4-22-2022

The Impact of Supplemental Proline on Stress Tolerance in Cucumbers and Tomatoes

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by

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An Honors Capstone submitted in partial fulfillment of the requirements for the Honors Diploma to

The Honors College of

The University of Alabama in Huntsville

April 22nd, 2022

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Abstract

Salt Stress is an increasing issue in growing crops across the globe. When undergoing salt stress, plants produce the amino acid proline to serve as an osmoregulator to ameliorate the effects of salt; however, the energy required to produce proline reduces plant growth and decreases the potential yield of the plant. Introduction of proline as a supplement through watering enables plants to uptake proline rather than having to allocate energy to its production. In this study tomatoes and cucumbers, plants with differing degrees of salt tolerance, were exposed to salt stress in order to observe the impact of proline supplementation to their growth. Plants in this experiment were watered using varying solutions of salt and proline with deionized water serving as a control. The plants watered with saline, but without proline, exhibited a notably stunted growth as compared to the controls. Plants receiving both salt and proline demonstrated decreased stress as compared to those receiving only salt at the majority of concentrations (Excluding a combination of high concentrations of salt and proline: 200 mmol of sodium and 100 mmol of proline). It was found that the greatest benefit—lack of wilting and increased growth when compared with the control cases—to the plants occurred when watered with a combination of 50 mmol of sodium solution and 25 mmol of proline solution. This can potentially be attributed to a combination of the osmoprotectant effects of proline and the improvement to water retention caused by the presence of a small quantity of soil salinity: The presence of the proline reduces the negative effects of the salt which normally outweigh the potential osmotic benefit to absorption.

Introduction

Land degradation is a significant threat to food security and quality of life in many parts of the world, particularly in rural communities (Saqib et al, 2019). Salinity, which is caused by the accumulation of salt when irrigation water is taken up by crops (and evaporation) leaving traces of salt behind, is one of the primary causes of land degradation. Salinity issues are becoming more significant as a result of land clearing, unsustainable irrigation practices, and through pressures created by increasing food requirements to utilize land that is marginally viable for agriculture (Munns and Gilliham, 2015). The general impact of salt degradation on plants is to "reduce the growth rate resulting in smaller leaves, shorter stature, and sometimes fewer leaves", which leads to decreased yield in plants over time (Shannon and Grieve, 1999). This can cause problems for farmers, particularly in the case of those growing necessary food crops. As of a 2014 UN report, nearly ⅕ of the world's farmland had been impacted by salt degradation, leading to a significant impact on the planet's food supply. It was found that this problem is continuously growing as more territory becomes degraded due to salt accumulation, estimating that nearly 8 miles of cropland is ruined due to salt degradation on a daily basis. The report notes that "the global annual cost of salt-induced land degradation in irrigated areas could be US $27.3 billion because of lost crop production." The issues of salt stress are particularly exacerbated in arid and semi-arid regions where irrigation occurs most (Qadir, 2016). For obvious reasons, seeking means of alleviating the effects of salt stress on agricultural productivity is increasingly necessary as cropland is continuously lost.

Salt is both an osmotic and cytotoxic stressor in plants. Osmotic stress leads to stomatal closure in an attempt to retain water to ameliorate the stress, while cytotoxic stress is caused by the accumulation of toxic ions which affect nutrient uptake from the soil. High salinity causes a two phase process, where in the first stage there is a high osmotic pressure between the root and the soil which causes a slow reaction due to accumulation of Na^+ and Cl^- in the leaves of the plant. This

leads to a reduction in shoot growth in the plant. In the second phase, toxic amounts of Na+ accumulate in the leaves, inhibiting photosynthesis and other processes due to stomatal closure and inhibition of gas exchange that is necessary to provide carbon for synthesis of carbohydrates. Cytotoxic stress can also motivate earlier senescence of plant leaves, which only exacerbates the other issues caused by these stressors (Hanin et al, 2016). Plants use osmotic adjustment to accumulate solutes and use them to decrease osmotic stress. When the ion concentration in the vacuole of plant species increases, solutes (called Compatible Solutes) accumulate in the cytosol to maintain water potential between the two spaces. Compatible solutes are osmotically active, but do not affect membrane structure or enzyme function, which allows plants to tolerate them in high concentrations without harming metabolic processes. One of these compatible solutes is the amino acid proline. When under stressful conditions, proline accumulates in the cell to serve as an osmoprotectant, encouraging water retention and providing protection from toxic byproducts produced under stress. It also serves as a source of carbon and nitrogen when the plant returns to normal conditions (Taiz & Zeiger, 2015). Stressors that can induce proline formation include salt, low temperatures, and heavy metal exposure. It is also utilized by soil fungal species as a nitrogen and carbon source, suggesting there may be valuable effects of proline supplementation on soil mycorrhizae (Chun et al, 2018). For this reason, proline accumulation is observed in plants that have undergone the discussed stressors, allowing for a mitigation of the stress caused. However, the synthesis of proline requires energy as an active metabolic process, which can consume a large amount of carbon: thus, the production of proline and other compatible solutes contributes to the reduction of crop yields. Of particular economic importance is the concern toward reduction of yields in plants of agricultural importance. With the increasing threat of land loss due to salt degradation, it is becoming critical for agriculture to seek methods of improving salt resistance in

plants, or in modification of plant responses to decrease the loss of yield caused by salt stress (Qadir, 2016).

