Calcium availability and the interaction of *Arabidopsis thaliana* and *Myzus persicae*

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Abstract

The reciprocity inherent to plant-animal interactions allows ecological systems to be influenced by a wide variety of environmental factors. Nutrient availability in soil is an important and extremely sensitive variable in the determination of successful plant performance. In particular, calcium is a macromineral that plays a role in both plant growth and facultative defence. Increased cytosolic calcium concentrations are correlated with an increase in callose deposition, a defence mechanism against piercing-sucking herbivores. In this experiment, we moderated the amount of calcium available to *Arabidopsis thaliana* samples, and assessed both direct effects on plant performance and indirect effects on the aphid (*Myzus persicae*) populations that were placed on the plants. Half of the plants in this study were inoculated with viviparous aphids, and all plants were treated with varying amounts of dolomitic limestone throughout the experimental timeline in a factorial design. Plant performance and aphid fecundity was evaluated through measures of aphid count, new leaf growth, old leaf growth, and plant height. The interactive effect of calcium treatment and time on the total number of aphids and the interactive effect of day and block on new leaf growth were analyzed. Calcium treatment negatively affected aphid population growth overall. Plants inoculated with aphids performed more poorly over time than plants that were not inoculated. This experiment indicates a negative relationship between aphid population growth and soil calcium levels, suggesting that calcium supplementation may enhance *A. thaliana* defence mechanisms. This overall negative trend could be explained by resource availability theory. Future studies could further explore ways in which human manipulation of the environment can have effects that propagate across many trophic levels.

Received: 02/28/2016 **Accepted:** 04/08/2016 **Published:** 04/08/2016
URL: https://journals.mcmaster.ca/iScientist/article/view/1151/1003

Keywords: Calcium, *Arabidopsis thaliana*, *Myzus persicae*, callose deposition, resource availability theory, micronutrient availability

Introduction

The reciprocity inherent to plant-animal interactions allows ecological systems to be influenced by a variety of external factors. The study of these factors can give insight into the mechanisms employed by both plants and animals in response to daily external stresses. In order to study the interactions of organisms with the biotic and abiotic components of their niche space, we investigate a simple model involving two trophic levels: a plant producer and an animal herbivore. The plant, *Arabidopsis thaliana*, is a popular model organism due to its rapid growth and simple, sequenced genome (National Institutes of Health, 2000). The herbivore used in this study is the green peach aphid, *Myzus persicae*, which exhibits phenotypic plasticity in response to changing environmental conditions (Peppe and Lomonaco, 2003). The interdependence between these two organisms is well studied (Louis and Shah, 2013). As a result, the effects of experimental manipulation will be apparent.

Nutrient availability in the soil is an important and extremely sensitive variable in the determination of successful plant performance. Micronutrients, such

as calcium, play very specific roles in plant physiological systems. Calcium is a macromineral required by all plants for the promotion of growth and structural vigour (Hepler, 2005). It is an important component of the middle lamella, which is the cellular glue that allows for expansive cell growth and nutrient exchange between adjacent cells (Kirkby, 1984). *A. thaliana* utilizes calcium cations as both a signalling element and a cellular constituent in one of its inducible defensive pathways. This facultative defence is triggered in response to certain exogenous stimuli, such as tissue damage from herbivory (Blume, 2000). The response, known as callose deposition, creates a physical barrier made of an amorphous, dense (1→3)-ß-D-glucose polymer at plasmodesmata (Luna, et al., 2011). Since aphids are sucking insects, they must pierce the epidermis to extract phloem sap, which is made more difficult by these thickened cell walls (Louis and Shah, 2013). Callose also provides a medium for the accumulation of antimicrobial compounds around sites of herbivorous attack (Luna, et al., 2011; Bieri, 2009).
While the mechanism is not completely understood, it has been experimentally demonstrated that an increase in cytoplasmic calcium is positively correlated with increased rates of callose deposition (Bhuja, 2004; Sivaguru, 2000). This study of the broader effects of micronutrient availability is extremely relevant, as it gives insight into the influence of chemical pollution by humans on surrounding ecological systems.

