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Article

The impacts of different $Al(NO_3)_3$ concentrations on the mitotic index of *Allium sativum*

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SUMMARY

Acid deposition, produced when NOx and SO_2 emissions react with water to produce HNO_3 and H_2SO_4, is regarded as a significant threat to biodiversity and agriculture. Soil pH conditioners have been developed to counteract this phenomenon; however, they sometimes fail to reverse nutrient imbalances caused by acid deposition. One of the problematic nutrient imbalances is the formation of aluminum nitrate, which dissociates to produce toxic Al^{3+} ions. In Basque country (Spain), a recent surge in metallurgical activities and the popularization of climate change denial threatens a drastic rise in NOx emissions. We explored whether returning to peak emissions of NOx, such as those in 1990, will increase $Al(NO_3)_3$ concentrations to a level where they significantly lower the mitotic index (the ratio of cells undergoing mitosis in a specific population of cells) of one of the most culturally significant vegetables of the region, *Allium sativum*. We grew cloves of *A. sativum* at different concentrations of $Al(NO_3)_3$. We processed tips of these cloves and placed them under a microscope to calculate their mitotic index. We hypothesized that a larger concentration of $Al(NO_3)_3$ would lower the mitotic index of *A. sativum*, since $Al(NO_3)_3$ dissociates in water to produce Al^{3+} ions, which are highly toxic as they inhibit cell division and elongation. Our results showed a negative exponential correlation between $Al(NO_3)_3$ and the mitotic index of *A. sativum*, as the higher the $Al(NO_3)_3$ concentration, the lower the mitotic index.

INTRODUCTION

International organizations have prioritized most of the globally damaging pollutants, such as ozone-depleting gases like chlorofluorocarbons (CFCs), hydrofluorocarbons (HFCs), and hydrochlorofluorocarbons (HCFCs); however, locally harmful contaminants such as NOx and SO_2 have flown under the radar of these organizations, and thus their regulation has been appointed to the individual countries (1,2). The popularization of climate change denial and the uprising of political parties that ignore environmental problems threaten an increase in pollutant emissions in Spain (3,4). Acid deposition has posed severe threats to the Basque countries' forests and especially agriculture, as the acid leaches nutrients and leads to soil infertility (5,6). To make matters worse, the low pH directly damages plant leaves, roots, and the microbiota that form symbiotic relationships with the local crops (7). To mitigate this, soil pH conditioners are frequently used to balance the low soil pH caused by acid rain, however, these soil conditioners fail to restore nutrients leached by acid rain, and also fail in reverting any toxic byproduct, that may have been formed as a result of the low pH (8,9). One of the most prominent toxic byproducts is aluminum nitrate $Al(NO_3)_3$, formed when insoluble mineral aluminum hydroxide (Al(OH)), reacts with nitric acid.

The $Al(NO_3)_3$ then dissociates to produce Al^{3+} ions, which are known for being toxic to plants as they inhibit root growth (10,11). This investigation aimed to discover whether an increase in NOx emissions, similar to those present in 1990 Basque country, could produce a sufficiently high concentration of $Al(NO_3)_3$ in soil for it to significantly impact the root apical (root tip) mitotic index of *Allium sativum* (commonly known as garlic). *A. sativum* is of great importance to Basque agriculture and tradition, as it is the protagonist of the Apostle Santiago festival (12).

We hypothesized that a larger concentration of $Al(NO_3)_3$ would lower the mitotic index of *A. sativum* as it dissociates in water to produce Al^{3+} ions, which are highly toxic as they inhibit cell division and elongation (11). However, it is possible that *A. sativum* is immune to $Al(NO_3)_3$ toxicity. This could be possible as it has shown to be capable of curing $Al(NO_3)_3$ poisoning in mammals (13), and there exists a wide range of Al^{3+} sensitivity amongst plants (14). It is also possible that the theoretical concentrations of $Al(NO_3)_3$ calculated to be formed in the soil (after the acid rain reacts with Al(OH)) are too low to create any significant impact on the mitotic index of *A. sativum*. In this case, the impact of a decrease in rain pH would be of little importance for *A. sativum*.

