JOURNAL OF
EMERGING INVESTIGATORS

Article

Biofortification of *Raphanus sativus* through irrigation with Ca^{2+} solutions does not increase calcium content

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SUMMARY

Micronutrient deficiencies, which affect more than two billion people globally, occur when an individual does not receive adequate amounts of essential vitamins or minerals. Biofortification aims to increase the nutritional content of food crops, and it is an important tool in decreasing the effects of micronutrient deficiencies. Specifically, hypocalcemia, or calcium deficiency, causes a wide range of symptoms that affect many different body systems. In this study, we tested the hypothesis that increasing the amount of calcium available to the food crop during growth would increase the amount of calcium present in the food crop. A total of 180 radish (*Raphanus sativus*) plants were grown in a controlled environment and irrigated with water of varying Ca^{2+} concentrations. The height of each plant was measured every five days beginning at day 0 to track the growth. At maturity, a random sample of four plants from each group were tested for calcium and magnesium content using an atomic absorption spectrophotometer. The group that received the highest concentration of Ca^{2+} grew significantly smaller than most other groups. The Ca^{2+} contents of leaf samples had no significant difference in Ca^{2+} or Mg^{2+} content; however, the Ca^{2+} content of root samples showed a significant decrease from the control in both Ca^{2+} and Mg^{2+} content across several experimental groups. These results provide preliminary evidence that irrigation with Ca^{2+} solutions does not increase the calcium content of mature plants, but further testing is needed to confirm these results. If increased calcium in irrigation water significantly increases the calcium content of mature radish plants, this technique could be used to increase the calcium content of other crops in areas with high rates of hypocalcemia.

INTRODUCTION

Global hunger is an issue that researchers around the globe have been investigating for decades. According to the United Nations Annual Food Report, 650.3 million people were undernourished in 2019 (1). The recent COVID-19 pandemic has worsened the crisis, increasing that number to 768 million undernourished people in 2020 (1). Micronutrient deficiencies, also known as hidden hunger, occur when the consumption of essential vitamins and minerals is not sufficient for full bodily function. According to the International Food Policy Research Center, more than two billion people worldwide suffer from a micronutrient deficiency (2). Hidden hunger has a wide range of symptoms in the human body, depending on the specific vitamin or nutrient in insufficient supply. Hypocalcemia, or calcium deficiency, can cause decreased bone and muscle mass. Diseases like osteoporosis and rickets, which negatively affect bone density, are caused mainly by hypocalcemia (3). Calcium deficiencies are especially prevalent in developing tropical and subtropical countries, where the estimated calcium intake of children is between ⅓ and ½ of the recommended daily intake (4). Crops grown in these areas tend to have less calcium due to the low natural calcium content of the soil and water (5). Most dietary calcium comes from dairy products such as milk and cheese due to the high bioavailability of calcium in these products (6). However, these products spoil quickly and cannot be easily transported over long distances. Finding a source of dietary calcium that can be transported easily and stored for longer periods of time is essential for addressing calcium deficiencies as well as the global hunger crisis.

Biofortification is a process that aims to increase the density of certain vitamins and minerals in food through selective breeding, genetic modification, or adjusted growing practices (7). It is an important tool in the effort to decrease micronutrient deficiencies. Previous research has shown that adding micronutrients to the soil can increase the nutrient density of the crops grown in it (8). All plants uptake calcium (Ca) and magnesium (Mg) from the soil in the form of the cations Ca^{2+} and Mg^{2+} (5). Due to their ionic charges being the same, Ca and Mg concentrations are inversely related in plants. Current methods of Ca fertilization rely mainly on Calcium carbonate (CaO_3), Calcium hydroxide ($Ca(OH)_2$), and Calcium oxide (CaO), commonly known as lime (9). While effective, these products are heavy and difficult to transport and apply to the soil without specialized equipment. These difficulties make it challenging to supplement the calcium-poor tropical soils in undeveloped tropical areas with these fertilization methods.

