The Age-Dependency of Jasmonate Levels Decay And Defense Metabolite Accumulation On Plant Insect Resistance

Research Question: Does the Jasmonate hormone levels change with the increasing age of *Lactuca sativa* in comparison with a young plant, (4-weeks old) and old plant (8-weeks old) and how does this contribute to the survivability of *Lactuca sativa* measured by its resistance against the larvae *Plutella xylostella* and *Helicoverpa zea* deduced by the change of weight of the insects after feeding?

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Research Question

Does the Jasmonate hormone levels change with the increasing age of *Lactuca sativa* in comparison with a young plant, (4-weeks old) and old plant (8-weeks old) and how does this contribute to the survivability of *Lactuca sativa* measured by its resistance against the larvae *Plutella xylostella* and *Helicoverpa zea* deduced by the change of weight of the insects after feeding?

Introduction

As a biology student, I was always fascinated by how plants defend themselves from herbivores. My interest derived from witnessing unusual event in which older plants in my house were usually more durable against herbivorous insects compared to younger plants which led me to research leading cause of this situation. First, I researched matter which contributes to plant defense and found out that it's a hormone named Jasmonate (JA)[1]. I was curious if JA production and signaling changed with plant's age or not as JA hormone seemed to be leading cause of plant insect defense and older plants seemed to be more durable. Turns out, not only does this hormone play a key role in plant defense, it is also involved in many other processes, such as production of defense metabolites (like phenolics, terpenoids and alkaloids) and activation of defense genes and signaling of cell death[2]. Furthermore, I also found out that response of JA hormone has a correlation with increasing plant age and is found to be semi-replaced with other defense metabolites which are accumulated during the growth stage of the plant[3] which I thought was the reason why my plants were more durable as they aged and were less likely to be a target for the herbivorous insects while my young plants were the main targets and therefore I decided to conduct this study and chose my topic as something that would allow me to observe even the smallest causes of my interest.

Background Information

As sessile organisms, plants have evolved a variety of mechanisms throughout many years for successful survival and reproduction. One of these mechanisms include the production of a phytohormone named Jasmonate which plays a role in a variety of processes including plant defense, growth, and reproduction[1]. JA hormone works as a signal molecule and is produced in response to stimuli, including being attacked by herbivores or pathogens, mechanical damage or environmental stress[4]. JA signaling is controlled through a variety of factors such as JA-ZIM domain (JAZ) proteins. These proteins bind to JA response elements (JAREs) in regions of JA-responsive genes and inhibit the accumulation of JA[5]. As plants grow older, the accumulation of another protein named Squamosa Promoter Binding Protein-Like 3 (SPL3) increases and prevents JAZ proteins from binding to JAREs which should increase the uptake of JA as the plant grows older[6]. However, SPL3 is a transcription factor, which means that it binds to DNA and regulates the gene expressions (genes that are involved in plant growth, development and stress response) through recruiting a complex of proteins (including Histone deacetylases (HDACs) and DNA Methyltransferases (DNTMs). HDACs typically remove acetyl groups from histones which makes the DNA more tightly packed which makes it harder for transcription factors to bind to it. DNTMs typically add methyl groups to DNA which results in a chemical modification that can silence genes and therefore makes it less likely for the genes to be transcribed)[7]. Since plants accumulate more SPL3 as they age, more SPL3 proteins bind to the promoters of JA signaling genes and prevent them from being transcribed, leading to a decrease in JA production. Even though this decrease should result in the plant being less durable, the plant also accumulates defense metabolites as it ages (phenolics, terpenoids and alkaloids).

These defense metabolites can semi-replace the JA hormone in defense and therefore result in the plant being more durable against herbivores compared to younger plants. Although these proteins are considered to be non-essential since their uptake can sometimes be toxic on high amounts, they are mainly considered positive since they perform a better job in defense compared to JA[8]. The function of these defense metabolites is vital for plant defense as they inhibit digestive processes and repel or reduce feeding activity from herbivorous attackers as explained in page 6. The decay of JA hormone and the build-up of defense metabolites in plants are proved to be age-dependent by young plants displaying a high level of JA signaling and a low level of defense metabolites and old plants displaying a low level of JA signaling and a high level of defense metabolites, yet old plants being more durable[9]. As plants age, the level of JA signaling reaches the bottom while the level of defense metabolites skyrockets (JA signaling, other than plant defense, takes part in the attraction of herbivores, as explained below). This age-dependent change in JA level and defense metabolite accumulation is the reason why older plants are more resistant to herbivorous insects than younger plants[10].