To test our hypothesis, we grew *A. sativum* at 4 four different $Al(NO_3)_3$ concentrations, each concentration corresponding to an acid rain pH, ranging from the average rain pH in Basque country´s least affected areas in 1889 (pH 4.9) to the average of the most affected areas in 1889 (pH 4.2), and an intermediate between the two (pH 4.5) (15). With these pH's and with the ratio of NOx to SO_2 emissions in Spain (39.56:60.43) (16), we calculated the theoretical concentrations of $Al(NO_3)_3$ present in the soil post-acid deposition (4.2/4.5/4.9) to be: 7.96×10^{-6} mol/L / 3.84×10^{-6} mol/L / 1.33×10^{-6} mol/L, respectively. Our results suggest a negative exponential correlation between $Al(NO_3)_3$ and the mitotic index of A. sativum, as the higher the $Al(NO_3)_3$ concentration, the lower the mitotic index. In other words, $Al(NO_3)_3$ negatively impacts *A. sativum*.

RESULTS

We used 32 ungerminated garlic cloves, 8 for each of the 4 $Al(NO_3)_3$ concentrations, and after nine days, we processed the tips of the roots of the garlic cloves, and examined them under a microscope to calculate the mitotic index

(Figure 1 and 2). We calculated the mitotic index by counting the number of root-tip cells observed with condensed chromosomes in defined patterns characteristic with mitosis, and then dividing this by the total number of cells observed **(Figure 3)**. Only the tips of the roots were used because mitosis occurs irregularly across the root, with the root's meristem (the root tip) containing the highest rate of mitosis (17). Thus with the higher rate of mitotically active cells, the effects of $Al(NO_3)_3$ would be more visible. We calculated the average mitotic index for each concentration and the standard deviation

Garlic bulb No 5 did not grow roots on any of the $Al(NO_3)_3$ concentrations, including the control group, consequently it was considered to be incapable of producing root meristems, thus it was not included in the processed data.

Furthermore, there appeared to be a negative correlation between the average mitotic index of *A. sativum* and $Al(NO_3)_3$ concentration **(Figure 4)**. The cloves grown in the solution containing the highest $Al(NO_3)_3$ concentration showed the lowest mitotic index of all, with an average of 0.519. This trend continued with the solution containing the second-highest $Al(NO_3)_3$ concentration resulted in the second-lowest mitotic index (0.949). The solution containing the third-highest concentration resulted in the third-lowest mitotic index (1.696), and finally, the solution containing no

Figure 1: Example of experimental setup. Each clove has a number beside it indicating from which bulb they originated. Cloves were pierced with a toothpick and their bases were submerged 1 cm into the water containing the $Al(NO_3)_3$.