Increased salt concentration in the soil is difficult to combat in the long-term, so many efforts toward ameliorating its effects focus on genetically modifying plants to allow for greater salt tolerance. However, issues arise due to the variability of genetic salt resistance in varying environments, and the fact that "crops adapted to alkali soils are usually tolerant of non-alkaline saline soils", but the opposite is untrue, which can cause issues when introducing plants genetically modified for salt stress to new areas (Shannon and Grieve, 1999). In addition to this, 30 species of plants comprise 90% of the food eaten, and thus genetic modification poses the risk of being simultaneously too broad and too specific to be fully effective (Zörb et al, 2018). Methods of improving salt tolerance via means such as application of proline and other osmolytes merit investigation to determine if they could provide additional assistance in resolving a worsening issue. A study in 2002 by Mani et al investigated the role of Proline catabolism in plants by examining the behavior of transgenic Arabidopsis plants. In that case, the plants that were mutated to have enhanced free proline levels did not show a difference in behavior to unmodified plants. However, the study found that an "addition of exogenous Pro increased survival rates of salt-stressed PDH-S plants by 30%". This study used a concentration of 100 mmol NaCl, at which the growth of control arabidopsis plants was heavily impacted. This motivated the use of this concentration of NaCl in these experiments. The Mani et al study used a lower concentration of proline than this analysis did, only utilizing 10 mmol concentration, but that concentration of proline did not affect growth and success of non-transgenic plants.

In this analysis, a two part study was carried out with tomatoes and cucumbers, known to exhibit high and low salt resistance respectively, to look at the potential effects on plants receiving pretreatment (Cuartero & Fernández-Muñoz, 1999). The pre- treatment supplementations were

delivered via application of varying proline concentrations during watering, to determine any beneficial effects in alleviating salt stress. Proline, also known to serve as a cold stress protectant, was also similarly tested in pretreatments of tomatoes and cucumbers with sodium chloride, proline and 50:50 v/v to see what effect(s) cold stress would have on plants. The objective of this experiment was to determine the impact that providing plants with supplemental proline would have on their development and growth when subjected to stressors such assoil salinity and cold temperatures.

Hypotheses

The Null Hypotheses are that proline addition will have no impact on plants undergoing salt or cold stress. Alternative hypotheses are that the addition of exogenous supplemental proline will help to mitigate the effect of stress from salt and cold on plants.

Methods

In studying the effect of supplemental proline on plant salt tolerance, two species of commonly accessible garden plants were selected, cucumbers and tomatoes. Cucumbers are recognized as highly salt susceptible (Zhu, 2004), with salt stress being one of the leading causes of decreased yield in cucumber farming, leading to a significant impact on farmers' income (Marium et al, 2019). Tomatoes, in contrast, have been found to be more salt-tolerant than many species of fruit-producing plants, and were selected to serve as an additional source of comparison concerning the impact of salt on growth (Shannon and Qualset, 1984). Two experiments were conducted, the first to confirm if there is any sign of effect from proline treatment, and a second to investigate differences between varying concentrations of salt and proline in the watering solutions.

Experiment 1:

Four conditions were used in the first iteration of this study: a) a 100 mmol solution of sodium, b) a 100 mmol solution of proline, c) an equal mixture (50:50 v/v) of these two solutions, and a control solution of distilled water. Plants in the series were watered weekly with 100 mL of the respective solution. In the case of both tomatoes and cucumbers, 4 replications were grown under each of 4 treatments, utilizing a total of 32 plants in the study: 16 tomato plants, and 16 cucumber plants. In the case of the cucumber plants, one seed did not germinate, leading to only 15 cucumber plants being examined in the results of this experiment.

The growth of the seeds used in this experiment was conducted in the UAH greenhouse, with the seeds being planted in mid-September. Temperatures during germination ranged between 29 and 30 degrees Celsius, and during the first two weeks of growth all the plants were given distilled water.

The application of the treatment solutions of salt and proline began one week after all of the seeds (excluding the aforementioned singular cucumber) had sprouted. The applications of these solutions continued until November 12th, a period of 7 weeks. Longer application may be beneficial in future studies to determine the impact of these supplements on fruit production.