The Role of JA Signaling in Plant Defense

JA signaling is a process that involves an assortment of proteins and genes. The signaling pathway is triggered when a herbivore damages a plant. The plant releases JA, which binds to receptors on the surface of cells. Initiating a cascade effect, this binding results in the expression of JA-responsive genes. These essential genes encode proteins which coordinate defensive activities such as creating barriers against potential threats whilst activating strong immune responses alongside producing defense metabolites.[11]

The Role of Defense Metabolites in Plant Defense

When plants undergo damage or experience stress, they generate an assortment of defense metabolites. These compounds exhibit a variety of impacts on herbivores like deterring them or inducing sickness and obstructing their digestion. Some of the most common defense metabolites include alkaloids, terpenoids and phenolics.[12]

Alkaloids: Alkaloids are a class of naturally occurring compounds with at least one nitrogen atom (Sinapine, $C_{11}H_{13}NO_3$, Sinalbin, $C_3OH_{42}N_4O_9S_2$) and they are mostly poisonous for herbivores. Most alkaloids have an effect of the insect's nervous system which results in paralysis or death. For example, Sinapine, an alkaloid that is found in ettuce contributes to plant defense in many ways. One way is it being a neurotoxin, which means that it can damage or kill nerve cells of insects by blocking the transmission of nerve signals[13].

Terpenoids: Terpenoids are a large, diverse class of natural compounds which are derived from isoprene units (C_5H_8). They are found in a variety of plants, including lettuce and that play many important roles, including defense against insects. Terpenoids act as insect repellents and feeding deterrents. They also interfere with insect development and reproduction. For example, limonene, a monoterpene (a type of terpenoid that is made up of two isoprene units), is a colorless liquid with a citrusy odor and a volatile compound that acts as a feeding deterrent against some insects. Limonene is toxic to most insects, it usually kills insects when it comes into contact with their bodies. This works by dissolving the insect's waxy exoskeleton, which then causes the insect to dehydrate and die[14].

Phenolics: Phenolics are a large, diverse class of chemical compounds that are characterized with the presence of a hydroxyl (OH) group which is attached to an aromatic ring. They play an important role in protecting the plant from environmental stresses, also responsible for the color of many fruits and vegetables. For example, Lignins, are also present in *Lettuce* and they are important for defense against pests. They make it so the cell barrier is more difficult for pests to penetrate, produce toxic compounds which are broken down by enzymes produced by pests which can kill them and can also attract predatory insects[15].

These compounds also have complementary effects (for example phenolics being able to deter insect feeding and affect their digestive while alkaloids affecting their nervous system) and indirect effects (referring to ways in which these compounds can affect pests and diseases without directly interacting with them, such as attracting natural predators of herbivorous insects)[16].

Furthermore, it must also be noted, not every plant may exhibit high levels of defense metabolites just because they are old, as this also depends on environmental factors. Plants who had low amounts of insect exposure may exhibit lower levels of defense metabolites and vice versa.

In this essay, I have decided to use the Lettuce plant and *Plutella xylostella* and *Helicoverpa zea* larvae to exhibit the relation between the decay of JA and plants age, and further explain it by using an insect feeding array with these insects.

Lettuce: This is a leafy, green vegetable which has originated in Mediterranean. I have decided to use this plant for my experiment due to it being available and easy to grow, having a fast growth rate and it being suitable for specific research questions.

Plutella xylostella: This is a small moth that is native to Eurasia and is a major pest of vegetables. I have decided to use this insect because it has high specialization on cruciferous plants, meaning that it's a good choice since Lettuce is also cruciferous.

Helicoverpa zea: This is a moth that is native to Americas and is highly adaptable as it has developed resistance to many insecticides. It is also a generalist, meaning that it feeds on a wide range of plants rather than a specific species, unlike *Plutella xylostella*.