In this experiment, we moderated the amount of calcium made available to different samples of *A. thaliana* and assessed both the direct effect on plant performance and the indirect effect on aphid fecundity. We attempted to answer the following questions:

1. Is *A. thaliana* plant performance affected by calcium availability?
2. Is *M. persicae* population growth affected by calcium availability to *A. thaliana*?

We hypothesized that an increase in calcium availability would allow plants to increase rates of callose deposition. Thus, plants would be better-defended, and aphid populations would be negatively affected through this interference with their penetrative feeding technique. The execution of this experiment through a simple, two-organism model allows for the results to be generalized and applied to larger and less-accessible communities.

Materials and Methods

Experimental Design

This experiment involved two blocks of 12 Colombian wild type *A. thaliana* samples in the rosette life-stage over a period of 11 days. In order to make a baseline calcium-deficient series of plants, all plant samples were treated with the fertilizer described in Table 1. This solution substitutes calcium nitrate with sodium nitrate, and this increase in sodium concentration reduces the ability of plants to uptake available calcium in the soil. Sub-irrigation took place in individual plastic dishes to apply 100 mL of fertilizer to each plant for exactly 3 hours. This was to ensure maximum fertilizer sequestration by the far-reaching roots and to avoid the physically disruptive process of super-irrigation (Cox, 2001).

Subsequently, plants were randomly assigned levels within the categorical designation of calcium availability and placed in two blocks (Figure 1). These levels – no calcium, low calcium, medium calcium, and high calcium - refer to the amount of calcium added to the plants during treatment.

After fertilization and placement of plants into blocks, the first twelve samples were each inoculated with 5 viviparous, green peach aphids. These aphids were reared on *Nicotiana attenuata*, which resulted in their red phenotype. They were placed at the basal rosette of the plants, which is the site of new plant

Table 1. Ratios of compounds added to create the calcium-deficient fertilizer administered to all *Arabidopsis thaliana* samples prior to experiment.

Chemical	Amount Added
KNO_3	11 mL of 1 M solution
$MgSO_4$	4.6 mL of 1 M solution
KH_2PO_4	2.2 mL of 1 M solution
Fe-EDTA	2.2 mL of Fe-EDTA solution (1 mL stock = 5 mg Fe)
Micronutrients	2.86 g of H_3BO_3, 1.81 g of $MnCl_2 \cdot 4\ H_2O$, 0.11 g of $ZnCl_2$, 0.05 g of $CuCl_2 \cdot 2H_2O$, and 0.025g of $Na_2MoO_4 \cdot 2H_2O$ in 1 L of solution
$NaNO_3$	11 mL of 1 M solution

growth, where varying concentrations of cytoplasmic calcium would be most apparent.

Upon establishment of a baseline calcium deficiency, solutions of varying calcium concentration were applied to plants. At three points during the experiment (Day 0, Day 4, and Day 8), plants were treated with a mixture of dolomitic limestone ($CaMg(CO_3)_2$) and water. The contents of these mixtures were 0.0 mg/mL, 18.2 mg/mL, 36.3 mg/mL, and 54.5 mg/mL for no calcium, low calcium, medium calcium, and high calcium treatments, respectively. Although dolomitic limestone is not very soluble in water, the mixtures were mechanically stirred during treatment to ensure uniform distribution. Calcium treatments were administered to plants using a Pi Pump Pipet Filler with a 10 mL syringe attachment. Each plant received 35 mL of their respective treatment level on each of the three days, applied evenly to the top of the soil. Care was taken to avoid exposed portions of the plant body.

Aphid and plant data were collected 6 times over the 11-day experimental timeline. Aphid fecundity was estimated through a count of aphids present on each plant and delineation of these counts into stage of life (nymph vs. viviparous) and phenotype (red vs. green). In addition to these measurements, observations were made regarding the position of aphids on plants, specifically whether aphids seemed to prefer feeding on upper leaves or at the basal rosette where they were placed.

