the results of experiment 1 and 2, we conducted an aggregation choice assay where a group of five beetles had to make a choice between leaves of similar treatment, but one of them had a founder beetle. This choice assay was followed up by a no-choice assay where we tried to understand how the beetles behave when only one of the choices from the previous assay was present (figure 10A and 10B).

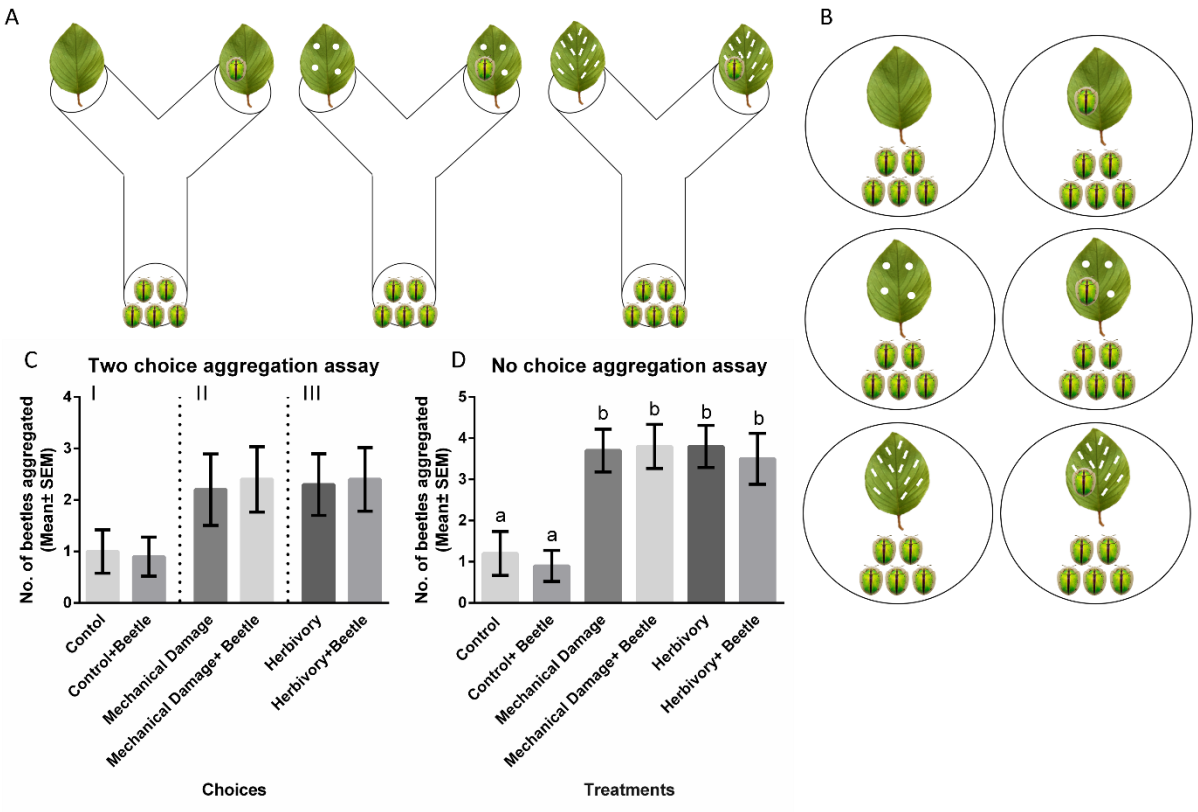


Figure 10- Two-choice and no-choice aggregation assays. *A. Schematic representation of two-choice assays in which five beetles were allowed to choose between Ipomoea elliptica leaves bearing the same treatment but one of them had a founder beetle. Assay ended when all five of the beetles have settled on any of the leaves. n=10 replicates for each assay B. No-choice aggregation assays. Treated Ipomoea elliptica leaves were left in a box with five beetles and number of beetles settled on the leaves were counted at the end of one hour. n=10 replicates for each assay. C. Dotted line separates two different assays, each having a pair of treatments. There is no significant difference in any of the pairs of treatments, unpaired t-test p>0.05. D. Different letters denote significant differences (p< 0.05, one-way ANOVA). Experiment done by Gauri; data compiled and analyzed by me.*

Two-choice aggregation data (figure 10C) suggest that the beetles cannot differentiate between the presence and absence of the founder beetle. In addition to this, no-choice assays (figure 10D) indicate that there is a statistically significant difference in aggregation on treatments involving damaged leaves irrespective of presence of founder beetle than the aggregation on control leaves. Hence, we can conclude that the founder beetles do not play any role in releasing the aggregation

signal, instead it originates in the damaged leaves irrespective of the cause of wounds.