Reason why I have used two different species of larvae is because it increases data robustness, addresses potential limitations and observe potential differences in response.

Hypothesis

Older plant's JA levels will be lower, but it will still be more resistant against *Plutella xylostella* and *Helicoverpa zea* compared to younger plant. As plant grows, it accumulates defense metabolites so even though JA level is lower, it is still more resistant than young plant, which is why larvae feeds less on it.

Variables

Independent Variable	**Explanation**	**Source**
Age of *Lactuca sativa*	Contribution of changing age of plant to decay of JA hormone	Sowing *Lactuca sativa* with ages 4 and 8 weeks.

Depenent Variable	**Explanation**	**Source**
JA Hormone Level	Change in JA hormone level as plant ages	Methanol extraction followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Controlled Variables	**Explanation**	**Source**
Identical picking of *Lactuca sativa*	Same types of *Lactuca sativa* without genetic disorders	Picking same plants from provider without genetic disorders
Time period of food exposure for larvae	Feeding larvae for same period of time to minimize error bars	Allowing every larvae to feed for a set period of time
Volume of methanol (10mL)	10 mL volume of methanol usage for every leaf mixture	10 mL of methanol in every flask
Sunlight	Same watts per square of sunlight in same room	Watts per square of sunlight decided by eye
Temperature	Room temperature, 20 C	Thermometer

Material List

1- 4x Lettuce Seedlings

2- 24x Lettuce leaves (Obtained from seedlings)

3- 3x Newly hatched *Plutella xylostella*

4- 3x Newly hatched *Helicoverpa zea*

5- Stopwatch (± 0.2)

6- LC-MS/MS device

7- Methanol (100mL) (± 0.01)

8- Grinding Mortar (4 inches)

9- Soil (231 cubic inches)

10- Pot (13 cm)

11- Tap Water (5L)

Method Development

My research, at first, was just me exploring into world of plant-insect interactions. I learned about important role of jasmonate (JA) as a signaling molecule in plant defense against herbivores. I was really interested by concept of age-related insect resistance, I further explored existing literature and discover that older plants often exhibit higher resistance compared to younger ones. This initiated my curiosity abou potential link between JA, plant age, and defense mechanisms.

To investigate this connection, I developed a specified approach. First, I cultivated lettuce plants, separating them into young and old groups. Then, I used a detailed process to extract jasmonate from leaves, asking for help from a university for LC-MS/MS analysis to measure its levels.

Finally, I conducted feeding assays, introducing specific insect larvae (Plutella xylostella and Helicoverpa zea) to separate young and old lettuce plants. By monitoring their weight gain and observing physical damage on plants, I wanted to discuss the effectiveness of the feeding behavior. Finally, to dive deeper into potential underlying mechanisms, I considered applying an optional protein-protein interaction analysis using a yeast-2-hybrid assay. However, I was aware of complexity and specialized equipment required, so I had to collaborate with a research lab for this specific step and managed to successfully conduct it.

Through this approach, I aimed to discover potential connection between jasmonate levels, plant age, and insect resistance in lettuce, contributing to a better understanding of plant defense strategies in nature.

Method

Measuring JA level

1- Buy 2 Lettuce seedlings and garden soil from a garden center.
2- Sow the seedlings.
3- Water them with 2.45 mL of water every day and grow them for 4 weeks.
4- When the plants reach 4 weeks of age, repeat the first 3 steps.
5- After the repetition, have 4 plants in total, 2 of them being 4 weeks of age and 2 of them being 8 weeks of age
6- Label the 4-weeks old plants “P1” and label the 8-weeks old plants “P2”.
7- Crop 6 leaves from each of the plants.
8- Have 12 leaves from P1 and 12 leaves from P2, 24 in total.
9- First, grind P1’s leaves in a mortar, then grind P2’s leaves.
10- After grinding for a minute, add 10 $\pm$0.1 mL methanol for each gram of leaves to both mortars.
11- Continue grinding both until a homogeneous mixture is formed.
12- Put the mixture from P1 in a tube, label it “T1”, label the mixture from P2 “T2”.

13- Centrifuge both tubes for 11500 RPM for 10 minutes.