Recent research suggests that increasing the mass flow of water to plant roots aids in the bioavailability of Ca^{2+} (10). According to the United States Geological Survey, any water that contains more than 60 mg/L of dissolved calcium or magnesium is considered 'hard' water (11). Additionally, water with between 1,000 and 3,000 mg/L dissolved minerals is considered undesirable for drinking, but it is safe for consumption and sometimes used in areas where softer water is unavailable (11). Calcium ions are added to natural water sources through dissolved limestone ($CaCO_3$) present in the ground around the water source (12). However, calcium ions can also be added to water by intentionally dissolving calcium sulfate, commonly known as gypsum ($CaSO_4$) (13,14).

This research aimed to determine the effect of calcium supplementation through irrigation with Ca^{2+} solutions on the nutritional calcium content of mature *Raphanus sativus*

plants. *R. sativus,* or the domesticated radish, is an edible root vegetable with hundreds of subspecies (**Figure 1**) (15). Radishes have been farmed for thousands of years and continue to be popular due to their quick growth, long storage time, and both the root and leaves being edible (16). We irrigated a total of 180 *R. sativus* plants with varying concentrations of Ca^{2+} solution from seed to maturity. This study aimed to understand the effect of increased calcium in irrigation water on the calcium content of *R. sativus* with respect to plant growth. We hypothesized that irrigating *R. sativus* with Ca^{2+} solutions would increase the bioavailability of Ca to the root and increase the total amount of Ca in the mature plant. Due to the inverse relationship between Ca and Mg previously observed in plant nutrient uptake, this study also examined the effect of increased calcium supplementation on the final magnesium content of the plants. Final nutrient analysis results revealed no significant differences in Ca or Mg content of leaf samples. However, the root samples did reveal a significant decrease from control in both Ca and Mg content. The control group contained more Ca than the group that received 0.23 M irrigation, and more Mg than the groups that received 0.23 M, 0.46 M, and 0.92 M irrigation. These results show preliminary evidence that irrigation with Ca^{2+} solutions is not a viable method of calcium biofortification for *R. sativus*, but further testing is needed to confirm this conclusion.

RESULTS

We tested the Ca and Mg content of a sample of 24 mature *R. sativus* plants belonging to six distinct groups irrigated with varying levels of Ca^{2+} solution. The sample was taken from a population of 180 radishes irrigated with the following concentrations of Ca^{2+} solution: 0 M (control), $CaCO_3$ (to simulate a production environment), 0.23 M, 0.46 M, 0.69 M, and 0.92 M. All collected data were analyzed using a one-way ANOVA test paired with a Tukey post-hoc test. A significance level (α) of 0.05 was used throughout.

R. sativus leaf samples from all 6 groups examined had an average calcium concentration of 12.37 ppm and a range of 7.76 ppm. The results of nutrient analysis on the leaf samples yielded no significant difference in Ca content between any two groups ($p > 0.05$). The *R. sativus* root samples had an average calcium concentration of 1.28 ppm and a range of 1.15 ppm. The control group had a significantly higher calcium concentration than the 0.23 M Ca^{2+} group ($p = 0.0238$). The control group's average calcium content in ppm was 46% higher than that of the 0.23 M group (**Figure 2**).

The *R. sativus* leaf samples had an average magnesium content of 1.83 ppm and a range of 1.26 ppm. Statistical analysis of the magnesium content of the leaf samples yielded no significant differences across groups ($p = 0.2983$). The *R. sativus* root samples had an average magnesium content of 0.54 ppm and a range of 0.80 ppm. The control group contained significantly more magnesium than the 0.23 M group ($p = 0.0010$), the 0.46 M group ($p = 0.0094$), and the 0.92 M group ($p = 0.0191$). The average Mg content in ppm of the control group was 60% higher than the 0.23 M group, 46% higher than the 0.46 M group, and 42% higher than the 0.92 M group (**Figure 3**).

Figure 1: Mature *R. sativus* plant. *R. sativus* plants were grown in individual 6" pots inside a controlled polycarbonate greenhouse from seed to maturity (approximately 45 days).