Results of experiment 4- Beetles aggregate similarly on herbivory and mechanically damaged leaves.

After discovering that the aggregation signal is olfactory, originates in the plant and does not require a founder beetle, we tried to set up an aggregation assay to demonstrate our field observations in controlled laboratory conditions.

A group of 5 beetles was subjected to a choice aggregation assays where they had to choose between all the combination pairs of *Ipomoea elliptica leaves* bearing three treatments- Control, mechanical damage and herbivory (figure 11A).

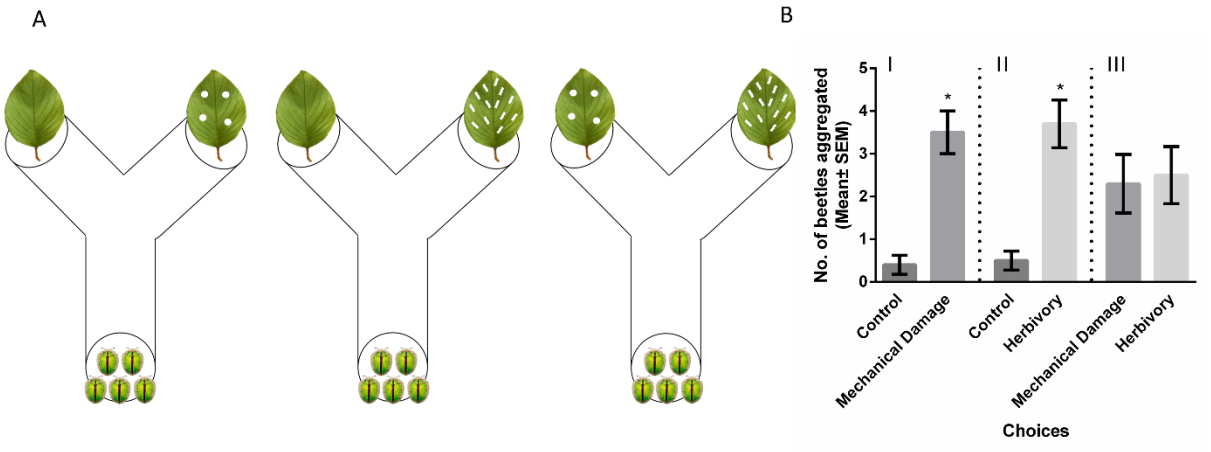


Figure 11- Controlled aggregation assay. *A. Schematic representation of controlled aggregation assays, five beetles were released in a Y-tube having choices as indicated. Assay ended when all five beetles had settled on some leaf in either of the arms. B. I- Control vs mechanical damage, unpaired t-test, p<0.0001, significant. II- Control vs herbivory, unpaired t-test, p=0.0002, significant. III- Herbivory vs mechanical damage, unpaired t-test, p=0.8370, not significant. Dotted line separates two different assays, and n=10 for each assay. Experiment done by Gauri; data compiled and analyzed by me.*

The controlled aggregation assays (figure 11B.I and 11B.II) suggest that damaged leaves release stronger signal than control leaves because they have a stronger aggregation response as compared to control leaves. Figure 11B.III suggests that a group of beetles cannot differentiate between the said aggregation signal from

herbivory leaves and mechanically damages leaves. This observation is consistent with one-beetle choice assays (figure 9B.III). We can also conclude that strength of the said aggregation signal from herbivory and mechanically damaged leaves is similar.

Results of experiment 5- Sesquiterpenes α-copaene, β-copaene and δ-cadinene are induced in damaged leaves upon herbivory and mechanical damage

Many plants respond to insect herbivory attack with an increased emission of volatile organic compounds (VOCs). These VOCs are signatures of insect herbivory and can play a role in mounting a defence against the herbivores (Unsicker et al. 2009) and sometimes insects can tap into this signature to locate an infested plant (Ichiki et al. 2011; Sullivan et al. 2000).

