The effect of salinity on hypocotyl and radicle length of *Arabidopsis thaliana*

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Abstract

To determine the effect of salinity on the hypocotyl and radicle length following germination of wild type *Arabidopsis thaliana*, we grew *A. thaliana* in three different concentrations of NaCl: tap water control, 0.01 mM, and 0.02 mM. The growth was measured by hypocotyl and radicle length. The results of our experiment indicated a mean growth of 0.737 mm ± 0.286 mm for the control treatment, 0.170 mm ± 0.254 mm in low salt treatment, and 0.242 mm ± 0.051 mm in high salt treatment on Day 5 of the experiment. The control experienced significantly more growth than the high salt treatment, as well as significantly more growth than the low salt treatment over the first five days of the experiment. Therefore, we are able to reject our null hypothesis. On Day 9, we found the growth to be 0.709 mm ± 0.077 mm for the control treatment, 1.019 mm ± 0.660 mm for the low salt treatment, and 1.563 mm ± 0.065 mm for the high salt treatment. These results show that the high salt treatment experienced significantly more growth than the control treatment during the final four days of the experiment. This is not consistent with previous studies in the field. This leads us to conclude that salt concentration does have a delaying effect on germination; however, after an initial period of stunted growth, the salt may enhance seedling growth. A likely explanation for the enhanced seedling growth is the production of salicylic acid by the seedlings, which is known to stimulate germination and have increased production in high salinity, as well as the production of proline, an amino acid, which increases salt tolerance in the organism following an applied salt stress.

Introduction

Arabidopsis thaliana is a plant belonging to the same family as mustard and cabbage plants (Hartwell *et al.* 2001). It is commonly known as "mouse-ear cress" and has a six week life span (Hartwell *et al.* 2001). Furthermore, the optimal growth conditions for *A. thaliana* are 23°C and light intensity between 120 and 150 micromoles/m^2 s (Rivero *et al.* 2014). The objective of our study is to learn how salt stress affects the growth of *A. thaliana* seedlings as measured by the hypocotyl and radicle length. This is important because irrigation can induce salinification, and has in fact affected more than 20% of arable soil worldwide (DeRose-Wilson and Gaut 2011). Understanding the effects of salinity on plants may lead to the development of specific salt-

tolerant species. Salinity affects *A. thaliana* in two ways; the first is ionic stress which is an increase of sodium ions inside the plant (DeRose-Wilson and Gaut 2011). This offsets the balance between sodium ion concentrations in the tissue and cytosolic potassium ion concentrations, thus disrupting homeostasis (Zhu *et al.* 1998). The plant therefore responds by storing the necessary ions in storage tissues to maintain normal ion concentrations which allows for the normal functioning of cells (DeRose-Wilson and Gaut 2011). The second way salinity affects the plant is osmotic stress, which is a reduced ability to absorb water (DeRose-Wilson and Gaut 2011). A previous study done by Xiong and Zhu (2002) found that salt concentrations above 50 mM will lead to plant death, and that young plants (especially those immediately following germination) are most susceptible to salt stress. Furthermore, DeRose-Wilson and Gaut (2011) conducted a study which found that 6 out of 16 replicates did not germinate at 150 mM NaCl concentration, and 13 out of 33 replicates did not germinate at 250 mM NaCl. In addition, the researchers found that 18 of the replicates at 250 mM NaCl germinated less than 10% (DeRose-Wilson and Gaut 2011). Subsequently, DeRose-Wilson and Gaut noticed that the percent germination at 250 mM concentration was significantly lower than the 150 mM treatment and the control (0 mM NaCl) (2011). Figure 1 shows how salt stress decreases the percent germination of *A. thaliana* seeds. Lo and Yamaguchi (1970) an abrupt decrease in per cent germination at NaCl concentrations greater than 130 mM.

The discrepancy between Xiong and Zhu's results (2002), and DeRose-Wilson and Gaut's (2011) results prompted our research in this field. Based on these studies, we formulated the following alternate hypothesis: increasing salinity will decrease the growth of *A. thaliana* seedlings, as measured by length of hypocotyl and radicle. Hereafter, the word "seedling" refers specifically to the hypocotyl and radicle sprouting from *A. thaliana* seeds. The null hypothesis is that increasing salinity will have no effect on, or will increase the growth of *A. thaliana*

seedlings. To expand on the literature and learn more about how salinity affects seedling growth of *A. thaliana*, seedlings were germinated in varying concentrations of NaCl and monitored over a period of nine days.