Plant growth was also monitored through the measurement of freestanding plant height. In addition, on the first day of the experiment, all of the plant leaves sprouting from the basal rosette less than 1.0 cm in length were marked with a black dot of Sharpie® pen ink to indicate old leaf growth. Base-to-leaf tip length of these rosette limbs was measured throughout the experiment to observe the effect of calcium availability on the growth rate of pre-existing leaves. Additionally, on Day 4 of the experiment, leaves within the basal rosette less than 1.0 cm in length were marked with a red dot of Sharpie® pen ink, and their lengths were subsequently recorded during data collection as a measure of new leaf growth. Finally, qualitative observations regarding general plant performance, including chlorosis of leaves, mechanical damage, and progression into the bolting stage of life, were noted individually across each day.

Statistical Analysis

A two-way analysis of variance (ANOVA) was used to analyze the interactive effect of calcium treatment and time on the total number of aphids and the interactive effect of day and block on new leaf growth. Dependent variables were log-transformed prior to analysis to satisfy the assumptions of the

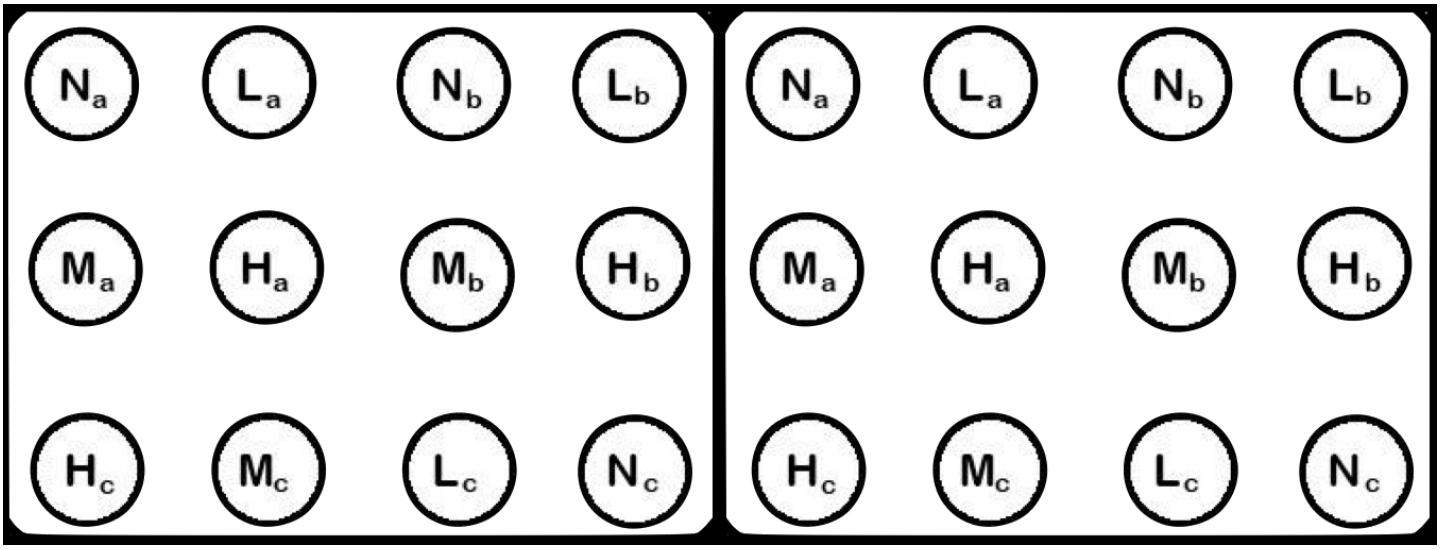

Figure 1: Spatial diagram of experimental setup. The *Arabidopsis thaliana* plants were separated into two blocks - one involved inoculation of each plant with five aphids (left), and the other had no aphids added (right). Within each block were four distinct levels of calcium treatment - None, Low, Medium and High. The placement of treated plants was randomly generated and duplicated for each block. Subscripts were used to differentiate between individual plants for purposes of data collection.

model. A post hoc Tukey Honestly Significant Difference (HSD) test was used to assess significance of pair-wise interactions of the independent variables.

All independent variables were cross-referenced with dependent variables in order to determine the effect of external and temporal modifications on plant performance and aphid fecundity. All statistical analyses were performed using R 3.0.3 (Urbanek, Bibiko and Iacus, 2012). Microsoft Excel was used to produce graphical representations of this data (Microsoft Corporation, 2011).

