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THE HYPERACCUMULATION OF ZINC IN SUNFLOWERS AND ITS EFFECT ON DISEASE RESISTANCE

by

RAYNER JACE SEAVEY

A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program
in the Department of Biology
in the College of Sciences
at the University of Central Florida
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ABSTRACT

Sunflowers are considered to be a part of a group of plants known as hyperaccumulators that share the ability to accumulate high amounts of heavy metals in the above ground organs, far in excess of the levels found in other species, often without suffering any phytotoxic effects. Quantifying the effects of zinc accumulation through the lens of the elemental defense hypothesis is essential for uncovering if there is a means to increase herbivore resistance in agricultural settings without the use of external interventions such as pesticides. A greenhouse study was conducted on four widely grown commercial cultivars of sunflower. Each cultivar was grown under multiple soil Zn concentrations ranging from 0 to 200 mg/kg of soil. Growth rate measurements were taken at evenly spaced intervals until maturity. Samples of leaves were taken from plants and tested for Zn concentration. A qualitative study using *Vanessa cardui* was conducted to observe the effects of zinc in the diet of caterpillars. Significant variation in the level of zinc accumulated in the leaves was observed as well as variation in overall biomass per treatment level. *V. cardui* experienced high rates of mortality at high zinc concentrations suggesting that further study may lead to significant evidence that Zinc accumulation is a form of herbivore resistance.

ACKNOWLEDGEMENTS

This project was funded by the UCF Office of Undergraduate Research and the Summer Undergraduate Research Fellowship. I would also like to thank Dr. Chase Mason, Dr. Eric Goolsby and Jordan Dowell for all their contributions and being an integral part of this process. This work could not have been completed without their support.

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INTRODUCTION

There are three hallmarks that distinguish hyperaccumulators: an increased rate of heavy metal uptake, a faster root-to-shoot translocation, and an improved ability to sequester heavy metals in leaves (Rascio et al., 2011). The strict definition of this trait is currently under debate as the lines between what can be classified as hyperaccumulators are blurry due to the fact that this trait is not limited to any specific taxonomic group but is found throughout the land plant phylogeny with wide variation in metal accumulation observed. One hypothesis for the repeated evolution of this trait is the elemental defense hypothesis.

Mechanisms

Heavy metals place oxidative stress on plant organs by increasing the output of reactive oxidative species (ROS), particularly in regard to chloroplast membranes by interacting with electron transport activities (Rascio et al., 2011). This oxidative stress leads to many modes of damage from ion leakage to DNA cleavage (Rascio et al., 2011). This creates a need for coping strategies in order to prevent damage and even death when exposed to large amounts of heavy metals from the environment.

When comparing hyperaccumulating to non-hyperaccumulating species, some of the key steps in hyperaccumulation rely on variation of expression in regulatory genes, particularly in genes that encode transmembrane transporter proteins (Rascio et al., 2011). Some plants employ an ‘excluder’ strategy where they hinder heavy metals from entering through the roots and prevent the metals from translocating to the leaves (Rascio et al., 2011). Alternatively, hyperaccumulators have an increased root to shoot translocation of heavy metals, as well as a greater ability to detoxify and sequester heavy metals once they have reached the leaves. This

improved root to shoot translocation is currently being suggested as a driving force for hyperaccumulation in the leaves by creating a deficiency in the roots, creating a gradient of heavy metals from the roots to the leaves (Rascio et al., 2011).

Once in the aerial organs, detoxification and sequestration must occur to avoid toxicity and damage. This is done away from regions in plant cells which perform vital functions like photosynthesis. Detoxification occurs mainly through the heavy metals interacting with small ligands, molecules attached to a metal atom, allowing them to become entrapped in vacuoles, cell walls, and other inactive compartments. In these compartments, heavy metals will be exposed to chelators which will bind to and prevent metals from floating around in a free ion form, reducing potential damage (Rascio et al., 2011).

Despite the possibility for phytotoxicity, many metals play a crucial role in normal metabolic functions of plants. For example, manganese (Mn) facilitates the oxygen evolution of photosystem II, as well as a series of other enzymatic activities (Page and Feller, 2015). Similarly, zinc (Zn) is essential for multiple enzymes such as metalloproteinase, carbonic anhydrase, and Cu-Zn superoxide dismutase (Page and Feller, 2015).

The typical journey of a heavy metal, when present within tolerable levels, begins in the roots. In many cases, heavy metals go through a process of insolubilization which can be caused by a number of reasons such as forming complexes with organic acids, phytochelatin or nicotianamine which prevent the heavy metals from being released into the xylem (Page and Feller, 2015). Once released, the heavy metals begin their trip upwards through the xylem where they are distributed to the aerial organs. Redistribution around the plant can occur through the help of the phloem (Page and Feller, 2015). While some heavy metals, such as Mn, have a hard time moving through the phloem, other heavy metals are shuttled to where they are needed

most, such as from a senescing leaf to growing vegetative parts or maturing fruits, which are called “sinks” (Page and Feller, 2015).

Classification of Hyperaccumulation Traits

One of the issues involving the definition of hyperaccumulation as a trait is whether we should view it from an evolutionary and physiological standpoint or from an ecological perspective. Often, the classification of hyperaccumulation as a trait is only looked upon in organisms that present higher than average levels of metal concentrations in naturally occurring environments rather than a controlled environment such as a greenhouse. It is argued by Goolsby and Mason (2015) that hyperaccumulation should be defined as a physiological trait and should be considered a separate and distinct trait from that of tolerance. This is backed by the reasoning that hyperaccumulation is an intrinsic and continuous ability of the plant. This is based on the presence of appropriate ions pumps, transporters, and other physiological mechanisms, which is separate from that of tolerance as shown by work in *Arabidopsis* which shows that uptake and tolerance have a separate genetic basis (Goolsby and Mason, 2015;Hanikenne et al., 2008). In contrast, Van der et al. frame their classification from an ecological standpoint basing their assertion on the idea that hyperaccumulation has evolved in response to selection from metalliferous soil and that metal accumulation and tolerance should be considered aspects of a single trait (Van der Ent et al., 2015). For the purposes of this thesis, hyperaccumulation is defined as the ability of a plant to accumulate high levels of heavy metals in above ground aerial organs.