Methods

We began our experiment by first labeling nine 60 mm x 15 mm polystyrene Petri dishes with three treatments: tap water as control, 0.01 mM NaCl and 0.02 mM NaCl concentration. We selected these particular salt concentrations because they would offer distinct differences in germination length, as highlighted by Lo and Yamaguchi's study (1970). Furthermore, the results of a previous study we conducted using 0.1 mM and 0.2 mM NaCl yielded no germination, so we concluded that a lower salt concentration was necessary. We placed filter paper at the bottom of each Petri dish and ensured it was completely covered. Figure 2 below is representative of our initial setup for a similar experiment; the setup for this experiment looked the same, with one exception: we used 3 replicates per treatment instead of 4. We dispersed ten seeds onto the filter paper and added 0.5 mL of the treatment corresponding to each Petri dish. On Day One, we photographed one seed from each treatment using the DinoXcope on the dissecting microscope at 32X magnification. We placed the Petri dishes onto trays as shown in Figure 1, and placed the trays in an incubator set to 17 °C, with fluorescent light bulbs at 1413 lux. The incubator was set on a fourteen hours light/ten hours dark photoperiod. All Petri dishes were kept under the same light intensity but slight variations were measured from day to day. The light intensity, measured with a photometer, ranged between 1409 and 1430 lux on each day.

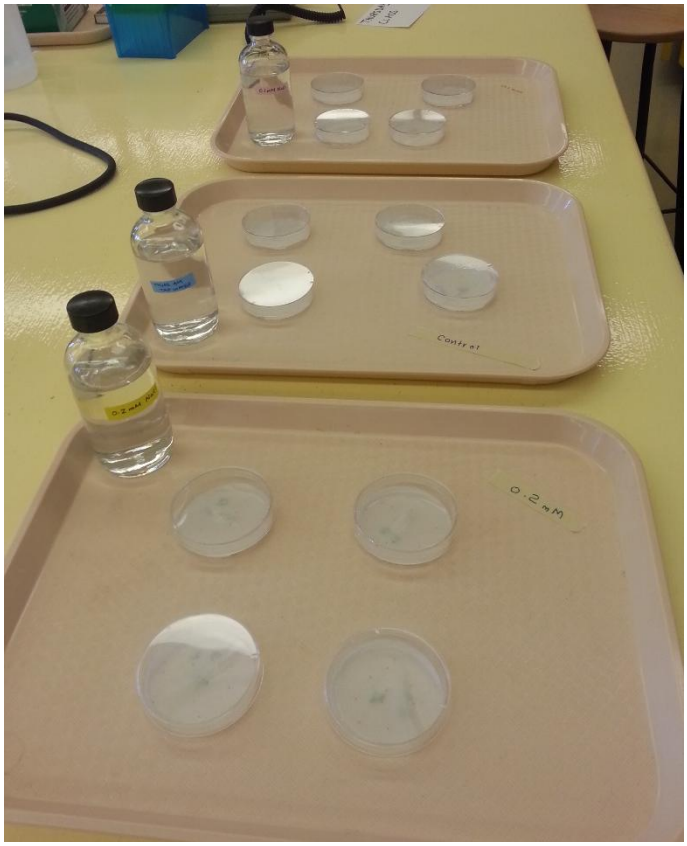

Figure 1. Experimental setup of the replicates and treatments for a previous experiment. Under the three treatments of tap water control, 0.1 mM NaCl and 0.2 mM NaCl, twelve Petri dishes in total were set up, where each treatment contained four replicates each. Each Petri dish contained ten seeds.

On the third day after planting, we watered each Petri dish with 0.5 mL of its respective treatment. We photographed seeds using a DinoXcope attached to a light microscope at 32X magnification. The fourth day after planting, we watered each Petri dish with 0.25 mL of its respective treatment because the filter paper appeared dry. The fifth day after planting, we watered each Petri dish with 1.0 mL of its respective treatment to ensure the filter paper remained moist throughout the weekend. We photographed seeds in the same fashion as we did on the third day. Our data of the seedling growth was obtained using the ImageJ software, where we calibrated the scale of 1 mm with a ruler viewed under the microscope to set up and then determined the relative size of the seedlings from the photographs taken with DinoXcope.