Results

Calcium Effect on Aphid Population Growth

The interaction between day and treatment level was analyzed using a two-way ANOVA. Calcium availability had a measurable effect on aphid population over time.

There was no interaction between day and treatment level on total aphid growth ($F_{3,52}$ = 1.9357, p = 0.14). No relationship was found between phenotype or stage of life and treatment. The relationship between aphid total and treatment alone was significantly different ($F_{3,52}$ = 3.2144, p = 0.030).

Application of a Tukey HSD to this ANOVA showed that the only significant simple effect was between the no calcium and low calcium treatment levels (df = 52; p_{adj} = 0.037). This relationship is seen for each of the four treatments (Figure 2). Aphid populations grew at the greatest rate on plants not supplemented with any calcium; however, increasing amounts of calcium did not have a sequential effect on aphid population. In general, calcium treatment was shown to negatively affect aphid population growth. Aphid population growth was lowest on plants that received the low calcium treatment. Plants that received medium and high levels of calcium had similar rates of aphid population growth, but both were less than growth rates on plants that received no calcium.

Calcium Effect on Plant Performance

Plants inoculated with aphids performed more poorly over time than non-inoculated plants (Figure 3). New leaf growth was significantly affected by the interaction of aphid presence and time ($F_{1,116}$ = 5.7639, p = 0.018). The correlation remained significant at each calcium level (dF = 116, p_{adj} = 0.031). The same model using old leaf growth as the dependent variable did not show significance. Height was not found to be significantly correlated with calcium treatment level, block designation, or even experimental day.

Discussion

This experiment indicates a relationship between aphid population growth and soil calcium level. Addition of calcium to soil in the form of dolomitic limestone negatively impacted the mean per capita population growth rate of aphids. However, this relationship was not linear and graduated, as expected. Therefore, although calcium levels were shown to negatively impact aphid performance, aphids on plants that received higher levels of calcium treatment were not negatively affected to a greater degree than those on plants that received lower calcium levels.

This overall negative trend can be explained by resource availability theory (Howe and Westley, 1988). Plants with no additional calcium treatment were unable to amplify defence mechanisms without the reduction of calcium available for general plant growth and repair. Plants treated with higher levels were no longer limited by calcium availability, and were thus able to employ defence mechanisms at a minimized cost to plant performance. The sensitivity of *A. thaliana* to calcium treatment level suggests of *A. thaliana* to calcium treatment suggests that increased availability of calcium for callose deposition is beneficial to the ability of resistant r-selected plants to defend against herbivory.