In order to investigate the olfactory aggregation signal originating from damaged leaves, we identified and quantified all the VOCs emitted by damaged *Ipomoea elliptica* leaves and studied their temporal kinetics at 0, 12 and 24 hours post onset of herbivory. Previous data from my semester project in the lab had identified 12 hours as the time point when these VOCs are significantly induced upon herbivory. I had also reported that this induction happened only for few selected sesquiterpenes.

Even though aggregation data from field states significant aggregation after 48 hours, we do not have information about the aggregation dynamics happening between 0 and 48 hours. The pilot study from my semester project included the 48 hours' time point but significant induction in concentrations of VOCs was found only at 12 hours post herbivory. It could mean that induction in VOCs of field plants does occur at 12 hours post herbivory by first visitors, which signals more beetles to infest that particular hostplant and this cycle continues to build up a large aggregation of beetles by 48 hours.

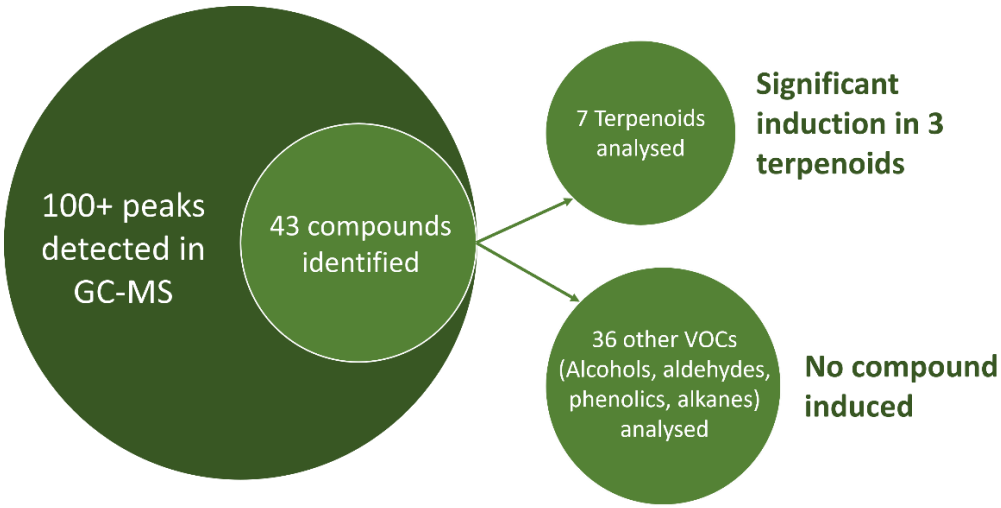


Figure 12- Solvent extraction and analysis of Ipomoea elliptica VOCs.

I. elliptica VOCs were first profiled by the solvent extraction method followed by GC-MS analysis, where we detected more than 100 peaks per sample after running control, herbivory and mechanical damage leaves collected at 0-, 12- and 24-hour timepoints. Of these, 43 compounds were identified by matching their mass spectra with that given in NIST11 and Wiley 8th edition libraries. These included terpenoids, phenolic compounds, alcohols and aldehydes, and long chain alkanes. Terpenoids are 18.18%, phenolics are 6.81%, aldehydes and alcohols are 13.63% and long chain compounds contribute a massive 61.36% to the metabolomic profile of *Ipomoea elliptica* leaves.

A significant induction in the levels of three sesquiterpenes was observed at 12 hours post onset of herbivory: α-copaene, β-copaene and δ-cadinene at retention times of 24.91 min, 27.62 min and 28.61 min. The levels of these sesquiterpenes in herbivory leaves and mechanically damaged leaves showed a similar trend (figure 13). A potential aggregation signal should be similarly induced in both mechanical damage and herbivory because aggregation occurs on both. Since these three compounds show similar induction in both types of damages, they are candidate signals.

The general trend in concentrations of VOCs is similar for herbivory and mechanically damaged leaves (figure 13), indicating that the blend is not affected by beetle oral secretions and frass deposited on the leaves.