14- Filter the supernatant from both.

15- Divide the supernatant from T1 into 25 samples, repeat for T2. Have 50 samples in total.

16- Put the labeled samples in separate places to avoid confusion. Follow steps 17 and 18 for both samples, separately.

17- Inject each sample once into the LC-MS/MS device.

18- Record the raw data of JA level after instrumental analysis performed by the device.

Feeding of Larvae and Damage Tests

19- Weigh 3 *Plutella xylostella* and *Helicoverpa zea* larvae, record data as initial weight.

20- Add 3 *Plutella xylostella* on one of the plants, name it group 1.

21- Add 3 *Helicoverpa zea* on the other plant, name it group 2.

22- Let the larvae feed on the plants for 5 days, keep watering the plants every day meanwhile.

23- Observe the damage done on the plants, such as irregular leaf shape and wounded body.

24- Re-measure the weight of larvae after feeding and record the data.

25- Compare it with initial weight to find the change of weight of larvae.

26- Re-measure JA levels from the plants after feeding

Showing the Interaction Between SPL3 and JAZ

27- Use a yeast-2-hybrid assay. Choose the GAL4 activation domain to show protein-protein interactions.

28- Bind SPL3 protein to the prey vector, bind the JAZ protein to bait vector.

29- Check on the machine to see the interaction, a white circular mark should appear if the prey vector takes is attracted by the bait.

Results and Analysis

Figure 1.1: Young Plant at Early Stages of Development

Figure 1.2: Old Plant at Early Stages of Development

Table 1.1: Raw Data Table

Plant Age	Trials	JA Level µmol (±0.01)	Plant Age	Trials	JA Level µmol (±0.01)
	1	0.0024		1	0.0002
	2	0.0032		2	0.0002
	3	0.0027		3	0.0003
	4	0.0031		4	0.0002
	5	0.0028		5	0.0003
	6	0.0025		6	0.0003
	7	0.0021		7	0.0002
	8	0.0034		8	0.0002
	9	0.0026		9	0.0004
	10	0.0033		10	0.0003
	11	0.0024		11	0.0003
Young P.	12	0.0028	Old P.	12	0.0002
(4-weeks	13	0.0031	(8-weeks	13	0.0002
old)	14	0.0029	old)	14	0.0004
	15	0.0025		15	0.0002
	16	0.0029		16	0.0003
	17	0.0021		17	0.0004
	18	0.0036		18	0.0002
	19	0.0024		19	0.0003
	20	0.0027		20	0.0002
	21	0.0027		21	0.0003
	22	0.0033		22	0.0002
	23	0.0028		23	0.0004
	24	0.0021		24	0.0002
	25	0.0025		25	0.0003

Table 1.2: Processed Data Table

Plant Age	Mean JA level	Standard Deviation	Standard Error
Young P. (4-weeks old)	0.002756	0.000405048	8.10096E-05

Plant Age	Mean JA level	Standard Deviation	Standard Error
Old P. (4-weeks old)	0.000268	7.33212E-05	1.46642E-05

Graph 1: Column Graph With Mean JA Levels of Young (4-weeks old) and Old Plant (8-weeks old)