Zinc

Zinc (Zn) has an atomic mass of 65.38 g/mol and is a group 12 (IIB) transition metal. While toxic in large doses, zinc is an essential micronutrient to plants and animals. Plant

requirements for Zn vary considerably across species and cultivars. Rather than a source of toxicity, Zn deficiencies are a common observation amongst plants, as much of the Zn in soils exists in bio-unavailable forms. Zinc becomes chelated, and potentially bio-unavailable, when interacting with organic molecules or minerals with a high surface area such as clay. This renders the Zn molecule unable to move through the soil and unable to be leached by plant roots under most conditions (Schulte, 2004).

With the addition of fertilizers, zinc sulfate ($ZnSO_4$) is the most common form of zinc found in agricultural soils due to its relatively high solubility (Schulte, 2004). Common soil Zn concentrations range from 20-70 ppm for common field crops (Schulte, 2004). When zinc is present in excess levels usually above 100 ppm (Schulte, 2004), plants may present symptoms of toxicity. These symptoms include: general chlorosis of the younger leaves, smaller overall leaf biomass, and in severe cases entire leaf death (Reichman, 2002).

Nutritional content of food crops is an important aspect of food production and breeding practices. Biofortification is an idea where either conventional breeding practices or genetic engineering can be used to increase the nutrient content of the plant. Biofortification could potentially be an approach to overcome nutrient deficiencies for humans or other animals (Page and Feller, 2015;Boyd, 2013). Regarding defense, evidence suggests that some herbivores prefer to eat leaves of *Thlaspi caerulescens* with a lower concentration of Zn as opposed to treatments with higher Zn concentrations (Pollard and Baker, 1997). This suggests a possible link between Zn concentrations and herbivory resistance.

Helianthus

Sunflowers are the fourth most important oilseed crop globally by value and acreage (Dowell et al., 2019). They are used mainly to produce sunflower oil and sunflower seeds.

Sunflowers have demonstrated hyperaccumulation ability amongst various metals (Cutright, 2010), with potential for increased herbivore resistance under the elemental defense hypothesis. The two main seed types used in this experiment are high oleic seed and confection seed. In general, high oleic seeds are used to produce sunflower oil while confection seeds are used for producing sunflower seeds for both human and animal use.

Elemental Defense Hypothesis

The elemental defense hypothesis states that plants can sequester high concentrations of heavy metals as a defense mechanism to protect against pathogens and herbivores (Poschenrieder et al., 2006). This hypothesis was originally suggested as plants grown in soil amended with nickel were found to have higher survival rates (Martens and Boyd, 1994). Recently, there have been many studies looking at the effects of various heavy metals such as Zn, Cd, Mn, Pb, Ni, and others in regard to potential trade-off effects associated with accumulation. A proposed trade-off posits that when defense is conveyed by metals, organic defenses can be decreased reducing energy expenditure of the plant (Boyd and Martens, 1998).

If defense can be conferred through the accumulation of zinc in sunflowers then this provides a potentially new target for study of disease and herbivore resistance. This context poses an interesting question: Can we breed crop sunflower to resist antagonistic effects, such as herbivores or pathogens, without the use external measures, such as pesticides? While the possibility is an appealing concept, there are many aspects to consider when looking at the plant as a whole. There is a possibility of toxicity when dealing with high levels of metal accumulation, which depending on the severity, may counteract all of the fitness and agricultural yield benefits conferred from that resistance. This toxicity may occur in the form of reduced growth rate or biomass, chlorosis of leaves, and even death of the plant (Reichman, 2002). These

effects need to be measured to identify possible disadvantages in an agricultural setting. If so, then this method should not be used in favor of a method that provides the greatest cost to benefit ratio for use in agriculture.

Questions

The information provided by the elemental defense hypothesis suggests that there may be a correlation between increased heavy metal levels in plant tissue and herbivore resistance. This study tests several aspects of the elemental defense hypothesis by addressing the following questions:

(1) Is zinc hyperaccumulation observed in cultivated sunflower?

(2) How is plant growth (a proxy for fitness and yield) altered by variation in soil zinc concentration and plant zinc uptake?

(3) What is the effect of plant zinc uptake on a model herbivore?

MATERIALS AND METHODS

Greenhouse

For this project four commercial hybrid cultivars were used. The four different cultivar types used were two high oleic seed varieties, N4HM354 and Hornet, and two confection seed varieties, Jaguar DMR and LD5009. The seeds were procured from Nuseed (Breckenridge, MN). Replicates of these cultivars were dispersed across three benches in an environmentally controlled greenhouse at the University of Central Florida. There were five treatment levels measured in mg of zinc to kg of soil. The treatment levels were: 0 mg/kg, 50 mg/kg, 100 mg/kg, 150 mg/kg and 200 mg/kg. The heavy metal concentrations selected straddle the known limit of phytotoxicity in several crop variants of *Helianthus*, where the effects of toxicity may or may not present themselves. Each cultivar was replicated six times per treatment level. The greenhouse was divided into an X and Y coordinate system where each pot was assigned to a position through a random number generator. Each pot was designated a unique coordinate position that served as a unique identifier (Figure 1).

The soil that was used was a 70% sand to 30% potting soil mixture in Azalea pots that were 20 cm in diameter at the top and 14 cm deep. The average of the amount of the soil mixture used in the pots was 2.83 kg. The Zn treatment came in a powdered form of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$). This treatment was applied topically to the soil using a liquid solution before the seeds were planted by mixing the $ZnSO_4 \cdot 7H_2O$ with water and pouring the corresponding amounts over the top of the soil. The treatment was left to sit for 48 hours.

After this time, two seeds were planted in pairs in each pot. Once the majority of the seeds began to germinate, there were a few pots that did not germinate on their own. The pots that did not germinate at this time did not have a correlation to any particular treatment nor

cultivar. Seeds for these pots were germinated by hand in the laboratory. Seed coats were removed and each seed was placed in a petri with a small amount of water and set under a growth light until each seed began to germinate. Each seed was then transplanted back to their corresponding pots.

Weekly measurements were taken every week starting the week after the first seed germinated. A measurement of the chlorophyll content was taken by using a chlorophyll meter (CHL STD, atLEAF). Three measurements were taken on the leaf, one at the tip, one in the middle and one at the base near the petiole. Height was recorded every week, measured from the soil level to the highest point on the stem during vegetative growth and to the point where the stem meets the head during reproductive growth.