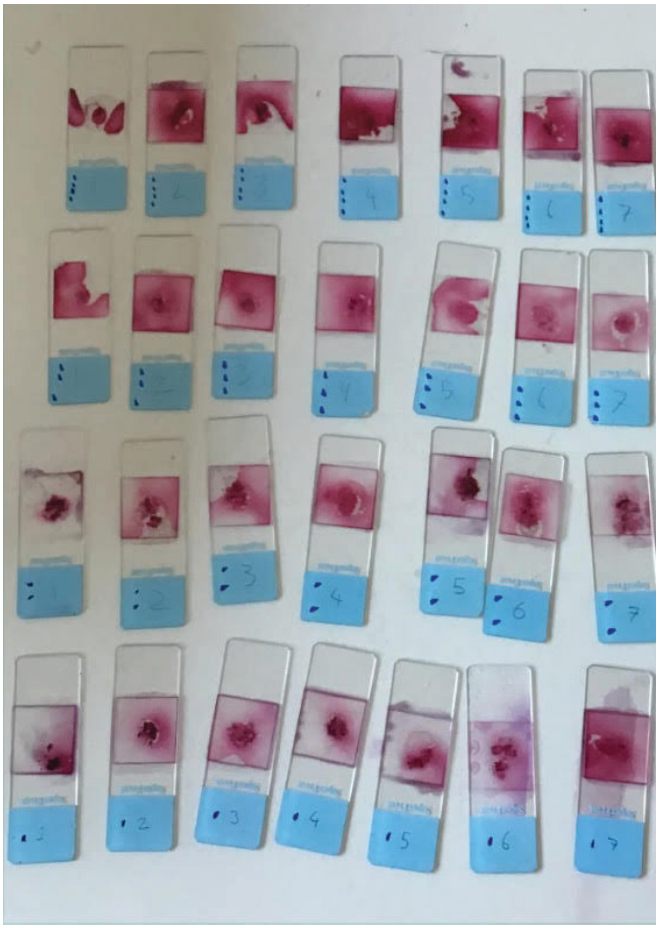

Figure 2: Slides after the Orcein DNA staining process. There are 4 rows representing different concentrations, and each row contains 7 columns representing the trial numbers. Therefore, there are a total of 28 slides.

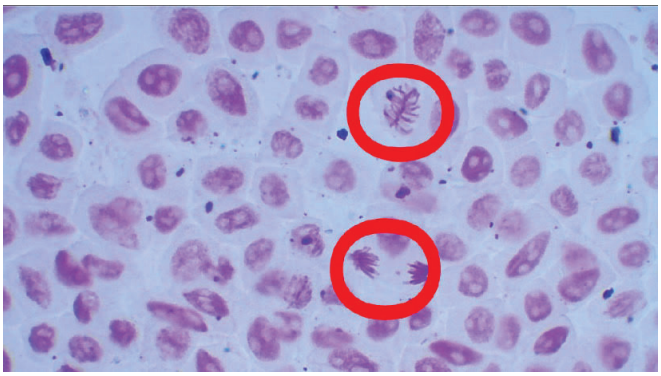

Figure 3: Example image of cells undergoing mitosis. 2 cells were undergoing mitosis (circled in red), whilst the image showed a total of 103 cells, thus the mitotic index was calculated to be 1.94%, 40x magnification.