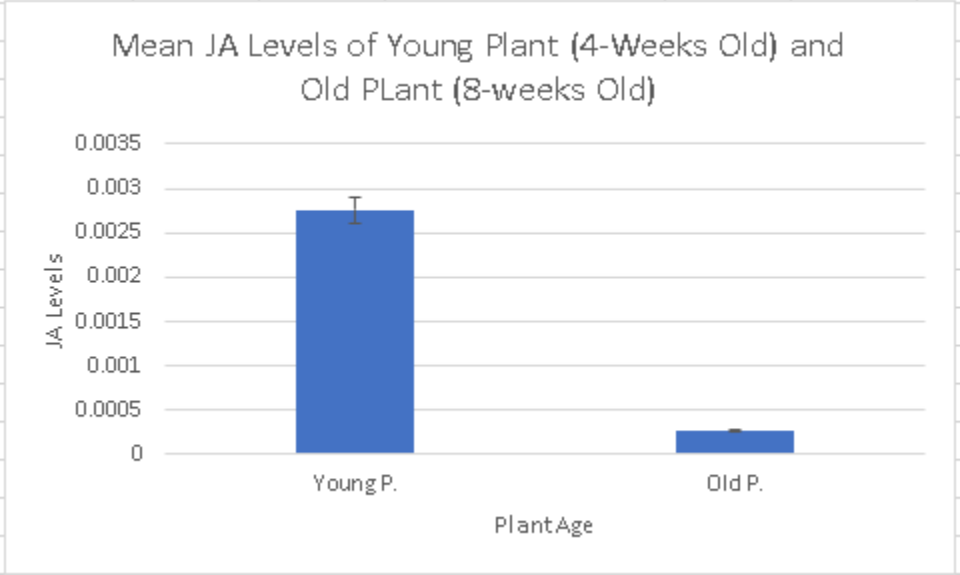


Table 2: Single-Factor ANOVA

Result Details				
Source	*SS*	*df*	*MS*	
Between-treatments	0.0001	3	0	*F* = 264.22311
Within-treatments	0	44	0	
Total	0.0001	47		

The *f*-ratio value is 264.22311. The *p*-value is < .00001. The result is significant at *p* < .01.

H0: There is no difference between JA level of young and old plants.

H1: There is a difference between JA level of young and old plants.

I used ANOVA test to check for differences between my data because it helped me understand if there are significant differences between means of JA levels from young and old plants, respectively. Because ratio of F/Fcrit is > than 1, meaning that value of F is larger than value of F crit, we can reject null hypothesis, meaning that JA response does decline with age.

Table 3: Initial and Final Weight of *Plutella Xylostella* and *Helicoverpa Zea* larvae on Young Plants

	Plutella Xylostella	Helicoverpa Zea
Initial Weight (g) (±0.001)	1.276	2.184
	1.342	1.957
	1.294	2.215
Mean	1.304	2.118

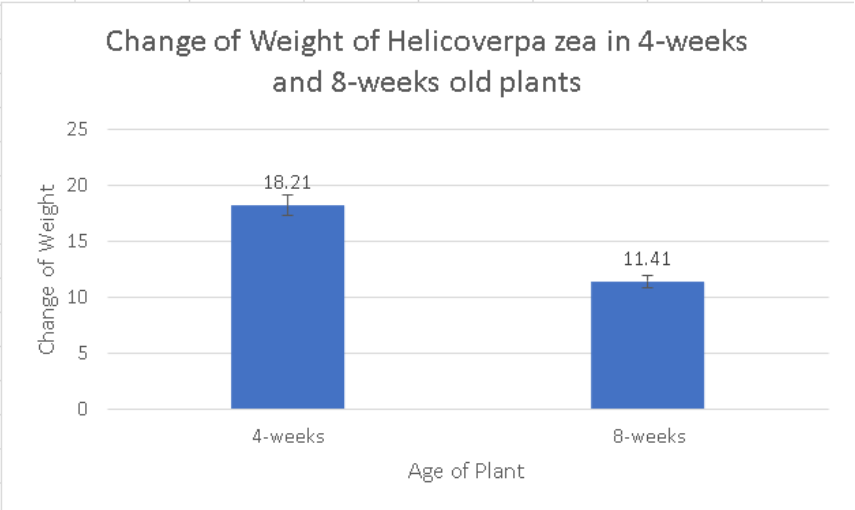
	Plutella Xylostella	Helicoverpa Zea
Final Weight (g) (±0.001)	6.456	20.77
	6.762	20.25
	6.284	19.97
Mean	6.501	20.33

Table 3: Initial and Final Weight of *Plutella Xylostella* and *Helicoverpa Zea* larvae on Old Plants