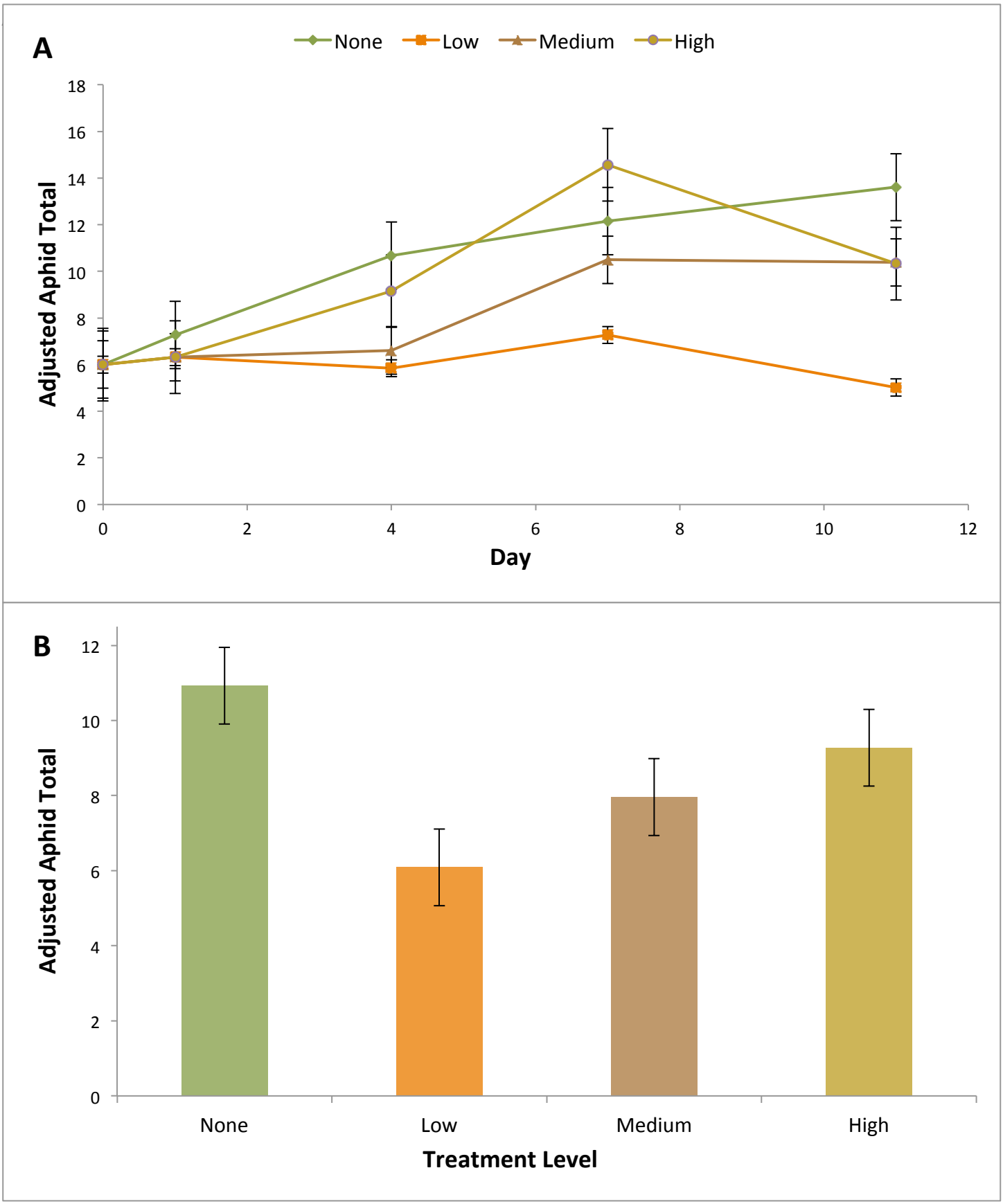

Figure 2: The effect of calcium treatment level on the total number of aphids through time (a) and in general (b). The adjusted aphid total was created by taking the base-10 logarithm of the total number of aphids on each plant across the experiment, finding the means of these values, and then back transforming the data to recreate the linear relationship. Standard error: None = 1.4411, Low = 0.3651, Medium = 1.0167, High = 1.5596. P-value: interaction term (treatment level and time) = 0.14; treatment level = 0.030.