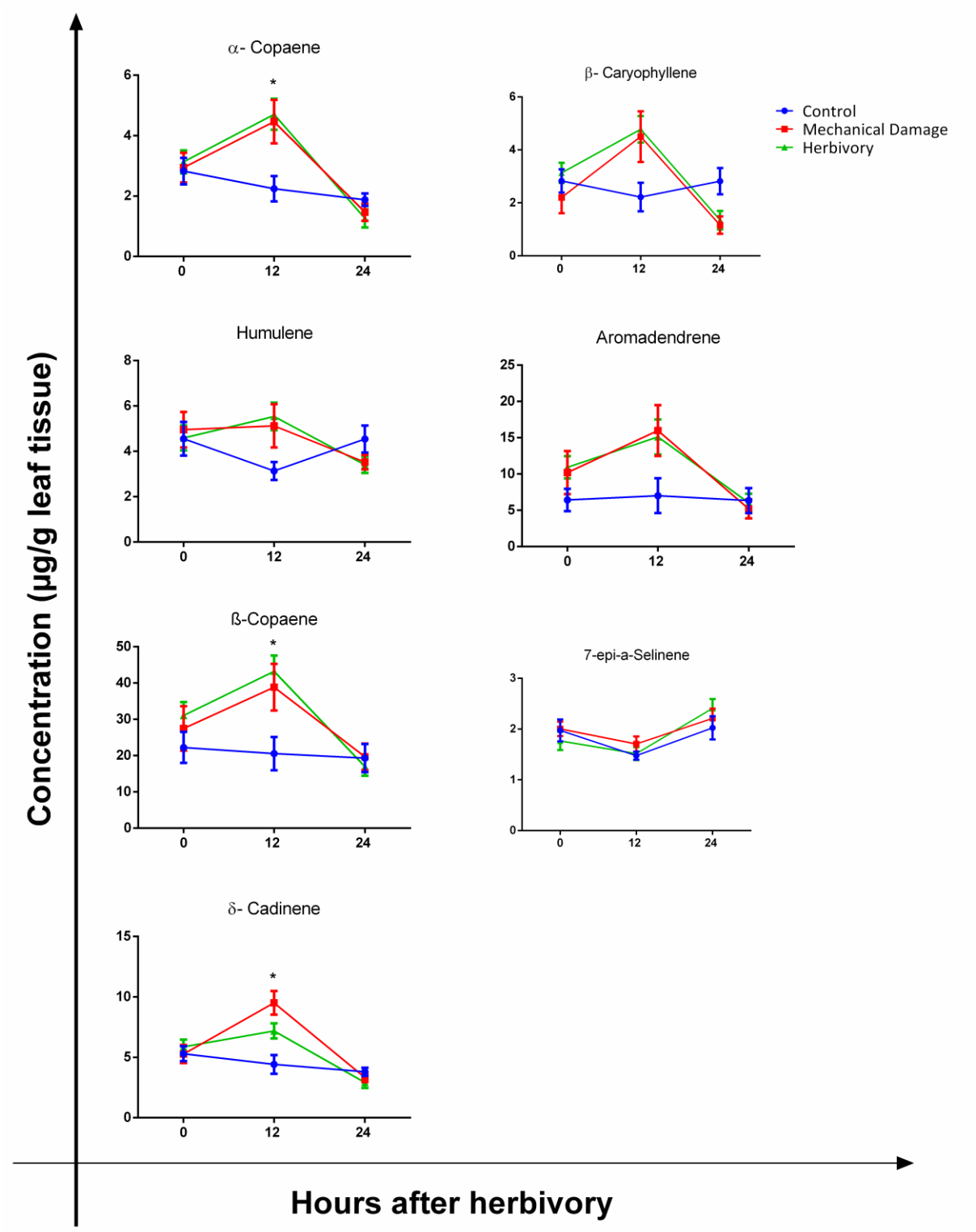


Figure 13- Temporal kinetics of terpenoid concentration. *A. Concentrations of terpenoids in control, herbivory and mechanically damaged leaves at 0, 12 and 24 hours after herbivory. Six replicates per time-point of each treatment were analyzed. * indicates significant induction at that time-point, One-way ANOVA.*

Solvent extraction analysis gives us information only about the chemicals that are present in the leaves. We do not know whether these induced compounds are released into the headspace or not. This criterion must be fulfilled for a compound to a part of olfactory signal. To confirm that these solvent-extracted leaf VOCs are indeed released into headspace, we tried to sample the headspace of the damaged leaves and checked for presence of these compounds.

Results of experiment 6- Change of method required because we could not detect any compounds.

Even after repeated trials using different incubation periods, we could hardly detect any compounds (figure 14) by this method. We also tried changing the material used to seal the mouth of the tubes, from PTFE membrane to stretched parafilm but no avail. There could be these problems-

1. The concentration of the compound in the 1 ml of air sucked in by the syringe could be lower than detectable levels.
2. The compounds could be leaking out of the tube as soon as the sealing material is pierced by the syringe.