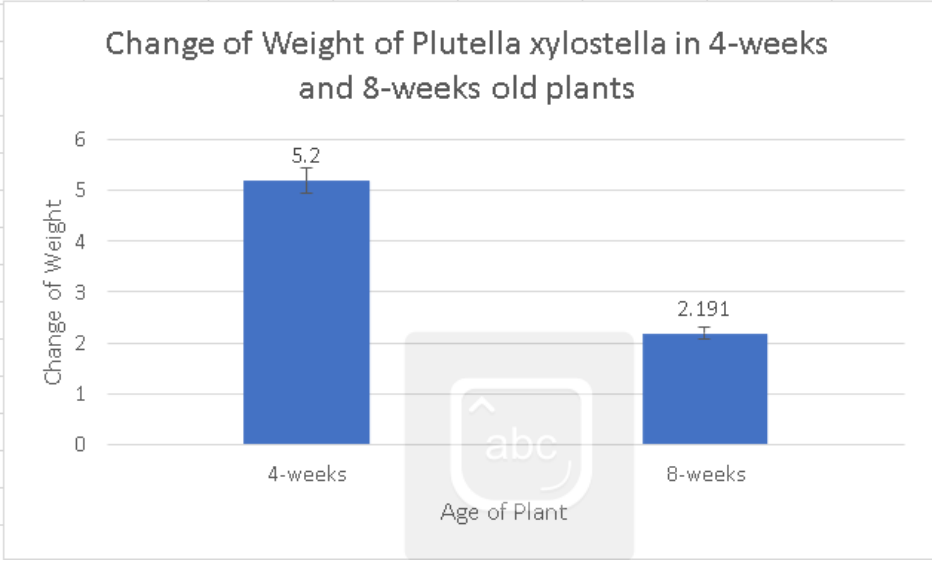
	Plutella Xylostella	Helicoverpa Zea
Initial Weight (g) (±0.001)	1.326	2.241
	1.413	2.135
	1.295	2.314
Mean	1.345	2.230

	Plutella Xylostella	Helicoverpa Zea
Final Weight (g) (±0.001)	3.587	13.58
	3.692	13.89
	3.328	13.45
Mean	3.536	13.64

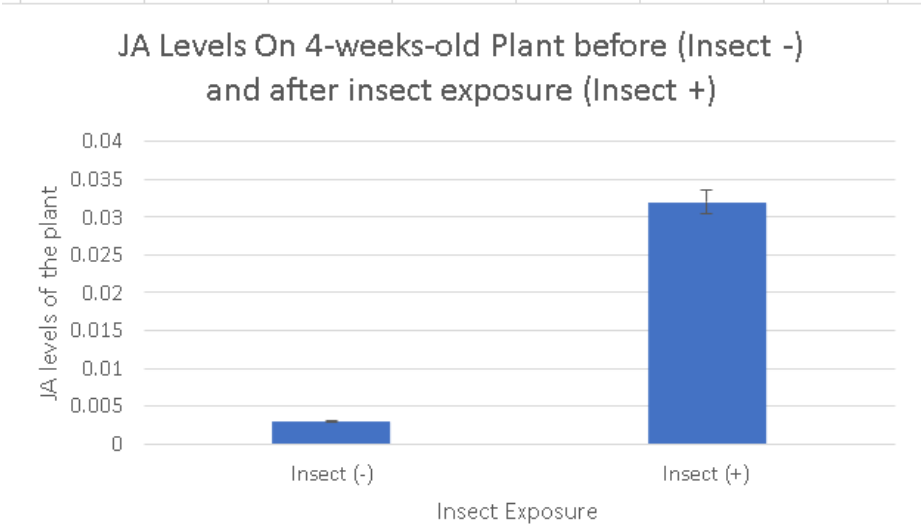
Graph 2: Change of Weight of *Helicoverpa zea* in 4-weeks and 8-weeks old plants