To analyze our data, we performed a set of calculations to first find the means, then the 95% confidence intervals. Firstly, we found the average length of the ten seedlings on each day in each Petri dish. Next, we averaged the mean growths for the three Petri dishes in each treatment where each Petri dish represented one replicate; for this calculation n=3. We then found the variance and standard deviation which we used to calculate the 95% confidence interval for each treatment. We assessed the significance of our results using the 95% confidence intervals. If the intervals for two treatment means overlapped, the results were interpreted as not significant. If the intervals did not overlap, the difference in mean growth between those treatments was deemed statistically significant.

Results

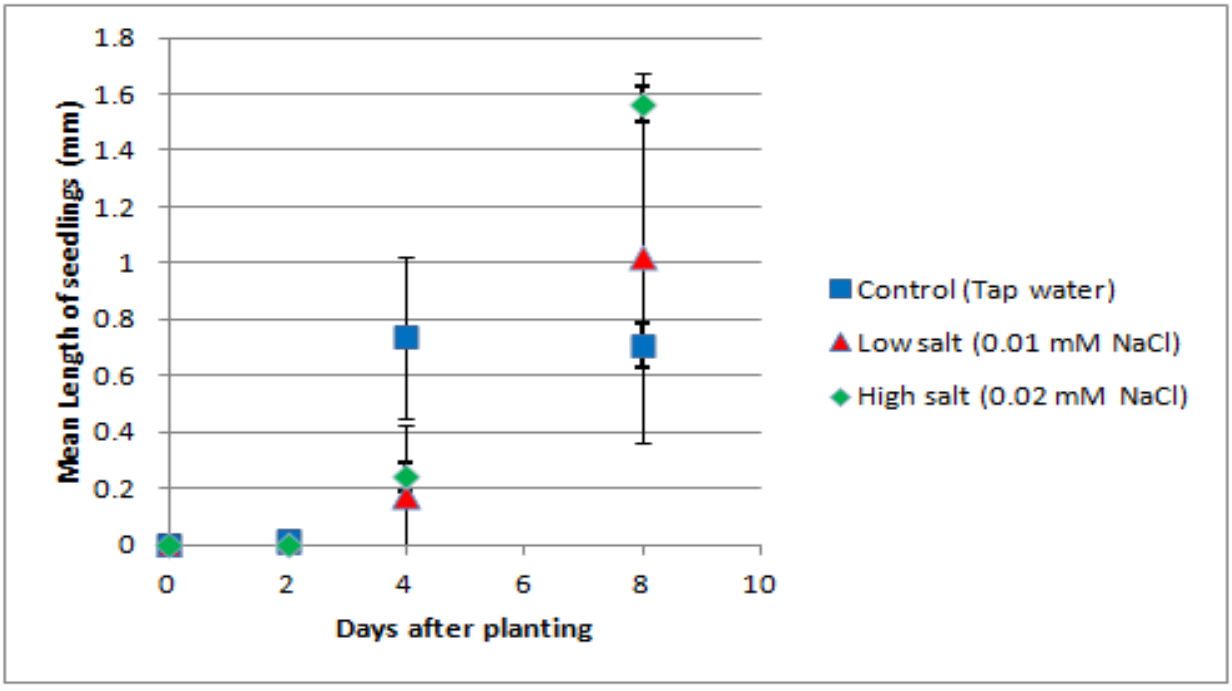

Figure 2. Mean length (mm) of *Arabidopsis thaliana* seedlings. 95% confidence intervals are shown for each mean. Seedlings were grown in three salinity treatments: control with tap water (n = 3), low salinity 0.01 mM NaCl (n = 3), and high salinity with 0.02 mM NaCl (n = 3).

Figure 2 summarizes the quantitative results of our experiment. On Day 1, when we planted the seeds, no germination was observed. Two days after planting (Day 3), no seeds in the high salt treatment had germinated, however, germination in the low and control treatments did occur, but was very slight. The germinated seeds had small white protuberances, which is the radicle sprouting from the seed capsule. Some of the seeds lacking radicles had cracks in them, indicating germination had occurred. Mean seedling length for the control treatment was found to be 0.012 mm ± 0.012 mm. On Day 3, there was no apparent germination in two of the low salt replicates (0.01 mM NaCl applied). However, in the third replicate, one seed had germinated. Mean seedling growth for the low salt treatment was 0.004 mm ± 0.007 mm.