After trying manual injection for about two months, we decided to shift to another method of headspace analysis—SPME analysis.

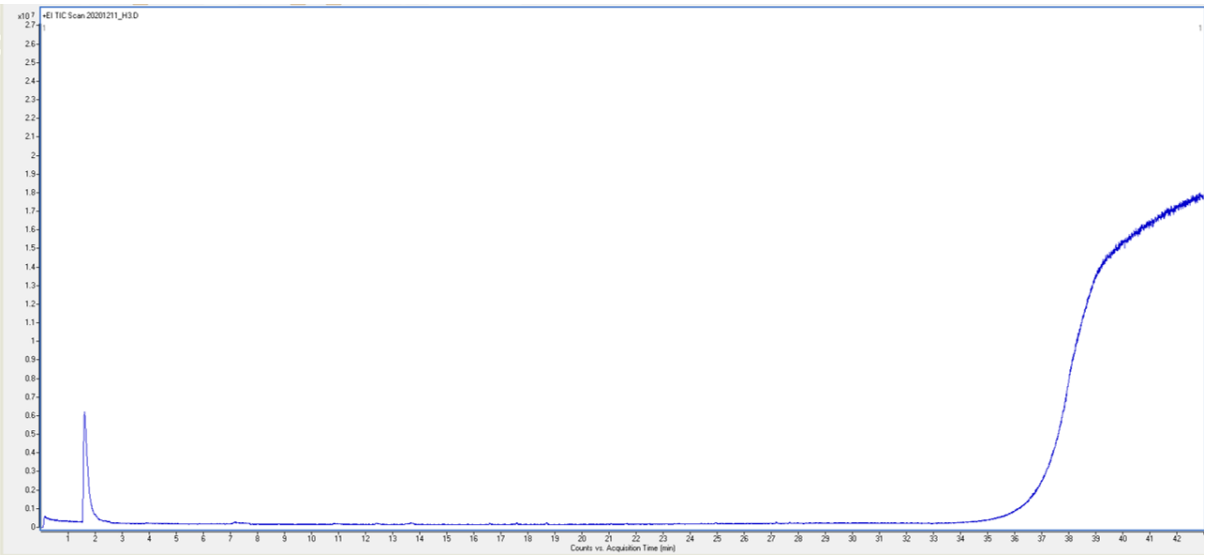


Figure 14- A sample chromatogram of headspace air injection. *We did detect any compounds in the headspace by this method, same is indicated by the absence of peaks.*

Results of experiment 7- α-copaene, β-copaene and δ-cadinene are present in the headspace of *Ipomoea elliptica*

In the process of standardization of SPME-HS method, we were able to detect various compounds in the headspace of *Ipomoea elliptica* after six hours of incubation with SPME fiber in a closed 1000 ml conical flask.