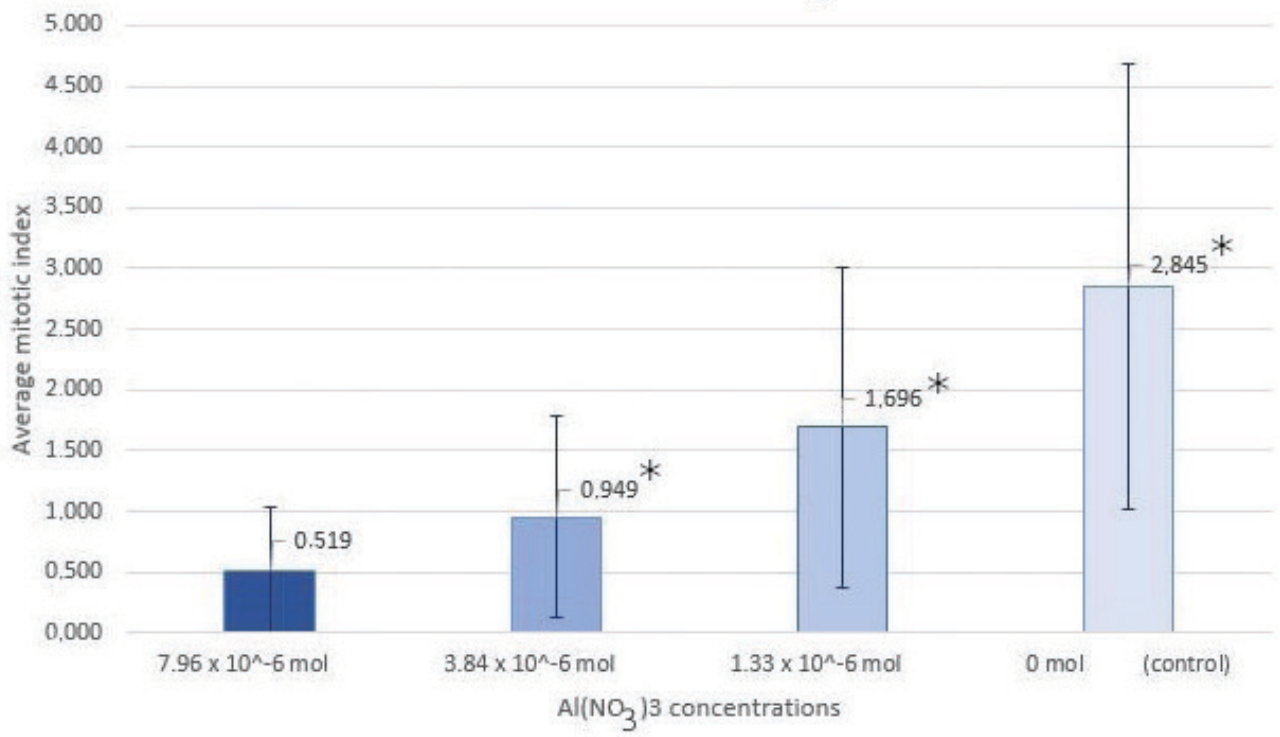

Figure 4: More concentrated $Al(NO_3)_3$ solution reduced the mitotic index of *A.sativum* root cells. Mean mitotic index is displayed above each bar for each $Al(NO_3)_3$ concentration, with the standard deviation between trials represented by the error bars. One-way ANOVA, ***$p < 0.001$.

$Al(NO_3)_3$ (control) resulted in the highest average mitotic index (2.845). Despite the average mitotic index showing a decreasing trend as $Al(NO_3)_3$ concentration increased, it should be noted that the range of mitotic indices for each concentration varies significantly. This is demonstrated by the large standard deviations, represented by the error bars **(Figure 4)**. However, after performing a one-way ANOVA and a Tukey Kramer procedure, all the average mitotic indices were statistically significant from the control groups, with all concentrations having $p < 0.001$, thus supporting the claim that the $Al(NO_3)_3$ concentrations formed by acid deposition in the Basque country would have significant negative impacts on the mitotic index of *A. sativum*.

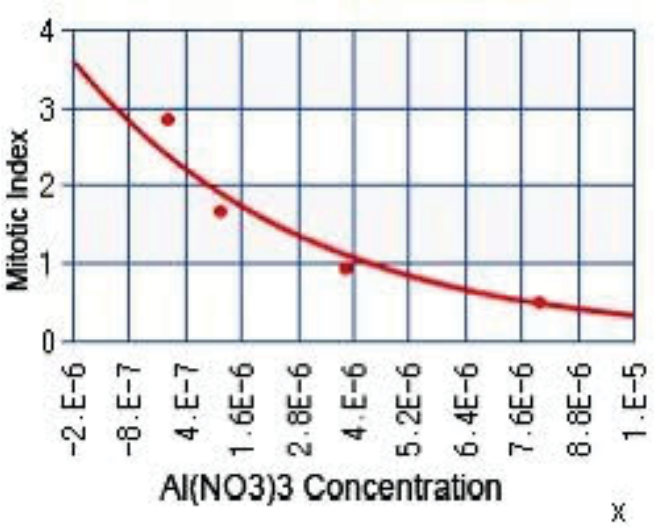

Figure 5: Scatter plot of obtained data plus the model regression function. The e-exponential function $y = 2.391e^{-202,799.801x}$ best modelled the impact of the different $Al(NO_3)_3$ concentrations on the mitotic index of *A. sativum*.

To understand the trend, we performed a regression analysis on the results we obtained. We used four types of functions to observe which model function best modeled the obtained results. The four model functions were linear, quadratic, e-exponential, and logarithmic. Of the four, the e-exponential model (with the function $y = 2.391e^{-202,799.801x}$) **(Figure 5)** showed the strongest correlation coefficient, meaning it more accurately represented the obtained data. Thus, it can be concluded that $Al(NO_3)_3$ negatively influences the mitotic index of *A. sativum* between the concentrations of 0 mol/L and 7.96×10^{-6} mol/L, following a negative e-exponential pattern.