Once flowered, the plants were harvested on the next date of regular measurement. First, a pair of the uppermost fully developed leaves were harvested where they were used for the analysis of zinc concentration. Each plant was then harvested by parts. Stems, heads, and leaves were placed into separate bags for drying. The dry mass of the separated parts was weighed, and the missing mass of the uppermost leaf pair was accounted for. Internal zinc concentrations for the separated plant parts were analyzed in by Louisiana State University via Inductively-Coupled Plasma Mass Spectroscopy. In order for the leaves to have been analyzed using this method they must have weighed at least 0.5 grams. Due to these physical limitations in the analysis procedure, only fifty leaves were therefore analyzed for zinc content, and only fifty plants are represented in the data.

Vanessa cardui

The analysis of the effect of zinc on the growth and development of caterpillars was done using *Vanessa cardui* in an incubator that was set on a twelve-hour diurnal cycle at 30°C with a

humidity around thirty five percent. The caterpillar eggs were supplied by Carolina Biological Supply (Burlington, NC). Caterpillar diet consisted of a handmade mixture of: 180g of water, 4.76g of agar, 61.5g of Painted Lady Diet (Product# F9698B, Frontier Agricultural Sciences, Inc; Newark, DE), 2g of liquid potassium hydroxide, 97mL of 25% acetic acid and the calculated amount of zinc sulfate heptahydrate.

First the 4.67g of agar was added to the 180g of water in a beaker and set on a hot plate with a stir bar until boiling. Once boiling, this mixture was poured into a blender along with the 61.5g of the food. This mixture was blended for twenty seconds. Then the 97mL of 25% acetic acid was poured into the blender and blended for twenty seconds. Then the 2g of potassium hydroxide was added and blended again for twenty seconds. Finally, the zinc was added and blended for the last time for twenty seconds. This mixture was poured into ten 1 oz. cups filling each cup a fourth of the way up. This procedure was repeated for each level of zinc.

Two trials were run. Eggs were placed in individual cups that had the previously placed layer of food at the bottom then was covered with a small piece of a tissue followed by a lid that had small holes around the top. In the first trail, three eggs were placed in each cup and all were allowed to grow to maturity. In the second trial, three eggs were placed in each cup but only one caterpillar was allowed to survive in each cup. Periodic checks for mortality were taken at various intervals until all were deceased. Caterpillars that formed a chrysalis were moved into a net to allow for further development.

Data Analysis

Zinc concentration in the leaves was analyzed using an analysis of variance (ANOVA) test as well as Tukey post-hoc test. Leaf zinc concentration by cultivar was analyzed by a repeated measures ANOVA. Total biomass and biomass fractions were analyzed using a two-

way ANOVA in conjunction with Tukey post-hoc test. Total biomass by cultivar was analyzed by a two-way ANOVA. Chlorophyll content and growth rate was analyzed using a repeated measures ANOVA. All analyses were performed in JMP Pro version 12 (SAS Institute; Cary, NC)

RESULTS

Zinc Uptake

Across all treatments and cultivars, zinc levels in the leaves ranged from 36 ppm to 699 ppm. The levels of zinc in the soil had a significant effect ($P<0.0009$) on the amount of zinc that was present in the leaves upon analysis. There was a trend of increasing levels of zinc in the leaves with increasing levels of zinc in the soil (Figure 2). To investigate further, the effects of leaf zinc uptake was separated by cultivar (Figure 3). A two-way ANOVA was run and showed that the different cultivars took up different leaf zinc levels as soil concentration changed as shown by a significant interaction term between cultivar and level with a p value of 0.0065. A repeated measures ANOVA was also run and showed a significant effect of level on all the cultivars (Figure 3).

Total Biomass

Visually, there was a trend of decreasing overall mean biomass across the cultivars as the soil Zn concentration levels increased (Figure 4). There was a range from less than 1g to upwards of 45g in overall biomass. From the plants in which total biomass was calculated, there was a significant effect of soil Zn level on total biomass (two-way ANOVA, $p<0.0001$).When separated by cultivar, however, this simple visual tend of decreasing biomass with increasing soil zinc is no longer present in three out of the four cultivars and there is a significant interaction between cultivar and soil zinc level with a p value of 0.0376 using a two-way ANOVA (Figure 5). Therefore, cultivars behaved differently with respect to the response of their biomass to soil Zn level. With respect to the allocation of biomass among shoot parts, the data suggests that there was a significant effect of soil Zn level on the fraction of total plant biomass allocated to the leaves (two-way ANOVA, $p = 0.0046$). Therefore, the level of zinc in the soil had a

significant effect on relative investment in leaves. There was no significant effect of soil Zn level on the mass fractions of the stems or heads.

Height over Time

Visually, the data showed a trend of increasing height over time (Figure 6). A repeated measures ANOVA was conducted using a mixed model framework, and it was determined that there was an interaction between week and soil Zn level for height over time that was significant within each cultivar (Hornet: $p<0.0001$, Jaguar DMR: $p< 0.0012$, LD5009: $p<0.0001$, N4HM354: $p<0.0004$). Therefore, height over time within each cultivar varied with zinc levels in the soil.

Chlorophyll Content

Figure 7 shows the chlorophyll content over time separated by cultivar. A repeated measures ANOVA was conducted using a mixed model framework, and shows a significant effect of level on chlorophyll content in all cultivars except Jaguar DMR (Hornet: $p<0.0039$, Jaguar DMR: $p< 0.0648$, LD5009: $p<0.0001$, N4HM354: $p<0.0003$). Week had a significant effect on all cultivars except Hornet (Hornet: $p<0.1048$, Jaguar DMR: $p< 0.0001$, LD5009: $p<0.0043$, N4HM354: $p<0.0002$).

Caterpillar Survival

From a qualitative perspective, the increased levels of zinc in the diet of *V. cardui* lead to high rates of mortality in all levels above 100 ppm (Table 1). Reduction in size as well as pupa deformation was common in these levels. In the first trial, all pupa formed in and above the 100 ppm level displayed a deformation where the head failed to detach during formation and thus remained on the chrysalis. In the second trial, this deformation was sporadic amongst levels but

common in the levels above 100ppm. In the second trial, the pupa that were formed in the higher levels failed to eclose and displayed deformation and discoloration such as the head remaining attached as well as turning black before caving in on itself resulting in mortality before eclosing.