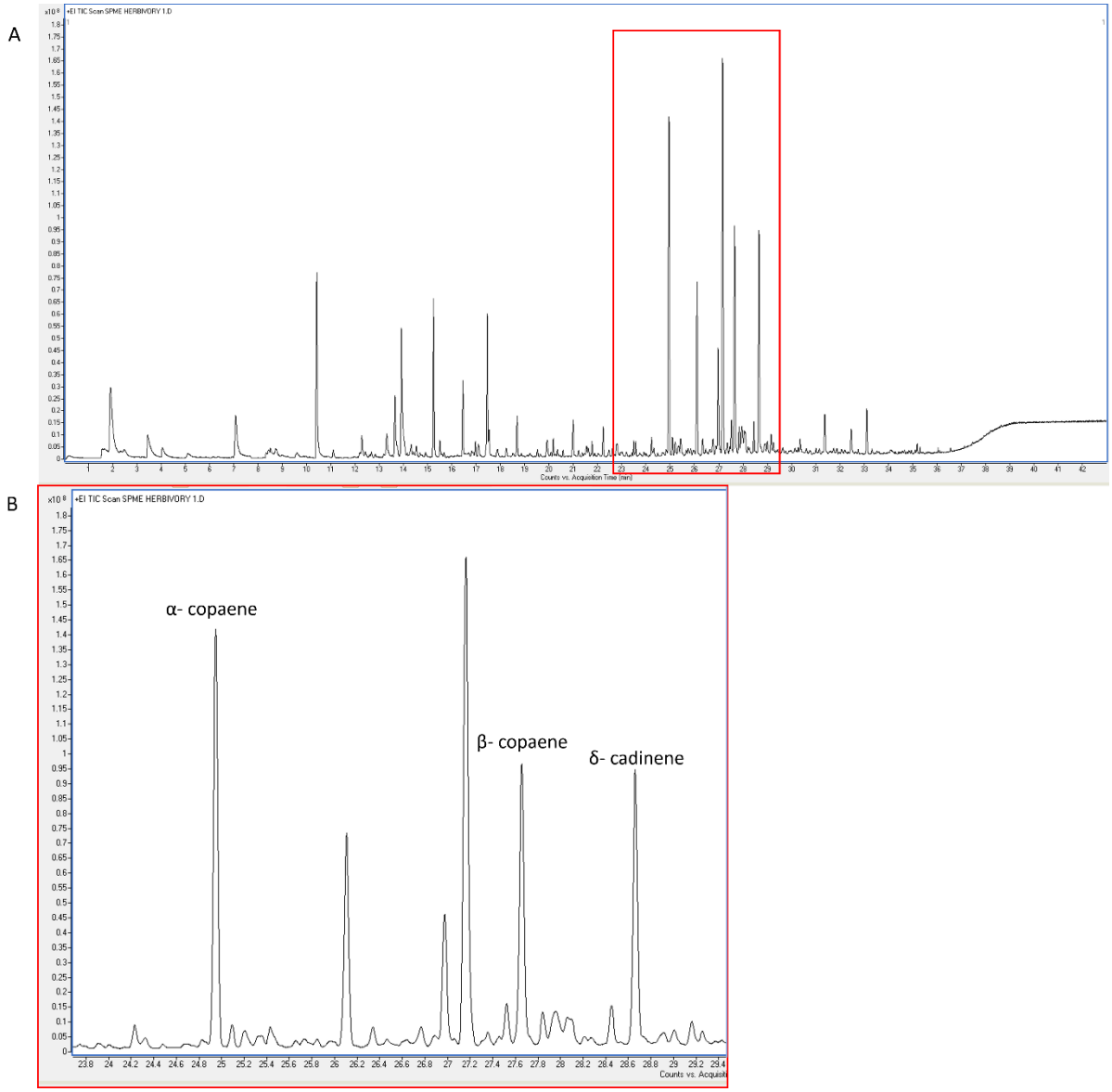


Figure 25- Sample SPME-HS chromatogram. *A. A typical chromatogram of Ipomoea elliptica headspace incubated with SPME fibre for 6 hours. Red box indicates the retention times of sesquiterpenes. B. Zoomed-in picture of the chromatogram with indicated peaks of α-copaene, β-copaene and δ-cadinene.*

After the *Ipomoea elliptica* headspace SPME assay, we detected all but one sesquiterpenes (figure 15) that were also reported by solvent extraction method. We also found some more compounds present in the headspace which were previously not detected during solvent extraction and analysis. One must note that SPME-HS is qualitative method and it only reports presence of compounds in the headspace. Hence from SPME-HS data we may not make conclusions about concentrations of compounds in the headspace. That must be correlated from the solvent extraction data.

Table 2- The summary of the compounds identified by SPME and solvent extraction.