On the fourth day after planting (Day 5), germination was observed in all seeds in the control treatment, many seeds in the low salt treatment, and most seeds in the high salt treatment. Germinated seeds had a radicle; no hypocotyls had yet developed. Average radicle length for the control treatment was 0.737 mm ± 0.286 mm. Average radicle length for the low salt treatment across all replicates was found to be 0.170 mm ± 0.254 mm. Average radicle length for the high salt treatment was found to be 0.242 mm ± 0.051 mm. The confidence intervals between the control treatment and the high salt treatment, as well as between the control treatment and the low salt treatment both do not overlap; therefore, the control seedlings are significantly longer than those grown in 0.02 mM NaCl, and are also significantly longer than those grown in 0.01 mM NaCl. There was no significant difference in growth between the high salt and low salt treatments. Overall, the growth and development of the radicles in all seeds was more apparent than on Day 3, as they appeared both larger and more translucent. The sprouting of root hairs from the radicle is also more apparent (Figure 3).

Figure 3. ***Arabidopsis thaliana*** **seedlings photographed at 32X magnification four days after planting. A.** Seedling from the control treatment. **B.** Seedling from the low salt (0.01 mM NaCl) treatment. **C.** Seedling from the high salt (0.02 mM NaCl) treatment.

On Day 9, the eighth day after planting, most seeds in all treatments had germinated. The most developed seedlings consisted of a radicle, a hypocotyl, and two small leaves at the tip of the hypocotyl (Figure 4C). These seedlings were most abundant in the high salt treatment. Some seedlings lacked leaves, and only had a hypocotyl and radicle (Figure 4A). All radicles had small root hairs growing from the tip. The control treatment had a mean seedling (measured as combined hypocotyl and radicle) length of 0.709 mm ± 0.077 mm. Mean seedling length for the low salt treatment was 1.019 mm ± 0.660 mm. Mean seedling length for the high salt treatment was 1.563 mm ± 0.065 mm. This was the longest mean seedling length observed. The confidence intervals between the high salt treatment length and control treatment length did not overlap, indicating that the high salt treatment seedlings were significantly longer than those grown in tap water. The confidence intervals in the low treatment overlapped those in both of the other treatments, indicating that the low salt seedlings did not experience significantly different growth than the other treatments.

Figure 4. ***Arabidopsis thaliana*** **seedlings eight days after planting. A.** Seedling from the control treatment, photographed at 32X magnification. **B.** Seedling from the low salt (0.01 mM NaCl) treatment, photographed at 16X magnification. **C.** Seedling from the high salt (0.02 mM NaCl) treatment, photographed at 16X magnification.

Sample calculation for 95% confidence interval for mean length of seedling, taken from high salt Petri dish 1 from Day 5:

Mean seedling length for entire treatment (n=3): Average = $\bar{x} = \Sigma x_i / n = 0.242$ mm

Standard Deviation of seedling length: $SD = \sigma = \sqrt{(\Sigma (x - \bar{x})^2 / (n - 1))} = 0.045$ mm

95% Confidence Interval of seedling length: $95\%\ CI = \bar{x} \pm (1.96 * (\sigma/\sqrt{n}))$

$= 0.242 \text{ mm} \pm (1.96 * (0.045 \text{ mm} / (\sqrt{10})))$

$= 0.242 \pm 0.051$ mm

Discussion

From Figure 2, on Day 5 the control seedlings were significantly longer than both the high salt seedlings and low salt seedlings (confidence intervals do not overlap); however there was no significant difference in length between the low salt treatment and the high salt treatment. Contradictory results were observed on Day 9; under the high salt treatment, seedlings experienced significantly longer growth than in the control treatment, and there was no significant difference between length of seedlings in the low salt treatment and the other two treatments.

The null hypothesis we formulated at the start of the experiment was: increasing the salinity of water used to germinate wild type *A. thaliana* seeds will increase or have no effect on the growth of the seedlings. The alternate hypothesis was therefore: increasing the salinity of water used to germinate wild type *A. thaliana* seeds will decrease the growth of seedlings. Since on Day 5 the control seedlings showed significantly greater growth than the high salt and low salt seedlings, we are able to reject our null hypothesis and provide support for the alternate hypothesis in early stages of germination. However, due to the fact that the high salt seedlings were significantly longer than the control seedlings on Day 9, it appears that salinity has a different impact in later stages of seedling growth.