DISCUSSION

The first experimental question asked whether zinc hyperaccumulation is observed in cultivated sunflower. With the significant effect of soil zinc concentration on the level of zinc found in the leaves it can be concluded that zinc uptake and accumulation occurred. With respect to current scientific literature opinions, hyperaccumulation is considered to have been reached at either the level of 1000 ppm or 10,000 ppm. The levels reached in this study fall below this limit and by these standards the observed phenomenon cannot be considered hyperaccumulation. However, as hyperaccumulation can also be considered a continuous trait (Goolsby and Mason, 2015), how can such a limit to the definition of hyperaccumulation exist? Through this contradiction of classifications, it can be concluded that the plants accumulated zinc at significant levels in a dose-dependent manner but whether the observed phenomenon is considered hyperaccumulation cannot be concluded.

In determining if hyperaccumulation of zinc may be a possible mode of disease or herbivore resistance it is vital to observe effects of toxicity presented during the developmental process. The second experimental question asked how plant growth (proxy for fitness) may be altered by variation in soil zinc concentration and plant zinc uptake. Unlike the first, the direct answer to this question is simple. The soil zinc concentration level had a significant negative effect on both the total biomass of the plant and growth rate over time. However, when looking at this data as a proxy for fitness, the answer becomes unclear. A plant may exhibit many signs when faced with toxicity. The data itself does not present any definitive evidence that toxic levels were reached. The decreased biomass and growth rate show a negative relationship with increasing zinc uptake, but this does not necessarily directly indicate that a toxic level was reached. Similarly, the results from the chlorophyll content does not indicate direct toxicity. There was slight variation in chlorophyll content as soil concentration level increased but due to

the interaction terms and the visual graph, the variation cannot be correlated directly with the changing soil level. Instead, these negative relationships may have been due to physiological tradeoffs in the plant such as changing nutrient allocation patterns based on the increased presence of zinc. There is much that is not understood when looking at the physiological effects of hyperaccumulation. Thus, a definitive answer as to whether toxicity occurred and to what degree based off the data alone, cannot be concluded. In opposition to this statement, based off visual observations that occurred during the developmental process of the sunflowers, there is evidence of toxicity. Chlorosis of the leaves and mortality in the higher levels was observed and suggests that some degree of toxicity was experienced (Reichman, 2002). The effects of the chlorosis were not picked up by the chlorophyll data because the chlorophyll measurements were taken on the upper most fully expanded leaf pair which were usually the healthiest while the leaves with chlorosis tended to be much lower on the plant. As a limitation to the design of this study, mortality rates and visual toxicity cues were not collected nor analyzed leaving a large component to the determining of toxicity out of the equation. It is crucial to include these measurements in future studies in order to better document toxicity.

As for the last experimental question that was asked, what does the data suggest about the effect of plant zinc on a model herbivore? Zinc had a devastating effect on the overall developmental process of *V. cardui* which caused high rates of mortality and common deformations in all levels above 100 ppm. Compared with the level of zinc accumulated in the leaves, 100ppm was accumulated in all plants in the soil zinc level 50 mg/kg and above. The plants in the 50 and 100 mg/kg soil zinc levels did not show a significant reduction in biomass indicating that the elemental defense hypothesis could be possible with little to no toxicity in this range. As stated previously, there are many other forms of toxicity that may have been present

that was not observed in these levels. The other forms of toxicity need to be studied more in-depth in these levels in order to conclusively state whether this is the range for herbivore resistance with little to no trade-offs.

This study used a compound of zinc dosed directly into the diet. Even though this is not directly answering the question at hand, using a compound of zinc in a formulated diet mixture that is designed specifically for *V. cardui* may actually provide more insight into the direct effects of zinc as opposed to using leaves that have accumulated zinc. This is because when using a formulated diet, zinc is the only factor that changes between samples. When working with an organism such as a plant, there are an incredible amount of biological and biochemical processes that make controlling for only one compound extremely difficult, if not impossible. Leaves may vary in the nutrients available, secondary metabolites present, or in the form that zinc has been converted into while being processed up through the roots and into the aerial organs.

This study touches on many key points but there are a few that need to be investigated further. While using a compound of zinc in a formulated diet may allow greater control of variables, using a leaf that has accumulated zinc needs to be the next step in determining if accumulation of zinc can provide herbivore or disease resistance. Using leaf tissue will shed light on potential effects compounded with the uptake of zinc, such as changes in secondary metabolite production, nutrient content, or general appeal to the caterpillars. Furthermore, expanding the range of observational parameters when looking at toxicity will give a better idea of if there are any tradeoffs that occur with the accumulation of zinc.

CONCLUSION

Based on the results of this study, can we can breed a type of crop sunflower that has an ability to resist antagonistic effects such as herbivores or pathogens without the use external measures such as pesticides? The data presented in this study suggests that within the range of 50 to 100 mg/kg of soil zinc level concentration there is the opportunity for herbivore resistance against *V. cardui,* but further investigation is needed and points us in the direction of focusing on the tradeoffs between toxicity and resistance. The possibility of using the mechanisms presented by the elemental defense hypothesis needs to be backed up by looking at these tradeoffs at any particular level of accumulation. If toxicity is occurring in the levels in which zinc accumulation is conferring herbivore or disease resistance, then the elemental defense hypothesis does not hold true in these plants meaning that this mode is no longer a viable option for herbivore or disease resistance in cultivated crop sunflowers. There is a way to combat this loss of fitness, however - zinc tolerance could be bred into zinc-sensitive sunflowers to allow zinc accumulation to confer pest resistance. However, if no toxicity tradeoffs are present in the levels in which resistance is conferred, or these tradeoffs can be overcome, then elemental defense may in fact be a possible mechanism for pest resistance.