RT (min)	Compounds	SE	SPME- C	SPME- MD	SPME- H	Air
3.05	Butanal, 3-methyl-					
3.12	Butane, 2-chloro-2-methyl-					
3.66	4-Methyl-2-hexene,c&t					
3.83	Pentane, 2-isocyano-2,4,4-trimethyl-					
4.41	Ethane, 1,1-diethoxy-					
5.68	2-Pentene, 2,3,4-trimethyl-					
6.56	2-Hexene, 2,5,5-trimethyl-					
7.1	2-Hexene, 2,5,5-trimethyl-					
7.34	1-Decene, 3,3,4-trimethyl-					
9.2	3-Octene, 2,2-dimethyl-					
10.38	Oxime-, methoxy-phenyl-_					
10.39	Methional					
10.51	Cyclopentane, 1,2,3,4,5-pentamethyl-					
12.25	Benzaldehyde					
13.25	Mesitylene					
13.98	: 4-Hexen-1-ol, acetate					
14.1	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-					
14.29	Carbonic acid, decyl 2-ethylhexyl ester					
14.37	Benzene, 1-methyl-3-(1-methylethyl)-					
14.49	D-Limonene					
15.12	Benzeneacetaldehyde					
15.2	Carene					
15.46	Dodecane, 2,6,11-trimethyl-					
15.63	Dodecane					
16.37	Benzaldehyde, 3-methyl-					
16.9	Dodecane, 2,6,11-trimethyl-					
17.42	(E)-4,8-Dimethylnona-1,3,7-triene					
18.81	Undecane, 2-methyl-					
20.03	Heptadecane, 2,6,10,15-tetramethyl-					
20.14	Decanal					
20.22	Carbonic acid, decyl nonyl ester					
20.29	Undecane, 2,5-dimethyl-					
20.54	Dodecane, 4-methyl-					
21.17	Octadecane, 1-chloro-					
21.32	2,6,10-Trimethyltridecane					
21.49	Pentadecane, 2,6,10,14-tetramethyl-					
21.59	4-Methyldodecane					
21.74	n-Heptadecane					
22.2	Hexadecene [14.197]					
22.42	Octadecane					
22.56	Heneicosane					
22.78	Hexadecane, 2,6,10,14-tetramethyl-					
23.44	Heneicosane					
24.91	**α-copaene**					
26.08	β-caryophyllene					
26.94	Humulene					
27.12	Aromandendrene					
27.62	**β-copaene**					
28.4	Gamma Cadinene					
28.52	7-epi-α-Selinene					
28.61	**δ-cadinene**					

Abbrevation	Meaning
SE	Solvent extraction
SPME- C	SPME control
SPME- MD	SPME mechanical damage
SPME- H	SPME herbivory
Air	Air control

Colour	Meaning
	Detected only in SPME
	Detected only in solvent extraction
	Detected in both
	Not present

Data from table-1 indicate that α-copaene, β-copaene and δ-cadinene are present in the headspace of *Ipomoea elliptica*, hence they were chosen as the candidate aggregation signals.

Identifying the aggregation signal-

1. Complementation assays- We have ordered the standards of these candidate compounds. Once they are procured, we will be conducting complementation assays. In these assays, a compound will be pasted on *Ipomoea elliptica* leaves such that the addition of concentration of the compound in the leaf and pasted compound together is same as the induced concentration. On the other hand, only solvent without the compound will be pasted on control leaves. Beetles will be then subjected to two-choice assays using these two types of leaves. Same assay will be conducted for other two compounds. Statistically significant preference to any one of the three compounds will confirm that it is the aggregation signal for *Chiridopsis nigropunctata.*
2. Another way to identify the signal is through reverse genetics i.e silencing the genes which transcribe enzymes responsible for the production of these three compounds and then check its effects on aggregation.

Results of experiments 8 and 9-

We proceeded to reverse genetics because of two reasons-

1. It was taking a long time for these compounds to ship and reach IISER Pune as they were being imported.
2. Since *Ipomoea elliptica* genome is not sequenced it would take lot more time to standardize a PCR using primers based on sequence of another *Ipomoea spp.*

After total RNA extraction from *I. elliptica and I. triloba* and cDNA synthesis, we tried to amplify the sesquiterpene synthase cDNA fragments using all the primer combinations (a to h; total 8 combinations and as many different PCR reactions) at 48˚C, 50˚C, 52˚C, 56˚C and 60˚C annealing temperatures, with 1X and 2X template concentrations, using genomic DNA template and also using different Taq enzymes. *Ipomoea triloba* cDNA template was used as a control because these primers were

designed over the predicted sesquiterpene synthase sequence of this species. Unfortunately, amplification was not observed in any of our reactions.

Once the sesquiterpene synthase silenced plants are obtained, they will be used for aggregation assays to test the hypothesis that the beetles will not aggregate on such plants.

Chapter 4 **Discussion and conclusion**

After this study we know much more about a relatively unknown system. From all the behavioural assays we understood that aggregation signal is olfactory in nature and originates in plants. To our surprise, we found that the founder beetles' role in this process is only limited to damaging the plant by herbivory, which in theory had the same effect on the induced on the damaged induced volatile profile as mechanical damage had. These two types of damages to the leaves resulted in significant induction in levels of three sesquiterpenes, namely α-copaene, β-copaene and δ-cadinene. By SPME-HS analysis we also confirmed their presence in the headspace of Ipomoea elliptica making them primary candidates for the said aggregation signal.