DISCUSSION

The goal of this study was to determine the effect that different concentrations of $Al(NO_3)_3$ would have on the mitotic index of *A. sativum*. We hypothesized that a higher $Al(NO_3)_3$ concentration would result in a decrease in the mitotic index, because other researchers have shown that aluminum ions are toxic to other plant species. Our hypothesis was supported, as we observed a statistically significant negative correlation between $Al(NO_3)_3$ concentration and *A. sativum* mitotic index.

There are two specific types of limitations in this experiment. The first type involves limitations that hinder

the ability to extrapolate the information gathered in this investigation to the Basque country´s real-life agricultural scenario, such as growing the garlic in water rather than soil, in which it is normally grown. Another limitation was not implementing any other nutrients or chemicals to the water in which the garlic cloves were grown, as garlic crop fields tend to contain other chemicals such as fertilizers, pesticides, herbicides, and soil conditioners. These chemicals could reduce the impact of $Al(NO_3)_3$ by reacting with it to produce a less harmful pollutant, analogous to a buffer, thus reducing its impact on *A. sativum*. The temperature, lighting, and humidity used could also hinder the experiment's applicability to the real-life problem, as the garlic cloves were grown in conditions that are not common in the Basque country: higher temperature (28°C), higher humidity (74%) and higher light intensity (18). These factors could have influenced the growth rate of *A. sativum*, and thus, influenced its mitotic index. However, because these conditions were kept equal for all trials, they do not impact the precision of the results.

Several assumptions have been made while performing this investigation, assumptions which could damage the extrapolation of this investigation's data to the real-life Basque agricultural scenario. Most assumptions were made in the process of calculating the theoretical nitric acid concentration in the different acid rain pH values, this concentration is important as it will help calculate the theoretical concentration of $Al(NO_3)_3$ that would be formed in the soil. We calculated the nitric acid (HNO_3) concentration by converting the three rain pH values to H^+ ion concentration. With the resulting proton concentration, 39.56% of the protons were assumed to be from HNO_3, as 39.56% of acid deposition pollutants originate from NOx, with the other 60.43% being H_2SO_4, being formed from SO_2. With this we assume that NOx reacts with water to produce HNO_3, at the same rate as SO_2 reacts with water to produce H_2SO_4, this assumption had to be made as no information was found regarding the percentage of emitted NOx that reacts to produce HNO_3 vs the percentage of emitted SO_2 that reacts to form H_2SO_4 (19). This assumption may have led to either an overestimation of $Al(NO_3)_3$ in the soil (if SO_2 reacts with water to produce H_2SO_4 more readily than NOx, or an underestimation (if NOx reacts with water to produce HNO_3 more readily than SO_2). Having now calculated the theoretical HNO_3 concentration, the stoichiometric ratio of the Al(OH) + HNO_3 reaction can be used to calculate the concentration of $Al(NO_3)_3$ that would be present in the soil. However, this concentration is obtained by assuming that HNO_3 is the limiting reactant and that the reaction goes to completion (100% product, 0% reactants), of course this is not how reactions usually occur, and this assumption probably lead to an overestimation of $Al(NO_3)_3$ concentrations.

This investigation focused on the effect that different $Al(NO_3)_3$ concentrations would have on the mitotic index of *A. sativum*; however, other aluminum ionic compounds are also formed when soil pH is lowered (such as aluminum sulfate, which also dissociates to produce Al^{3+}), meaning the Al^{3+} concentration would likely be much higher than this experiment has considered (20). Thus, the impact of a lower rain pH would be much more severe than this investigation has interpreted, as these additional Al^{3+} ions would also be toxic to plant root systems. Furthermore, we only measured the effects of $Al(NO_3)_3$ on *A. sativum*; however, the mineral imbalances caused by acid deposition do not solely impact *A. sativum* cropland. It also affects soil quality in forests, parks, and other croplands, which are populated by a variety of other plant species, with each possibly having different sensitivities to Al^{3+} ions.