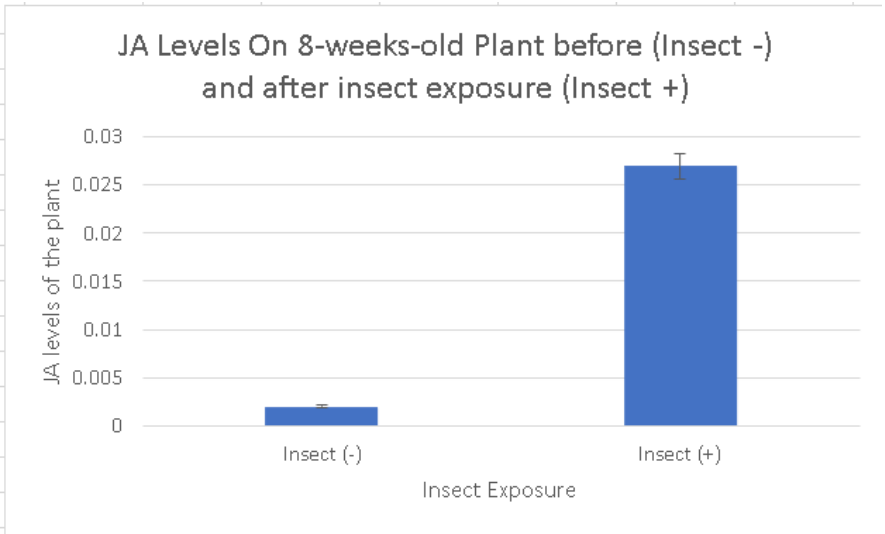
Graph 2.1: Change of Weight of *Plutella xylostella* in 4-weeks and 8-weeks old plants



Graph 2.3: JA levels on 4-weeks-old plant on insect exposure (Insect +) and before exposure (Insect -)



Graph 2.4: JA levels on 8-weeks-old plant on insect exposure (Insect +) and before exposure (Insect -)



SPL3 Can Interact With the JAZ Proteins

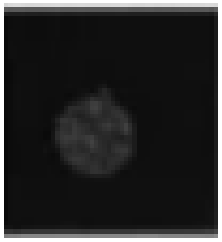
In yeast-2-hybrid assays SPL3 had a direct interaction with JAZ, as observed. Additionally, other JAZ proteins (JAZ2, JAZ9, JAZ6) were also put in test, all showed a direct interaction with SPL3.

Figure 2.1: SPL3 Interaction With JAZ2

Figure 2.2: SPL3 Interaction With JAZ1

Figure 2.3: SPL3 Interaction With JAZ9

Figure 2.4: SPL3 Interaction With JAZ6



Comparing to accepted theory of yeast-2-hybrid[15], when SPL3 shows an interaction with JAZ on assay, there should be a visible dot (each of them representing a successful colony under a microscope), which appears in every trial, meaning that these two proteins interacted with eachother.

SPL3 Promotes Accumulation of JAZ

As plants grow, SPL3 levels are increased, while miR156 (a microRNA molecule that negatively regulates SPL3 levels) levels decrease. In addition to this, we know that SPL3 can bind and interact with JAZ proteins. This interaction stabilizes JAZ proteins, which prevents their degradation and therefore increases level of JAZ proteins overall.

Discussion and Evaluation

When I placed *Helicoverpa zea* larvae on 4-weeks-old and 8-weeks-old Lettuce for 3 days, larvae which was placed on young plants grew faster than ones placed on older plants. When another specialist pest, *Plutella xylostella*, for whom we can use Lettuce as a host, comparable results were obtained. larvae of *Helicoverpa zea* and *Plutella xylostella* gained more weight from young plants than older plants.

Therefore, results suggest that plants develop better resistance against insects as they age. Additionally, JA levels measured from leaves of each plant shows a correlation between its decline and plants age.

Plants face attacks from many species of insect herbivores at different ages. In this essay, I explored change of JA response, which is very high in early stages of a plants life and continues to decline as plant ages.

In contrast to this behaviour of JA, defense metabolites like alkaloids, terpenoids and phenolics are accumulated (even though this couldn't be directly measured, it can be accepted true as the old plant is still more resistant even though the low levels of JA, further proven by scientific context shown on page 6), contributing to insect resistance in old plants.

JAZ proteins are the key to JA signal output, and my data from Figure 2.1 – Figure 2.4 shows that SPL3 can stabilize JAZ through protein-protein interactions. I also showed that increased amount of SPL3 in old plants result in higher levels of JAZ proteins, which inhibits JA responses, as shown in Graph 3 and Graph 4, with the older plant having a lower JA response, further proven by the statistical analysis shown in Table 2.