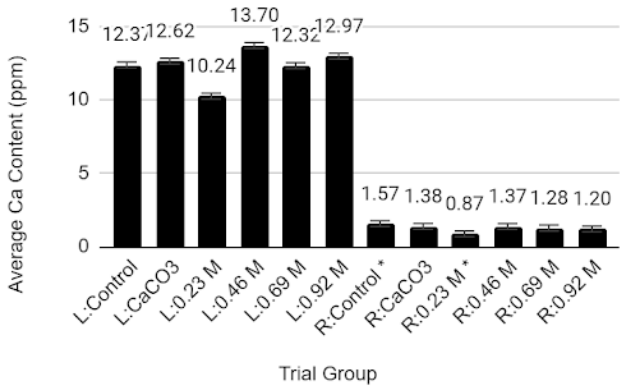

Figure 2: Calcium contents of *R. sativus* root and leaf samples (n = 4). The Ca contents of each sample was determined using atomic absorption spectrophotometry. The bars labeled "L" represent leaf samples and the bars labeled "R" represent root samples. $p < 0.05$ is shown as *. R:Control is significant compared to R:0.23 M.

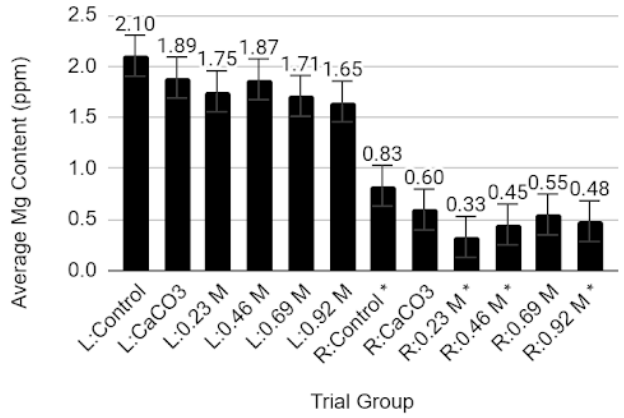

Figure 3: Magnesium contents of *R. sativus* root and leaf samples (n = 4). The Mg contents of each sample was determined using atomic absorption spectrophotometry. The bars labeled "L" represent leaf samples and the bars labeled "R" represent root samples. p < 0.05 is shown as *. R:Control is significant compared to R:0.23 M, R:0.46 M, and R:0.92 M.

When the final height measurements of all *R. sativus* plants were analyzed, it was found that the 0.92 M group, which received the highest concentration of Ca^{2+} irrigation, was significantly smaller than every other group besides the 0.23 M group (control: $p = 0.0010$, $CaCO_3$: $p = 0.0010$, 0.46 M: $p = 0.0010$, and 0.69 M: $p = 0.0022$) (**Figure 4**). Compared to the 0.92 M group, the control group grew on average 34% taller, the $CaCO_3$ group 31% taller, the 0.46 M group 34% taller, and the 0.69 M group 23% taller. Despite the initial difference in heights, once each plant was dried there was no significant difference in the dry weight of each group.

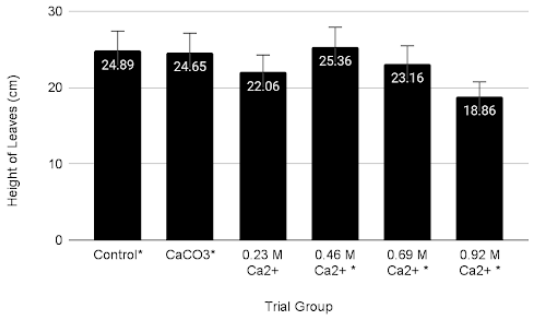

Figure 4: Average height of R. sativus leaves at harvest (day 45) (n = 30). Plant heights were measured after 45 days of growth when irrigated with water containing different concentrations of Ca^{2+}. $p < 0.05$ is shown as *. 0.92 M Ca^{2+} is significant compared to Control, $CaCO_3$, 0.46 M Ca^{2+}, and 0.69 M Ca^{2+}.