Figure 1: Plants prior to application of salt and proline solutions, following 2 weeks of growth. Solutions were applied to the plants at this point.

Following 6 weeks of Salt stress analysis, one sample plant of each species and treatment group was isolated and subjected to 24 hours of cold stress via being placed into refrigeration at 1.1 degree Celsius. Comparisons were drawn between the plants subjected to this stress and those remaining in the greenhouse.

Figure 2: Plants prior to cold stress. From left to right, the plants shown are the control batch, the salt treated batch, the batch treated with both salt and proline, and the batch treated only with proline.

Experiment 2:

Another series of experiments were performed the following summer, with an increased array of conditions to further investigate the effect that proline supplementation had on varying levels of salt stress. Again, treatments were performed on both cucumbers and tomatoes, with 4 pots allocated to each treatment for each plant (for a total of 96 pots). The following combinations of salt and proline were utilized:

Table 1: Solution Treatments

200 mmol Sodium 100 mmol Proline	100 mmol Sodium 100 mmol Proline	50 mmol Sodium 100 mmol Proline	No Sodium 100 mmol Proline
200 mmol Sodium 25 mmol Proline	100 mmol Sodium 25 mmol Proline	50 mmol Sodium 25 mmol Proline	No Sodium 25 mmol Proline
200 mmol Sodium No Proline	100 mmol Sodium No Proline	200 mmol Sodium No Proline	Control - Only Water

Once again, plants were watered with 100 mL of solution, utilizing equal combinations of these solutions. A hardening process was utilized, beginning by watering with lower molarities and working up to the intensities above. Additionally, during this secondary experiment, the treatment applications were rotated with dosages of only water, to mitigate the likelihood of solute accumulation in the soil that could lead to skewed results. The samples were planted in early May of 2021, and allowed to grow for a full month prior to treatment. There was a lack of germination in some cases (particularly the cucumbers), leading to reduced numbers of test cases for some treatment types, though there was a minimum of 2 of each plant undergoing each treatment. The hardening process began at the end of the month of May, with a 20% increment of concentration until reaching the total desired molarity. The plants grew while receiving treatments for 2 months, with data collection at the end of July. Due to the increased temperatures of this trial period (summer temperatures in Alabama, ranging up to 36°C), watering and treatments were conducted every other day rather than once

weekly.

Results

Experiment 1

Figure 3: plants after 6 weeks of growth. From left to right: control study, salt stressed plants, plants receiving mixed solution of salt and proline, plants receiving only proline.

In the image it can be clearly observed that the cucumbers that were subjected to salt stress with no alleviation are smaller than the other 3 treatments, averaging a height of approximately 1.5 inches while the controls, the plants receiving a mix of salt and proline, and those receiving only proline were approximately 5 inches in height. All the cucumber plants, including those receiving salt stress, developed blossoms that indicated that yield would not be completely compromised by the stress. There was no discernible difference between the plants receiving proline treatment and the controls during this stage of the experiment. In the tomato plants, there was less discernible difference between the treatments. This is due to the higher salt tolerance of tomatoes as compared to cucumbers, and indicates that the 100 mmol concentration of salt did not break the tomatoes salt tolerance threshold. In future experiments, it could be worthwhile to increase the concentration of salt used to observe whether the same behavior occurs in tomatoes, and to determine whether the ameliorating effects of proline supplementation continue to function at higher sodium concentrations. This would also help in determining whether proline supplements are a viable tool for helping in areas of varying salinity. The NaCl treated plants presented a higher degree of leaf loss than any of the other three treatments, indicating the impact of NaCl accumulation on leaf senescence was at work to some degree.

Figure 4: Plants from all treatments post 24 hours of cold stress in a 1.1 degree C refrigerator. Top left image is control, top right is salt treated, bottom left is treated with salt and proline, bottom right is treated only with proline.

Proline in the concentration used did not appear to impact the plants' reaction to cold stress. However, the tomatoes subjected to salt stress appeared to have a slightly more severe response (both in the case of those treated only with salt and with a combination of salt and proline) than those that did not undergo any salt exposure. The four cucumber treatments appeared to have similar responses to cold stressing.