We took notice of some trends in our qualitative observations. For the first two days, all 3 treatments looked the same, as can be seen in Figure 5.

Figure 5. Photos of the seeds taken on Day 1 at 64X magnification. A. Seed from control treatment. **B.** Seed from low salt 0.01 mM NaCl treatment. **C.** Seed from high salt 0.02 mM NaCl treatment.

In the early stages of germination (Day 5), we noticed more growth in the control group, and the least amount of growth in the high salinity group, as shown in Figure 2. However, by Day 9, we noticed that both the high salinity treatment group and the low salinity treatment appeared to have experienced more growth than both of the other treatments, indicated in Figure 2. This led us to believe that the salt had a stunting effect on the growth of the young seedlings; however, after a period of time, the salt enhanced seedling growth.

Our results up to Day 5 are in agreement with the literature. A similar experiment was conducted by DeRose-Wilson and Gaut (2011), in which they found higher salt concentrations negatively affected germination of *A. thaliana*. Salt negatively affects germination because it limits the seed's ability to absorb water (Dodd and Donovan 1999) and also affects mobilization of reserves stored within the seed (Bouaziz and Hicks 1990). The salt concentrations we used in our study were much lower than those used by DeRose-Wilson and Gaut (2011); we used 0.01 mM and 0.02 mM, while they used 150 mM and 250 mM. This difference in salt concentration could be one explanation for why the findings on the last day of our study varied from the study results of DeRose-Wilson and Gaut (2011). Furthermore, DeRose-Wilson and Gaut germinated the seeds at 22 °C, while our seeds were kept at 17 °C. Additionally, different lines of *A. thaliana* will exhibit variability in salt tolerance (Carrasco *et al.* 2007).

A possible reason for the discrepancy between our results after Day 5 and those found by DeRose-Wilson and Gaut (2011) is the potential presence of salicylic acid, which enhances seed germination under high salinity conditions, as demonstrated from a study by Lee *et al.* (2010). Within the genome of *A. thaliana* there encodes two isochorismate synthase genes; isochorismate synthase is an enzyme that acts as a catalyst in the conversion of chromate to salicylic acid (Chen *et al.*, 2009). This process makes it possible for salicylic acid to have been produced by our seedlings. As a phytohormone, commonly known as a plant hormone, salicylic acid regulates the growth of plants, and it is possible that the seedlings produced the compound as a response to the salt stress (Lee *et al.* 2010). The mechanism behind salicylic acid's surprising function is that it acts as an antioxidant when in the presence of a highly salinized environment (Rajjou *et al.* 2006), inhibiting the effects of oxidative damage from reactive oxygen species (Lee *et al.* 2010). In addition, salicylic acid alleviates osmotic stresses by increasing the absorption of water from plants under salted conditions (Lee *et al.* 2010). Another function of salicylic acid is its role in

triggering pathogenic response during infections in plants (Borsani *et al.* 2001), where they induce the activation of pathogenesis-related genes (Lee *et al.* 2010). As shown in DeRose-Wilson and Gaut's (2011) study and the research conducted by Borsani *et al.* (2001), salt supposedly has a negative impact on *A. thaliana* seed germination. The introduction of salt in the water used to water the plants may have posed as an alien factor similar to pathogens that the organism has adapted to defend against, and in response the seeds produced the phytohormone salicylic acid within them. Since the small seeds were growing on filter paper where water cannot freely float around or escape, the shrouding of the seeds with salinized water may have stimulated the processes of hypersensitive response and systemic acquired resistance to defend against the 'pathogenic' feature of NaCl (Borsani *et al.* 2001). Although no literature has indicated a possible salt stress threshold for *A. thaliana*, Kreps *et al.* (2002) were able to single out a set of mRNA's within the species that responds to salt at a concentration of 100 mM. This concentration is not necessarily representative of the threshold for salt stress, but it is a concentration that was found to have produced a response in the organism against oxidative stress (Kreps *et al.* 2002). Due to the large difference in NaCl concentrations that were used in their experiment in comparison to our own (Kreps *et al.* 2002), it is possible that the difference in growth of the radicle and hypocotyl observed in the low treatment seeds and the high treatment seeds were instead because of *A. thaliana*'s natural variability in tolerance of salt under polygenic control, as highlighted by the research from Carrasco *et al.* (2007). This fluctuation in salt tolerance may have contributed to the high variation in seedling growth of low treatment seeds as observed on Day 9. Thus, it is possible that the buildup of salt in the Petri dishes under high salinity treatment over the course of the experiment may have resulted in levels high enough that surpassed a salt stress threshold in *A. thaliana* to trigger the salicylic acid response in the later days of the experiment, but not the first five days. Consequently, the low salt treatment may not