DISCUSSION

In the year 2020, 768 million people across the globe were undernourished (1). Biofortification is an important tool in lessening that number, focusing on increasing the nutritional value of food crops through genetic manipulation, fertilization, or adaptive growing practices (11). *R. sativus,* or the domesticated radish, has many varieties and is grown worldwide (7). This study examined the effects of increased calcium supplementation through irrigation water on the calcium content of mature *R. sativus* plants. This study aimed to determine the viability of irrigation with Ca^{2+}-enriched water as a biofortification strategy for developing countries with high rates of calcium deficiency.

The results of nutrient testing suggest that increasing the amount of Ca^{2+} ions available to *R. sativus* plants via irrigation water does not significantly increase the amount of calcium in the mature plant. The only significant change in calcium content observed was a decrease in root calcium content in the plants irrigated with 0.23 M Ca^{2+} ($p = 0.0238$). This data rejects our original hypothesis that increased Ca in irrigation water would increase the calcium content of the plant. However, only 24 mature *R. sativus* plants, 13% of those grown for the study, were tested for Ca and Mg content due to equipment and time limitations. For that reason, further study is required to confirm the data as the number of samples tested may not have been enough to fully reveal trends in the larger population.

Unlike the Ca content results, Mg analysis yielded significant decreases from the control in the root samples of the groups that received 0.23 M Ca^{2+}, 0.46 M Ca^{2+}, and 0.92 M Ca^{2+}. These differences were unexpected, especially noting the insignificant results of Ca analysis. It is well known that Ca and Mg are closely related in plant nutrition, as both elements have the same charge (2+) when taken up by plant roots. Often increased calcium concentrations decrease the bioavailability of magnesium and vice versa (17). The significant decrease of magnesium content found in the 0.23 M, 0.46 M, and 0.92 M groups alone would suggest that these groups absorbed significantly more calcium, but this was not the case. The contradictory results of the calcium and magnesium testing highlight the need for further testing and a greater sample size.

The results of the plant height measurements taken throughout the experiment yielded a significant decrease in the growth of one group of *R. sativus* plants. The group that received the 0.92 M Ca^{2+} was found to be significantly smaller than all but one other group, which may have been due to the presence of SO_4^{2-} ions in the Ca^{2+} solutions. When gypsum ($CaSO_4$) is dissolved in water, it splits into calcium and sulfate ions. Sulfur is essential to plant nutrition, playing an important role in response to stress (18). However, too much sulfur can be harmful to plants and cause decreased sugar yield (19). The difference in growth observed in this study could be attributed to excess sulfate concentrations in the irrigation water.

Calcium biofortification is essential to solving calcium deficiencies, and research into its future prospects needs to continue. We could pursue several avenues of research to further this study. Firstly, the study could be repeated with a larger population and sample size. Increased replicates could reveal patterns in the data that were lost within the small sample size used in this study. Secondly, different methods of adding Ca^{2+} to the irrigation water could be investigated to achieve greater concentrations of Ca^{2+} without the danger of excess SO_4^{2-}. Finally, biofortification through irrigation water could be employed with different nutrients or other species within the *Brassicaceae* family.

Based on our results, we determined that there is preliminary evidence that irrigation with Ca^{2+}-fortified water is not an effective means of biofortification in *R. sativus*. No significant increase in Ca content was observed in the plants irrigated with increased calcium. Furthermore, the SO_4^{2-} ions created by dissolving gypsum in water may have harmed the plants at higher concentrations. Despite these results, magnesium content analysis indicated a possible trend towards increased calcium content in irrigated plants. Additional testing is needed to confirm the results observed in this study. While the data did not support our original hypothesis, this study offered valuable insight into biofortification through irrigation and opened new opportunities in this field of study. Biofortification may be the key to solving micronutrient deficiencies, and thus, it is imperative that the agriculture industry continues to research and modernize to and eliminate the hunger crisis.

MATERIALS AND METHODS

Plant Growth

The *R. sativus* seeds we used in this study were bought from Gurney's Seed Company (item number 14965). We grew plants in individual six-inch pots in a climate-controlled polycarbonate greenhouse (**Figure 5**). The daytime temperature remained at a constant 21ºC, and the nighttime temperature remained at 19ºC. A total of 200 *R. sativus* seeds were planted; a total of 191 germinated. Of these 191

plants, we used 180 plants in the study. Plants were split into six experimental groups of 30 plants each. We recorded the height (in cm) of each plant every five days, beginning with the first visible sprout (Day 0) and ending on the harvest date (Day 45). We collected all height measurements by hand, measuring from the base of the plant to the tip of the tallest leaf. All irrigation was administered through hand watering when the top layer of soil became dry, and all plants received equal irrigation.