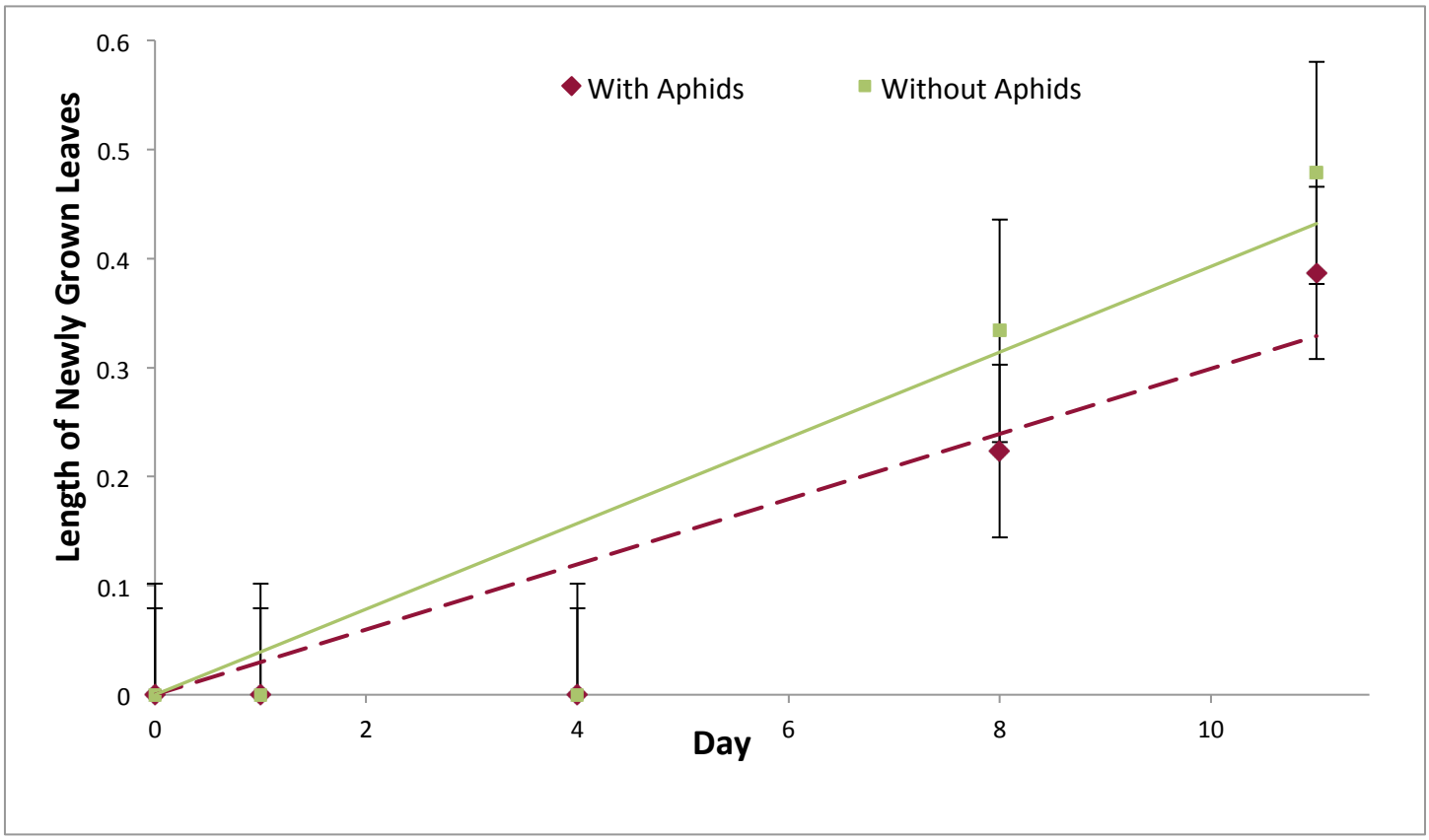

Figure 3: The effect of presence of aphids on new leaf growth through time. Values for new leaf growth transformed using a base-10 logarithm. Standard error: With aphids = 0.2862, Without aphids = 0.4107. P-value: presence of aphids = 0.018.

Additionally, Figure 3 shows that plants incoluated with aphids did not grow to the same extent as those that were free from herbivory. Length of dotted red leaves was measured as a proxy for plant performance, as it represented the ability for the plant to grow after calcium treatment had begun. This observation can also be explained by resource availability theory (Howe and Westley, 1988). Plants that needed to defend themselves against herbivory were unable to dedicate as many resources to new plant growth. This shows that plants have the ability to actively partition resource use in response to external stimuli.

Finally, it is worthwhile to comment upon trends observed during data collection. During the counting of aphid populations, we observed a qualitative difference in main location of aphid feeding on plants between different treatment levels; on the majority of the plants not treated with calcium, aphids congregated in the centre of the basal rosette at the site of newest leaf growth. This was especially true for aphid nymphs. This aphid placement is likely selective, as this area of the plant is most sheltered and contains high amounts of sugar to promote cellular growth (Wiese, et al., 2007). In contrast, aphids tended to move further away from the basal rosette on plants that received increasing levels of calcium. Both viviparous adult and nymph aphids were found much more often on the underside of taller and more mature rosette leaves for plant systems treated with high levels of calcium. This suggests that plants treated with higher levels of calcium sequester more of this micronutrient within the plant. Because calcium is required for new leaf growth, it will be most highly concentrated at the rosette of the plant, and we can infer that callose deposition is greatest in these areas. Since this defence makes it more difficult for an aphid to pierce the plant epidermis with its stylet, the aphids migrated away to the edges of mature leaves. Since these leaves are the most slow-growing portions of the plant, less calcium is likely to be transported to this area. This would thus decrease the plant's ability to build up its structural defence of callose (Coley, Bryant and Chapin, 1985).