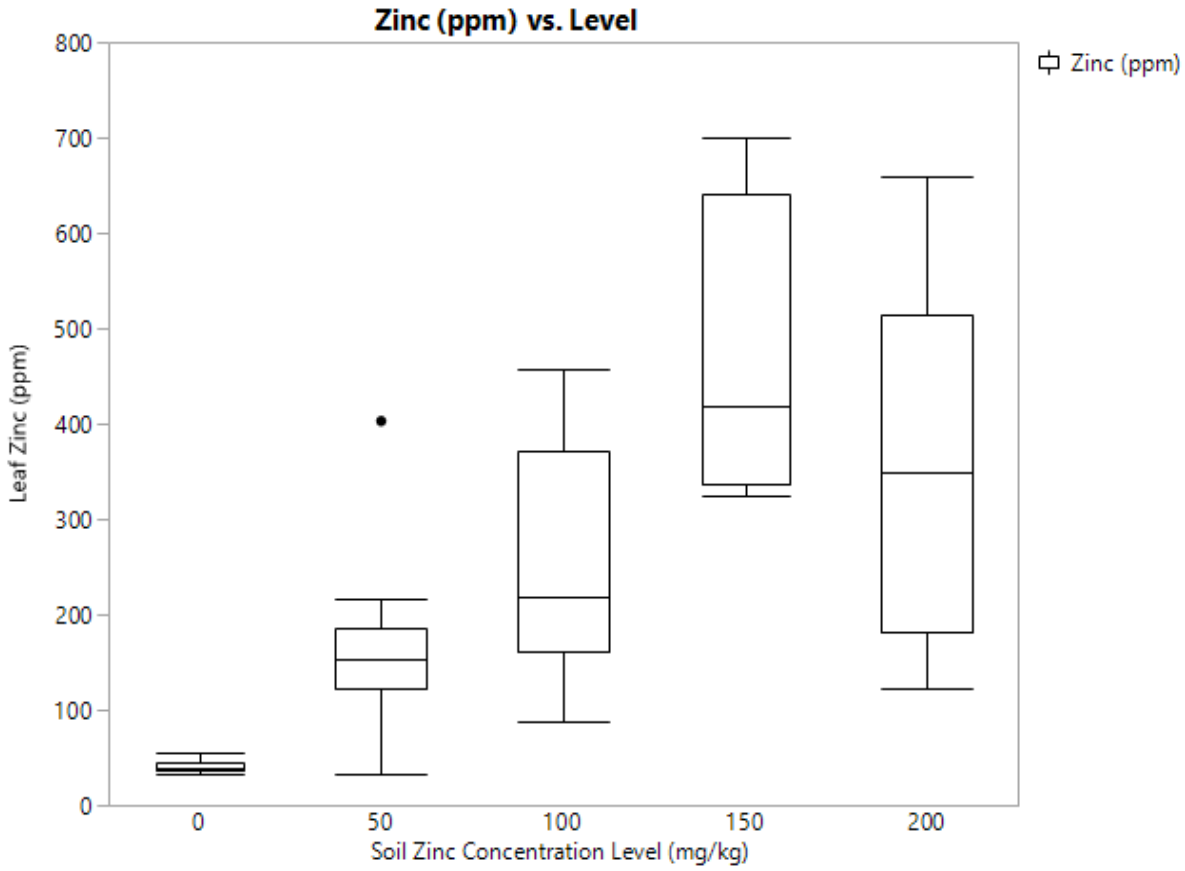
APPENDIX

Figure 1

A layout graph of the three greenhouse benches. Each box represents an individual plant and contains its unique identifier which was assigned by its coordinate position in the form of a letter followed by a number. The cultivar type, zinc soil level, and replicate number sit below the identifier in the form of [abbreviated cultivar name] [zinc soil level]-[replicate number]. The colors correspond to the zinc soil level: red = 200 mg/kg, orange = 150 mg/kg, light blue = 100 mg/kg, green = 50 mg/kg, tan = 0 mg/kg (control).

Figure 2

Variation observed in leaf zinc concentration across the five soil zinc treatments. An ANOVA table shown below shows the significant p-value for soil zinc concentration level (mg/kg) on leaf zinc (ppm). A Tukey range test was preformed showing which levels are different from one another and is shown in the connecting letters report below.



Analysis of Variance

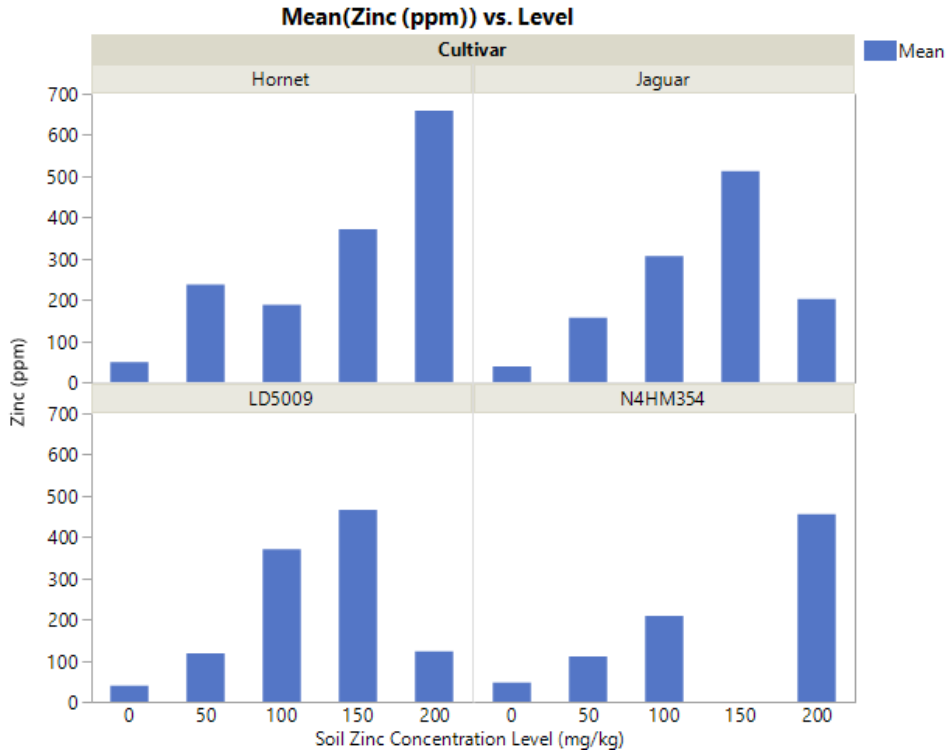
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Level	4	8642864	216072	16.7510	<.0001*
Error	45	580454.9	12899		
C. Total	49	14447414			

Connecting Letters Report

Level				Mean
150	A			465.27775
200	A	B		364.59929
100		B	C	263.70064
50			C D	161.01287
0			D	41.36715

Figure 3

Variation observed in leaf zinc concentration across the five soil zinc treatments separated by cultivar. A two-way ANOVA was performed where a significant p-value of 0.0065 was observed as an interaction term meaning that the cultivars take up different leaf zinc levels. Therefore, a repeated measures ANOVA was performed and the fixed effect of level is shown below and is grouped by cultivar type.