On receiving the standards of α-copaene, β-copaene and δ-cadinene, we will proceed with complementation assays as mentioned in the results section. It may happen that the beetles do not show significant preference to any of these compounds. In that case we will proceed with more complementation assays using a blend of two or all three compounds. In the extreme event of beetles not showing significant preference in these trials, we will proceed by using the complete induced blend in the complementation assays. General volatile blend post herbivory has also been shown to be attractive for certain insects (War et al. 2011). Figure 13 indicates that β-caryophyllene (another sesquiterpene) also shows a similar trend of increase in concentrations 12 hours post herbivory, but it turned out to be statistically not significant. But it may be the case where it is biologically significant to the herbivore beetles. These experiments were carried out in the month of December and January, but these beetles are at the peak of their activity from July to September. Owing to the aspect of seasonal variation, a pilot herbivory assay should be done in the peak season just to compare the induced blends. It will be interesting to know the levels of β-caryophyllene in the season of peak activity. An educated guess would be that β-caryophyllene will join α-copaene, β-copaene and δ-cadinene in being significantly induced 12 hours post herbivory.

Plants and their insect herbivores have been up against each other in the evolutionary arms race. Plants respond to insect herbivory by various defensive

strategies. One of the strategies is to deter the insects by ramping up the production of VOCs after herbivory, and terpenoids are a prime constituent of these VOCs.

But here in the *Ipomoea-Chriridopsis spp.* system we observed that the terpenoids induced after herbivory function as an aggregation signal for the conspecifics which must be maladaptive for the plant as these beetles completely devour the plants in matter of days (field observation). Hence, this specific plant-herbivore system is at an intriguing turn of the evolutionary arms race because a response that is meant to deter the herbivore is in fact attracting more of them, ultimately resulting in more damage to the plant. The beetles have in fact hijacked the plant defence system to their own benefit.

In many cases herbivory induced plant volatile blend is an attractant for the parasitoids of the herbivore (Gols et al. 2011; Ichiki et al. 2011; Sullivan et al. 2000; Turlings and Erb 2018; Uefune et al. 2020) and not the herbivore itself; and these beetles do not have any known parasitoids. There are only few other cases in which induced volatile blend of the plant played the role of an attractant to its herbivore. In *Eucalyptus grandis*, induction of monoterpenes α-pinene and γ-terpinene attracted more pests *Leptocybe invasa* to the plant (Naidoo et al. 2018). In another example, rice-field weed *Ludwigia octovalvis* infested with the larvae or adults of *Altica cyanea* (Flea beetle) became more attractive to the conspecific females as the volatile blend changed qualitatively as well as quantitatively (Mitra et al. 2017).

This phenomenon can be exploited in sustainable pest management strategies by setting up decoy insect-traps in agricultural fields. These decoys would attract the pests because of an artificial volatile blend imitating the blend of herbivore infested crop. *Chridopsis bipunctata* is a reported pest of *Ipomoea batatas* (sweet potato). This plant-herbivore pair is phylogenetically close to the pair studied here. If these results are universally applicable to the *Ipomoea-Chiridopsis spp*. (figure 1) then the terpenoids induced in *I. batatas* by *C. bipunctata* herbivory can be used to set up bio-traps to lure the beetles away from the sweet potato fields. Similar concept is being used to set up traps to attract the parasitoids (Jones et al. 2011; Turlings and Ton 2006; Yu et al. 2008) to herbivore infested fields to oviposit in the herbivores, ultimately killing them and reducing the damage to the crops (Heraty 2009; Yang et al. 2014).

Through this study we have tried to understand facts about a lesser-known system and there is still a large unknown. Next step would be trying to understand why one hostplant species is infested only by one species of beetle at a given time. This question is intriguing in the light of the fact that the aggregation signal is of plant origin and the plant cannot differentiate between herbivory and mechanical damage (at least from the VOC blend point of view), implying that it is highly possible that *Ipomoea elliptica* will respond in a similar way to *C. bipunctata* herbivory as it did to *C. nigropunctata* herbivory. To explain this peculiarity, we hypothesize that cues for territorial demarcation could possibly be of insect origin and separate set of assays are required to test this hypothesis.

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