A prolonged study would be very helpful in determination of the effects of calcium treatment on aphid population. Aphid populations increase on plants with both medium and high calcium levels until day 7, at which point the populations decline (Figure 1). This decline is greatest for the high calcium treatment. As hypothesized, overall aphid population growth rates on plants treated with higher calcium levels were lower than those for aphids on untreated plants. However, this critical decrease in growth rate was a delayed result, rather than a lessened growth rate of calcium-treated plants throughout the entire experiment. This may give insight into the conditions required for a plant to increase callose deposition. One proposed mechanism is that cytosolic calcium concentration must exceed a certain threshold before the plant begins to increase callose deposition (Will, et al., 2007). Another possible explanation could be that plants increase callose deposition as soon as more calcium is available, but the effect is only seen on aphid populations after plant defence has significantly decreased aphid fecundity. A longer study with more plant samples could give further insight into the legitimacy of these trends or any larger trends that we failed to observe.

Callose imaging techniques could be implemented in a prolonged study in order to substantiate our observations. Throughout this extended experiment, leaves from *A. thaliana* plants within each treatment level would be periodically removed for testing. These leaves would then be stained with aniline blue and imaged immediately under fluorescent and UV light (Yim and Bradford, 1998). Software packages, such as CalloseMeasurer, could be used to quantitatively measure the callose within these images and the extent of its spread throughout the plant (Zhou and Robatzek, 2012). This would determine whether callose deposition throughout the plant had a definite correlation with a decline in aphid fecundity and aphid preference for feeding location.

Callose deposition, as a physical barrier and repository for antimicrobial defences, occurs not only in *A. thaliana*, but also in many other plant species (Luna, et al., 2011). Varying environmental calcium levels, especially as a result of pollution, could affect plant defensive ability. Limestone erosion, construction, industrial activity, and wastewater treatment can release calcium into the natural environment (Health Canada, 1997). This increased soil calcium may negatively impact insects with similar feeding techniques to aphids. Conversely, low-lying ecosystems may have lower soil calcium levels as a result of water filtration through downhill subsurface flow. This could limit the ability of some plants to properly defend themselves, and thus increase the risk of a level of herbivory that may be fatal to the plant population.

Conclusion

Manipulation of calcium levels and aphid presence on *A. thaliana* plants had a measurable effect on plant performance and success of resident aphid populations. Aphid population growth rate on plants treated with calcium was low in comparison to aphids on untreated plants. However, the correlation was not sequential; aphid population growth did not decline linearly with increasing calcium dosage. *A. thaliana* uses callose deposition as a defensive mechanism against resident aphids, and increased calcium levels provide plants with an abundance of resources to build up this defence and promote new leaf growth. New leaf growth was greater in plants without aphids. This was likely due to selective allocation of resources by *A. thaliana*, as plants free from herbivory did not need to expend their limited resources on facultative defence. Overall, it is clear that micronutrient availability has both a direct effect on plant health and an indirect effect on the animals that depend on these plants. Further studies of this interdependence would indicate the ways in which human manipulation of the environment, including altering macronutrient balance through agriculture or pollution, could have effects that propagate across many trophic levels.

Acknowledgements

We would like to thank Drs. Chad Harvey and Susan Dudley for their assistance and guidance. The help of Russ Ellis and Geneviève van Wersch in the lab was greatly appreciated. Sebastian Irazuzta helped to clarify many of the underlying concepts essential to this report and provided guidance in the experimental procedures. Dr. Elizabeth Weretilnyk provided instructions for the mixing of fertilizers, and

Vera Velasco aided in the preparation of mixtures. Art Yeas of the McMaster Biology Department Greenhouse supplied the dolomitic limestone used to create the treatment levels in this experiment. Many thanks go to our loving families that have remained supportive despite long nights spent working and missed video calls.