Hornet

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Level	4	4	270871.97	8.3399	0.0195*

Level		Least Sq Mean
200	A	658.80300
150	A B	371.52500
50	B	237.24050
100	B	187.45500
0	B	49.08250

Jaguar

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Level	4	4	417485.69	8.8960	0.0007*

Level			Least Sq Mean
150	A		511.98200
100	A	B	306.10160
200	A	B C	201.81050
50		B C	156.70320
0		C	38.13767

LD5009

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Level	4	4	188506.14	39.5294	0.0018*

Level		Least Sq Mean
150	A	465.62200
100	A	370.75500
200	B	122.70800
50	B	117.45867
0	B	39.06200

N4HM354

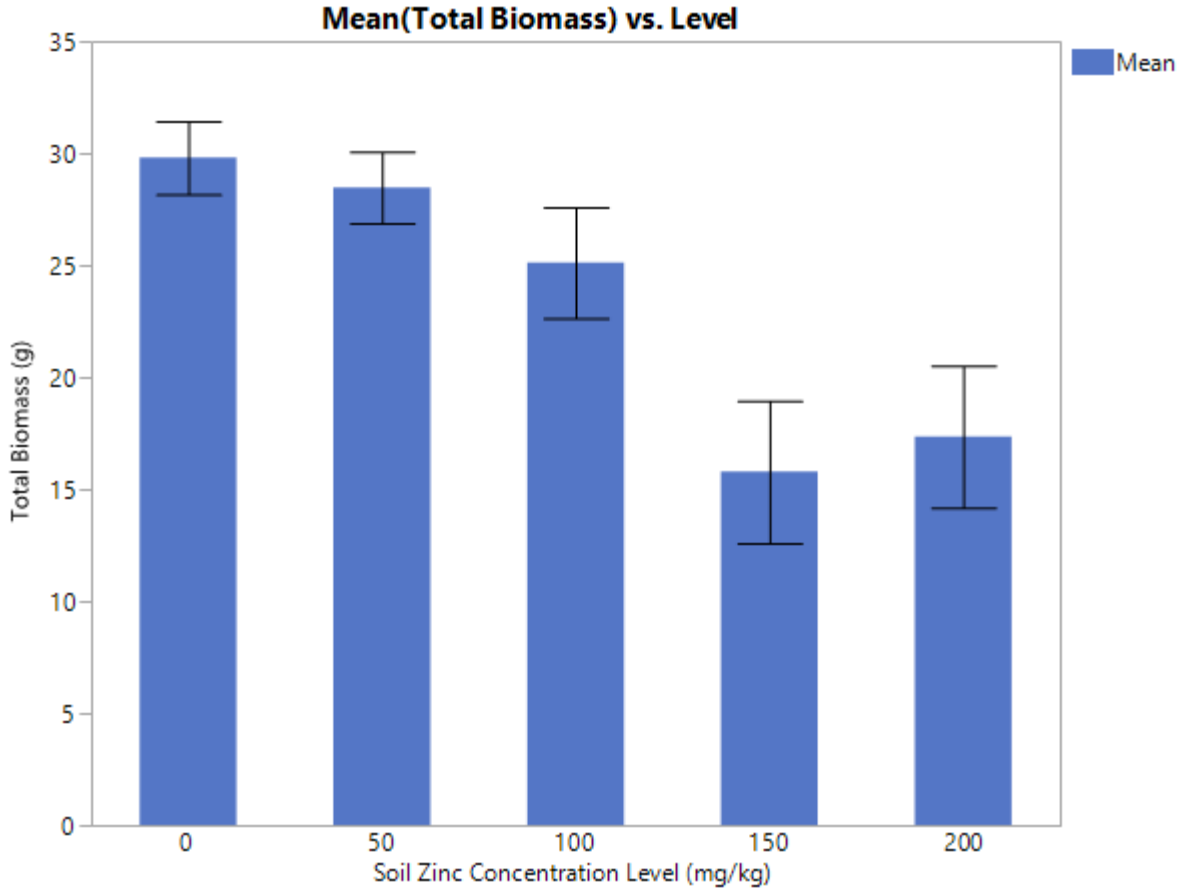
Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Level	3	3	263286.82	13.7715	0.0025*

Level		Least Sq Mean
200	A	455.68767
100	B	208.17800
50	B	110.11300
0	B	46.79800

Figure 4

Variation observed in the mean total biomass of all cultivars across the five soil zinc treatment levels. An ANOVA table shown below shows the significant p-value for soil zinc concentration level (mg/kg) on mean total biomass (g). A Tukey post-hoc test was preformed showing which levels are different from one another and is shown in the connecting letters report below.



Analysis of Variance

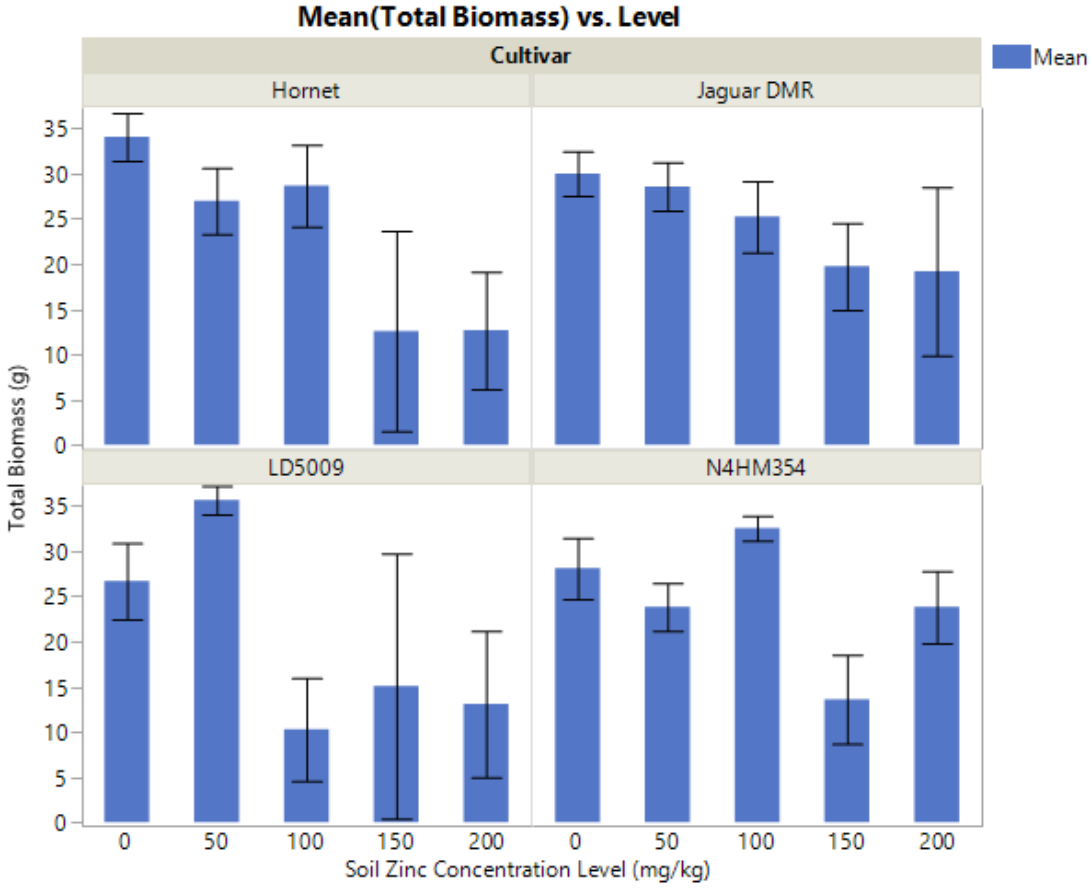
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Level	4	3071.502	767.875	6.8572	<.0001*
Error	97	10862.107	111.980		
C. Total	101	13933.609			