Experiment 2

Table 2A: Heights and Branching information of Tomatoes

Treatment	Average Height (mm)	Height Percent Variance from Control	Standard Deviation	Average Branches
200 mmol Sodium 100 mmol Proline	20.75	-17%	2.9	6
200 mmol Sodium 25 mmol Proline	26	4%	2.4	7.25
200 mmol Sodium No Proline	12.75	-49%	2.5	4.75

100 mmol Sodium 100 mmol Proline	28.25	13%	1.7	6.25
100 mmol Sodium 25 mmol Proline	26.5	6%	2.1	6.25
100 mmol Sodium No Proline	20.5	-18%	3.0	5.75
50 mmol Sodium 100 mmol Proline	25.25	1%	4.6	8
50 mmol Sodium 25 mmol Proline	30.75	23%	2.2	6.75
50 mmol Sodium No Proline	26.25	5%	9.43	6.25
No Sodium 100 mmol Proline	26.75	7%	3.9	8.75
No Sodium 25 mmol Proline	29	16%	2	7
Control	25	0%	3.7	7.5

Table 2B: Heights and Branching information of Cucumbers

Treatment	Average Height (mm)	Standard Deviation	Average Branches
200 mmol Sodium 100 mmol Proline	15.3	3.5	7
200 mmol Sodium 25 mmol Proline	20.3	2.1	8.6
200 mmol Sodium No Proline	16	7.1	6.5
100 mmol Sodium 100 mmol Proline	22	2.8	9
100 mmol Sodium 25 mmol Proline	25.6	1.2	8
100 mmol Sodium No Proline	24.5	4.9	7.5

50 mmol Sodium 100 mmol Proline	18	1.4	6
50 mmol Sodium 25 mmol Proline	31.7	4.7	9.7
50 mmol Sodium No Proline	17.25	6.0	7.25
No Sodium 100 mmol Proline	17.5	6.0	9
No Sodium 25 mmol Proline	17.5	0.9	8
Control	9.2	0.8	5.6

Observing Height Variance based on Controls for Cucumbers would be ineffective, due to underperformance of the Cucumber Controls in general.

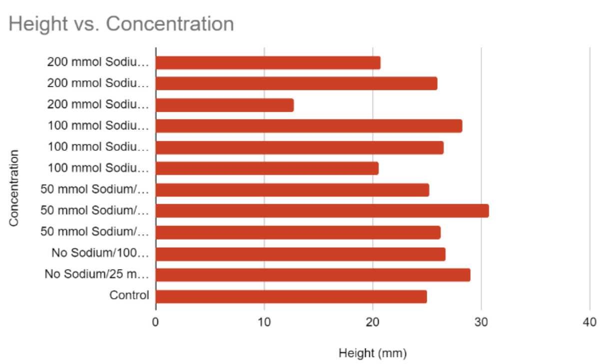

Figure 4A: Height Comparison of Tomatoes at Varying Concentration Treatments

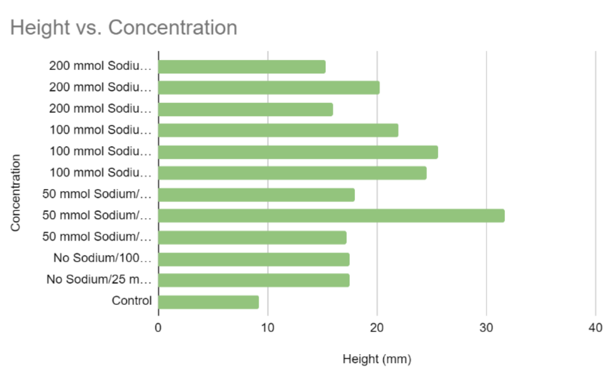

Figure 4B: Height Comparison of Cucumbers at Varying Concentration Treatments

Figure 5: Tomatoes (Left) and Cucumbers (Right) watered with a solution combining 50 mmol saline and 25 mmol Proline

Figure 6: Tomatoes (Left) and Cucumbers (Right) watered with a solution combining 100 mmol saline and 100 mmol Proline

Figure 7: Tomato (top) and Cucumber (bottom) controls

Conclusion

The categories that showed improved performance when compared to the controls were combinations of 50 mmol saline/25mmol proline and 100 mmol saline/100 mmol proline (see figures 5, 6, and 7), while many other tested categories had comparable results to the control value.

Combinations of 200 mmol Saline/100 mmol Proline, 200 mmol Saline/No Proline, and 100 mmol Saline/No Proline showed significantly lower average heights when compared with the controls for tomatoes, while Cucumber controls showed poor growth overall and thus had a lower average height than any of the treatments. In both species, plants showed the greatest improvement at a combination of 50 mmol of Sodium solution and 25 mmol of proline solution. This is likely due to the improvement to salt stress caused by proline supplementation, and exceeds the behavior of only-water samples due to providing increased osmotic pressure within the plants, allowing them to more efficiently uptake water and other resources. It is notable that in all cases, plants that were receiving sodium treatment *without* supplemental proline performed more poorly than those receiving the same molarity of sodium along with supplemental proline, and that throughout the series 25 mmol of proline provided greater improvement than 100 mmol of proline. This is

indicative of proline's beneficial impact, but suggests that over-supplementation of proline may lead to issues as well, so provision of a moderate level is more effective than strongly dosing crops.

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