Experimental Groups

Our study consisted of six distinct trials, two control groups and four experimental groups. We watered the control groups throughout the experiment using deionized water. One of these groups received no calcium supplementation, and the other received a one-time treatment of calcitic lime at the agricultural standard rate of 3.0 g $CaCO_3$ per 1 L of soil at germination. We created the calcium solutions by dissolving gypsum ($CaSO_4 \cdot 2H_2O$) in deionized water. The amounts of gypsum per liter of water were 1.0 g, 2.0 g, 3.0 g, and 4.0 g to create 0.23 M, 0.46 M, 0.69 M, and 0.92 M Ca^{2+} solutions, respectively. We dissolved the gypsum powder in the water by mixing the solution on a magnetic stirrer for approximately one minute per gram of gypsum. The irrigation solutions were mixed in the lab and transported to the greenhouse in sterile containers. Over the course of the experiment (45 days), each plant received a total of 1.35 L of irrigation.

Harvest

The *R. sativus* plants were deemed mature 45 days after the first visible sprout when the tops of the roots began to show above the soil. On day 45, we measured the height of the plants a final time and then uprooted them. Then, we chose a random sample of four plants from each experimental group. The selected plants were triple-washed in deionized water and then weighed. After the weight was recorded, we carefully separated the root from the leaves, and each part of the plant was placed in its own paper bag. The paper bags containing the samples were then placed in a 60ºC oven for 36 hours to dry. When each sample was fully dried, we took the weight of both plant parts again to determine the dry weight of each plant. Finally, we stored the dried samples in their respective paper bags in a cool, dry place until nutrient analysis could be performed on all samples at once.

Figure 5: Mid-growth *R. sativus* plants in the greenhouse. *R. sativus* plants on Day 30 of growth.

Figure 6: HCl nutrient extraction method in preparation for analysis. The left vial contains an extracted and strained leaf sample ready for analysis. The right beaker contains a single root tissue sample and HCl acid before being rested and strained.

Nutrient Analysis

We used an atomic absorption spectrophotometer to analyze the plants used in this study for calcium and magnesium content. Prior to analysis, each sample was extracted using an HCl extraction method (20). First, we finely ground the dried plant matter using a mortar and pestle, and each ground sample was combined individually with 0.5 M HCl. The mixture was stirred and let sit for 5 minutes. When the sample was fully extracted, we filtered the liquid through a medium flow rate filter paper and transported it to the atomic absorption spectrophotometer for analysis (**Figure 6**). We created a set of calcium and magnesium standards using 0.5 M HCl as a background to create a standard curve. Each sample was diluted at a 1:40 ratio with 0.1% lanthanum oxide to be within the instrument's range. We then analyzed each sample twice, first to determine calcium content in ppm and second to determine magnesium content in ppm. The percent of each element in the plant tissue was calculated using the following formula:

$$\%N = \left(\frac{mg^N}{L}\right) \times (dilution\ factor) \times \left(\frac{sample\ vol.}{sample\ weight}\right)$$

In this equation, *N* = nutrient. Once the percentage of each element was determined, the dry weight measurements of each sample were used to calculate the total amount of each element, in grams, present in each sample.

Statistical Analysis

We analyzed all the data through a one-way ANOVA with a Tukey post-hoc using the Astatsa online calculator. All data was organized and stored in Google Sheets. A significance level of 0.05 was used to determine significance.

ACKNOWLEDGEMENTS

Special thanks to Dr. Melissa Bledsoe of Missouri State University for her mentorship and the use of her lab. Many

thanks as well to Mr. Justin Slye of Lebanon High School for his continued support and Miss Hayley Miller for access to the greenhouse.

Received: February 11, 2022
Accepted: June 8, 2022
Published: September 6, 2022

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