Connecting Letters Report

Level			Mean
0	A		29.804783
50	A		28.479130
100	A	B	25.119167
200		B	C 17.352667
150			C 15.782941

Figure 5

Variation observed in the total biomass separated by cultivar across the five soil zinc treatment levels. A two-way ANOVA was run and the results are shown in the Effects Test box below that shows the significant p-value for soil zinc concentration level (mg/kg) on total biomass (g) as well as the interaction term. A Tukey post-hoc test was preformed showing which levels are different from one another and is shown in the connecting letters report below.



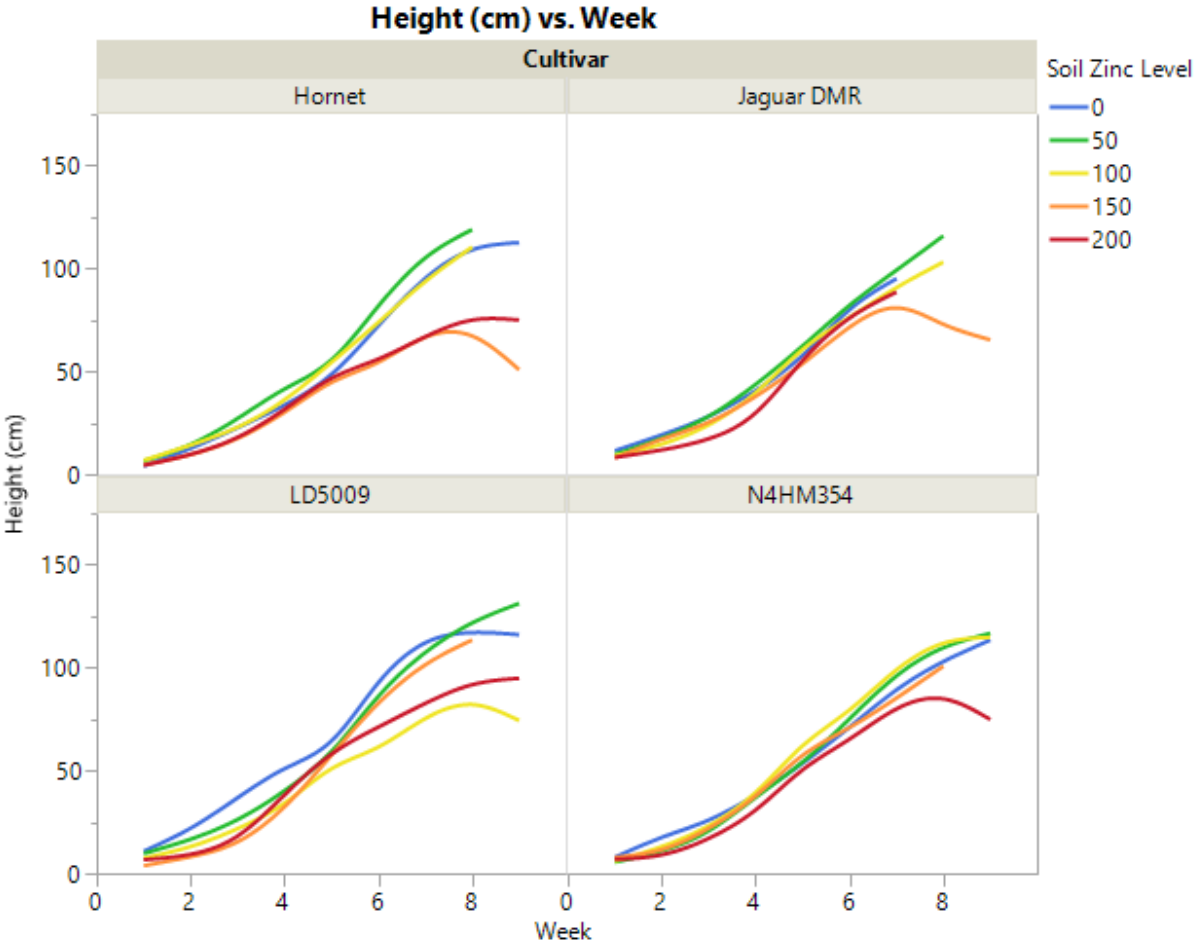
Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Level	4	4	2904.3720	7.2801	<.0001*
Cultivar	3	3	246.2851	0.8231	0.4848
Cultivar*Level	12	12	2357.4650	1.9697	0.0376*

Level		Least Sq Mean
0	A	29.734083
50	A	28.778583
100	A B	24.190786
200	B	17.204250
150	B	15.270417

Figure 6

Variation observed in the growth rate across time grouped by cultivar using a smoothed curve line graph. Each individual line on the graph represents a different soil zinc concentration level indicated by a specific color. A repeated measures ANOVA was performed and the fixed effects of level and week are shown below and are grouped by cultivar type.