I have shown that in the early stages of a plant, specifically when they have little biomass and insufficient amount of defense metabolites, the active response of JA is a crucial factor for the plant to be able to endure and resist herbivores and pathogens. As it can also be seen in Graph 2 and 2.1, insects have gained more weight from the young plant compared to the young plant. The reason why *Helicoverpa zea* gained more weight is because it is a generalist compared to *Plutella xylostella*, a specialist, as explained in background information. It must also be noted that larvae were not harmed and were refunded to buyer after experiment.

As defense metabolites are accumulated by age, they may provide a better level of resistance, and thus may decrease the work needed to be done by active defense of JA.

Additionally, even though JA provides the plants with resistance against herbivores and pathogens, it also inhibits plant growth by interfering with growth hormone signals. This decay of JA is potentially a strategy of plants to ensure development, in which SPL proteins contribute to the balance between growth and defense.

Defense metabolites serve as defense mechanisms for plants against infections and herbivorous insects. It's interesting to note that they might also employ the same protective chemicals. For instance, cotton plants store gossypol and related aldehydes while simultaneously stimulating their production.

Finally, changes in defense metabolites may also have an effect on insect resistance and therefore act as a compensation for the decayed JA response in adult plant. However, this deserves further investigation.

Method Evaluation

The methodology has strengths, which includes well-structured approach that progresses from seed germination to insect feeding and molecular analysis without any problems.

Considering both insect feeding and yeast-2-hybrid components provides a better understanding of the plant's response at physiological molecular levels. The use of quantitative measurement, them being weighing the larvae and applying LC-MS/MS for JA analysis improves the precision of the results I have obtained. I have also included multiple plants at each age group which contributes to the statistical strength of my findings. Additionally, choosing two different insect species also expands the scope of my study.

However, there are also many weaknesses, which include an absence of a control group without insect infestation may limit the ability to understand the age-related effects and those excited by insect infestation.

Additionally, there is a subjective nature of damage assessment based on physical properties, which could be addressed by implementing a scoring system.

Monitoring and reporting the environmental conditions and ensuring the consistent weights of larvae and addressing ethical considerations is essential for a stronger methodology.

Further molecular techniques such as co-immunoprecipitation could also strengthen truthfulness of protein interactions which could be analyzed more in depth in future studies.

Applications

The findings of my study has many implications for agriculture and plant protection strategies. Understanding the age-dependent dynamics of plant defense mechanisms can help in the development of targeted pest management approaches that change natural defense mechanisms. Farmers can use this knowledge to optimize crop protection strategies, such as timing pesticide applications or implementing crop rotation practices to focus on the natural durability of older plants. Furthermore, my research contributes to the field of plant biology and biotechnology by enhancing our understanding of the molecular mechanisms that change plant-insect interactions. This knowledge could expand the development of genetically modified crops with enhanced resistance, or design of plant-based insecticides from natural defense metabolites.

Overall, my study shows the importance of research in explaining biological processes for sustainable agriculture and pest management.

Conclusion

In conclusion, my findings provide ideas of the age-regulated dynamics of plant defense mechanisms in Lettuce against herbivorous insects. Through trials of experimentation and analysis, I have shown that changes in JA hormone levels and accumulation of defense metabolites contribute a lot to observed resistance patterns. Older plants had lower levels of JA hormone but higher concentrations of defense metabolites (in theory)[12,13,14], which correlated directly with their increased resistance to *Plutella xylostella* and *Helicoverpa zea* larvae compared to younger plants. This age-dependent change in defense strategies shows a complexity between hormonal signaling and secondary metabolite accumulation in plant defense responses. My experiments provided further evidence of interaction between SPL3 and JAZ proteins, showing a potential mechanism under the age-related decline in JA.

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15- Fig. 5. SPL interacts with the TCP transcription factors. A: Y2H assay. . . (n.d.). ResearchGate. https://www.researchgate.net/figure/SPL-interacts-with-the-TCP-transcription-factors-A-Y2H-assay-shows-pairwise_fig3_269820080

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To Whom This May Concern,

[redacted] has done his IB diploma programme extended essay experiment by himself with the supervision of our staff in the laboratories of the Department of Chemistry (Faculty of Arts and Sciences) at Middle East Technical University (METU).

This document has been provided at the request of [redacted]

Best regards,

Assoc. Prof. Dr. Serhan Türkyılmaz