References

Bieri A.P., Härri S.A., Vorburger C. and Müller C.B., 2009. Aphid genotypes vary in their response to the presence of fungal endosymbionts in host plants. *Journal of Evolutionary Biology,* 22, 1775-1780.

Bhuja P., 2004. Accumulation of (1➔3)-ß-D-glucans, in response to aluminum and cytosolic calcium in Triticum aestivum. *Plant Cell Physiology*, 45(5), 543-549.

Blume B., Nürnberger T., Nass N. and Scheel D., 2000. Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. *Plant Cell,* 12, 1425-1440.

Coley P.D., Bryant J.P. and Chapin F.S., 1985. Resource availability and plant antiherbivore defense. *Science,* 230, 895-899.

Cox, D.A., 2001. Growth, nutrient content, and growth medium electrical conductivity of poinsettia irrigated by subirrigation or from overhead. *Journal of Plant Nutrition,* 24, 523-533.

Health Canada, Water Quality and Health Bureau, 1997. *Environmental and Workplace Health: Calcium*. Available at: <http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/calcium/index-eng.php> [Accessed October 11, 2014].

Hepler, P.K., 2005. Calcium: a central regulator of plant growth and development. *Plant Cell,* 17, 2142-2155.

Howe H.F. and Westley L.C., 1988. *Ecological Relationships of Plants and Animals.* New York: Oxford University Press.

Kirkby E.A. and Pilbeam D.J., 1984. Calcium as a plant nutrient. *Plant, Cell and Environment,* 7(6), 397-405.

Louis J. and Shah J., 2013. Arabidopsis thaliana - Myzus persicae interaction: shaping the understanding of plant defense against phloem-feeding aphids. *Frontiers in Plant Science,* 4, 213.

Luna E., Pastor V., Robert J., Flors V., Mauch-Mani B. and Ton J., 2011. Callose deposition: a multifaceted plant defense response. *Molecular Plant Microbe Interactions,* 24(2), 183-193.

Microsoft Corporation, 2011. *Microsoft Excel for Mac*. (Version 14.3.9).

National Institutes of Health, 2000*. Arabidopsis.* Available at: <http://www.nih.gov/science/models/arabidopsis/> [Accessed October 9, 2014].

Peppe F.B. and Lomônaco C., 2003. Phenotypic plasticity of Myzus persicae (Hemíptera: Aphididae) raised on Brassica oleracea L. var. acephala (kale) and Raphanus sativus L. (radish). *Genetic Molecular Biology*, 26, 189-194.

Rippy J.F.M., Peet, M.M., Louws, F.J., Nelson, P.V., Orr, D.B. and Sorensen, K.A., 2004. Plant development and harvest yields of greenhouse tomatoes in six organic growing systems. *Horticultural Science,* 39(2), 223-229.

Sivaguru, M., 2000. Aluminum-induced (1➔3)-ß-D-glucan inhibits cell-to-cell trafficking of molecules through plasmodesmata. *Plant Physiology,* 124, 991-1006.

Urbanek S., Bibiko H.J. and Iacus S.M., 2012. *R.* (Version 3.0.3). R Foundation for Statistical Computing.

Wiese A., Christ M.M., Virnich O., Schurr U. and Walter A., 2007. Spatio-temporal leaf growth patterns of Arabidopsis thaliana and evidence for sugar control of the diel leaf growth cycle. *The New Phytologist,* 174, 752-761.

Will T., Tjallingii W.F., Thönnessen A. and van Bel A., 2007. Molecular sabotage of plant defense by aphid saliva. *Proceedings of the National Academy of Sciences of the United States of America,* 104, 10536-10541.

Yim K. and Bradford K., 1998. Callose deposition is responsible for apoplastic semipermeability of the endosperm envelope of muskmelon seeds. *Plant Physiology,* 118, 83-90.

Zhou J., Spallek T., Faulkner C. and Robatzek S., 2012. CalloseMeasurer: a novel software solution to measure callose deposition and recognise spreading callose patterns. *Plant Methods*, 8, 49.