Hornet

Fixed Effects Tests

Source	Nparm	DFNum	DFDen	F Ratio	Prob > F
Level	4	4	25.8	3.5388648	0.0197*
Week	1	1	196.7	1260.0803	<.0001*
Level*Week	4	4	196.4	17.000087	<.0001*

Jaguar DMR

Fixed Effects Tests

Source	Nparm	DFNum	DFDen	F Ratio	Prob > F
Level	4	4	26.7	1.1945999	0.3360
Week	1	1	169.2	1314.7326	<.0001*
Level*Week	4	4	168.2	4.7508731	0.0012*

LD50009

Fixed Effects Tests

Source	Nparm	DFNum	DFDen	F Ratio	Prob > F
Level	4	4	28.8	3.972584	0.0109*
Week	1	1	173.0	974.54238	<.0001*
Level*Week	4	4	171.8	7.6093447	<.0001*

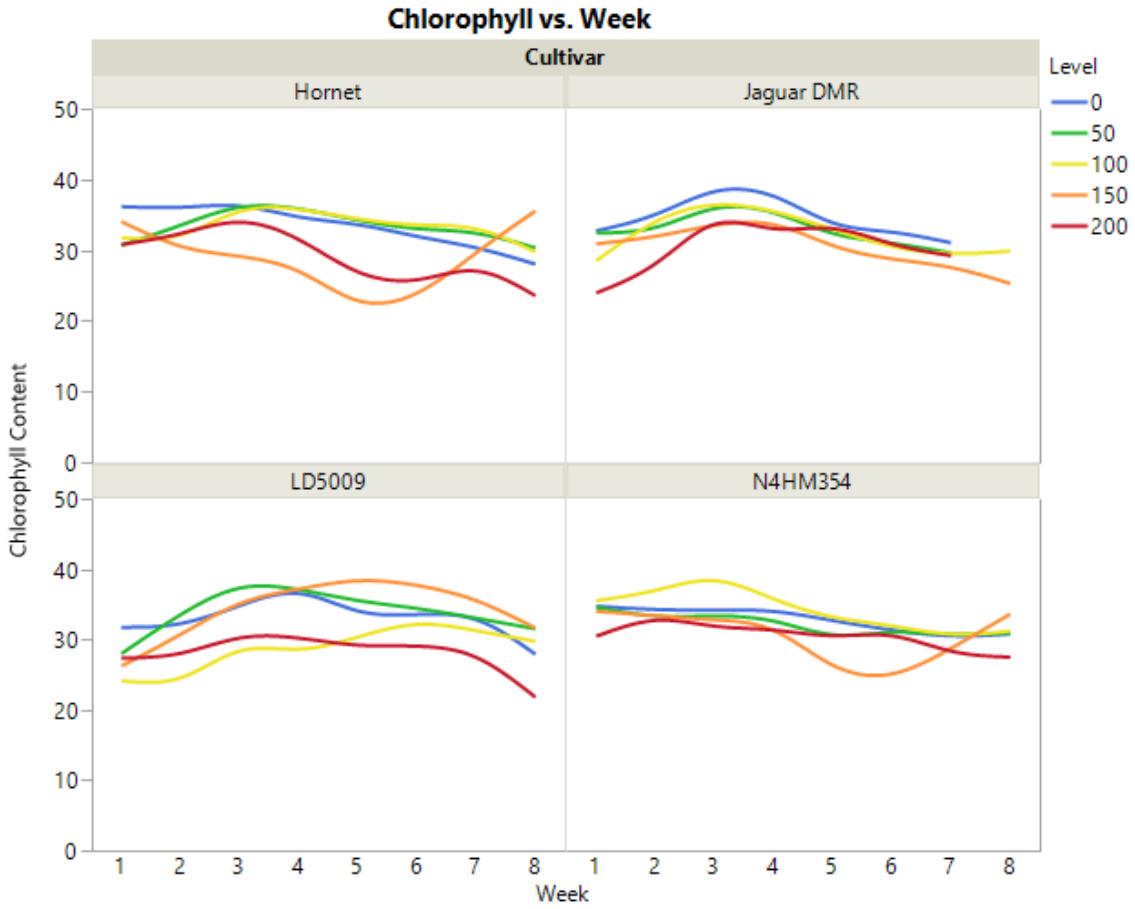
N4HM354

Fixed Effects Tests

Source	Nparm	DFNum	DFDen	F Ratio	Prob > F
Level	4	4	24.9	1.7549024	0.1697
Week	1	1	206.5	1746.825	<.0001*
Level*Week	4	4	206.5	5.3977007	0.0004*

Figure 7

Variation observed in the chlorophyll content across time grouped by cultivar using a smoothed curve line graph. Each individual line on the graph represents a different soil zinc concentration level indicated by a specific color. A repeated measures ANOVA was performed and the fixed effects of level and week are shown below and are grouped by cultivar type.



Hornet

Fixed Effects Tests

Source	Nparm	DFNum	DFDen	F Ratio	Prob > F
Week	6	6	154.0	1.7889318	0.1048
Level	4	4	154.0	4.0344304	0.0039*
Week*Level	24	24	154.0	0.7101629	0.8358

Jaguar DMR

Fixed Effects Tests

Source	Nparm	DFNum	DFDen	F Ratio	Prob > F
Week	6	6	151.0	5.7783385	<.0001*
Level	4	4	151.0	2.2653649	0.0648
Week*Level	24	24	151.0	1.1557602	0.2921

LD5009

Fixed Effects Tests

Source	Nparm	DFNum	DFDen	F Ratio	Prob > F
Week	6	6	110.0	3.37621	0.0043*
Level	4	4	110.0	6.4929085	<.0001*
Week*Level	24	24	110.0	0.5133492	0.9691

N4HM354

Fixed Effects Tests

Source	Nparm	DFNum	DFDen	F Ratio	Prob > F
Week	6	6	164.0	4.7363105	0.0002*
Level	4	4	164.0	5.5061865	0.0003*
Week*Level	24	24	164.0	0.6061031	0.9250

Table 1

Mortalities observed during the development of *Vanessa cardui* across both trials. Mortalities were counted in the larva and pupa stage. The fourth column shows how many eclosed into adulthood. Total population size indicates how many individuals were hatched.

Trial 1

Level (ppm)	Died in Larva	Died in Pupa	Eclosed	Total Population Size
0	2	4	38	44
100	5	0	10	15
250	18	0	0	18
500	19	0	0	19
1000	21	0	0	21
1500	17	0	0	17
2000	19	0	0	19

Trial 2

Level (ppm)	Died in Larva	Died in Pupa	Eclosed	Total Population Size
0	1	3	36	40
100	0	5	5	10
250	3	5	2	10
500	10	0	0	10
1000	10	0	0	10
1500	8	2	0	10
2000	8	2	0	10

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