have accumulated sufficient salt to reach said threshold in order to initiate the response, resulting in data that did not reflect a significant difference between the low salt treatment and the control treatment.

In two other studies conducted by Nanjo *et al.* (1999) and Thakur and Sharma (2005), both teams of researchers found that proline (Pro), an amino acid, accumulates as a metabolic response to osmotic adjustment for abiotic stresses applied in the environment. Particularly when the environmental stress is due to high salinity, Pro will attempt to counteract dehydration by acting as a mediator to the osmotic pathways in the seed (Mattioli *et al.* 2009). An analysis of the transgenic cDNA encoding for proline dehydrogenase (ProDH) in *Arabidopsis* plants, which catalyzes Pro degradation was carried out (Nanjo *et al.* 1999). To compare the effect of proline in plants, a transgenic plant with anti-ProDH, having the opposite function of ProDH, was constructed and placed under NaCl salt stress treatments. The researchers concluded that the plants with the anti-ProDH had a significantly higher tolerance to salinity than ProDH plants, suggesting that Pro does play a role in regulating salt stresses, and may consequently be affecting our replicates under high salinity treatment by building more tolerance, resulting in higher growth in the high salt treatment in the last four days of the experiment (Nanjo *et al.* 1999). However, as the accumulation of proline takes time, it did not immediately counteract the effects of the salt treatments; therefore, leading to no germination in the first two days. The findings for a possible salt stress threshold that triggers proline accumulation may also be a likely reason for why the replicates under the low salinity treatment developed a lower salt tolerance than in the replicates under high salinity treatment.

Our low salt treatment had significantly shorter seedlings than the control treatment on Day 5, but due to large confidence intervals (Figure 2) on Day 9, it did not exhibit significantly different growth from either of the other treatments. These large confidence intervals were due to

large variation in seedling length across the replicates. This variation could possibly be attributed to experimental error.

Possible sources of error throughout our experiment could include variation in measuring the length of the seedlings. The seedlings were measured using ImageJ. Due to the large quantity of seedlings, measurements were made by three members of the research team. This may have resulted in measurement error, stemming from individual differences in the judgement calls about how to measure seedlings. A large source of error comes from the positioning of the seeds in the photos. Many of the seedlings sprouted in the upward direction, or bent as they grew. The pictures using DinoXcope were taken from the top of the seeds, so the part facing upwards was not accounted for in the measurements which may have resulted in inaccurate seedling length data. Another possible source for error could have come from how the Petri dishes were watered; salt and water may have been unevenly distributed on the filter paper, causing some seeds to have slightly different environments than the others within their treatment.

In the future, our research team would like to further investigate the role of salicylic acid in increasing seed germination under salinity stress. To do this, we could repeat the experiment we did, but this time have a treatment that receives additional salicylic acid to the seeds at three different salinity concentrations, and a treatment that does not receive extra salicylic acid at the varying concentrations of NaCl. This will give us valuable insight about the effect of salicylic acid in the results we obtained from this experiment.

One more avenue for further research could involve carrying out our experiment over a longer period of time, to observe whether the high salt treatment continued to grow more than the control treatment, or if eventually the control treatment would grow more than the high salt.

Conclusion

In this experiment, we observed significantly longer *Arabidopsis thaliana* seedlings in the control (no NaCl) treatment than in the high salt (0.02 mM NaCl) treatment in the first five days of germination. This allows us to reject our null hypothesis, and provides support for our alternate hypothesis that increasing salinity decreases growth of *A. thaliana* seedlings. However, by the last day of our experiment, the seedlings in the high salt treatment were significantly longer than those in the control treatment, indicating that different mechanisms may be at play in later stages of germination.

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