Finally, the obtained mitotic indices were compared to the mitotic indices obtained from published literature. However, because there are no other investigations researching the effect of aluminum nitrate on *A. sativum*, only the mitotic index of the control group could be compared to other published values. In one study by Shaymurat *et al.*, the mitotic indices obtained in the control group fell near the 9% mark, far from my obtained average of 2.845% (21). It is possible that this discrepancy is due to the fact that their root tips were only 24h hours old, compared to the tips used in this investigation which were 9 days old. Thus, the younger age correlated to a more active root meristem. It is also possible that Shaymurat and his team were more experienced in identifying cells going through mitosis, and thus identifying more cells and obtaining a higher index.

MATERIALS AND METHODS

Eight *A. sativum* bulbs from the brand “Ajos Pittirri” were purchased one day before experimentation. This brand was chosen as it cultivates garlic in the northern central region of Spain (Portillo) near the Basque country. This would make the experimental results more applicable to the Basque agricultural scenario, as garlic from different regions or countries may vary in $Al(NO_3)_3$ sensitivity.

Eight trials were performed for each $Al(NO_3)_3$ concentration. Each trial consisted of a garlic clove from one of the eight garlic bulbs purchased. Thus, each concentration had one clove from each of the eight bulbs. This was done to maintain fair testing, as the slight genetic diversity between the bulbs could potentially cause them to be either more resistant or susceptible to $Al(NO_3)_3$.

The $Al(NO_3)_3$ concentrations were calculated by first calculating the theoretical nitric acid concentration in the rain, which in turn was calculated by converting the three rain pH values to hydrogen ion concentration, for example (4.2), $10^{-4.2}$ = 0.0000631 mol. Because rain already has a low pH (5.6), due to CO_2 naturally present in the atmosphere, 0.000002511 mol/L ($10^{-5.6}$) was subtracted from 0.0000631 mol/L, as these protons would be originating from the carbonic acid formed, and carbonic acid does not react with Al(OH) to produce $Al(NO_3)_3$. With the resulting proton concentration (0.0000606 mol/L), 39.56% of the protons were assumed to be from HNO_3, as 39.56% of acid deposition pollutants originate from NOx, with the other 60.43% being H_2SO_4, being formed from SO_2. Having now calculated the theoretical HNO3 concentration in the 4.2 acid rain (0.0000239 mol x /L), the stoichiometric ratio of the Al(OH) + HNO3 reaction was used to calculate the concentration of $Al(NO_3)_3$ that would be present in the soil, that being 7.96 x 10^{-6} mol /L.

To treat the garlic cloves with $Al(NO_3)_3$, the solutions were prepared and placed into 40 mL plastic cups, the cloves were then impaled with a toothpick so that the base of the clove would hover in the $Al(NO_3)_3$ solution.

To measure the mitotic index of the *A. sativum* roots, the following procedure was conducted to stain the nucleus of the cells, thus allowing us to determine whether a cell was in mitosis or not. First, 2 mm from the tip of the two longest roots of each clove were cut and placed on a watch glass. Following

this, 0.5 ml of Orcein A was pipetted on top of the two root tips. The tips were then placed on a hotplate for 5 minutes until the Orcein A evaporated. The tips were then placed on the center of a microscope slide (at 40x magnification), where a drop of Orcein B was applied. One minute after applying the Orcein B, a cover glass was placed on top of the roots and pressure was applied to flatten the roots, and for light to be able to pass through.

For the calculation of the mitotic index, any cell which contained visible chromosomes was considered to be undergoing mitosis and any round dark purple circle (the nucleus) was considered a cell. This information was necessary to calculate the mitotic index with the following formula:

$$\frac{Number\ of\ cells\ undergoing\ mitosis}{Total\ number\ of\ cells} \times 100$$

Mitosis occurs irregularly across the root, with the root's meristem containing the highest rate of mitosis (19). Thus, the location where the microscope pictures are taken could drastically influence the experiment's results. Therefore, three pictures were taken for each slide. Each picture was randomly taken near the tip of the flattened root. This was done to reduce the random error produced by the uneven distribution of cells undergoing mitosis. To ensure true randomness, a system was devised which consisted of localizing the center of the root tip. Then, utilizing a random number generator, a random number was selected indicating the millimeter to move the microscope stage, first horizontally then vertically. Afterwards, a picture of the frame was taken. If this resulted in an image where there were no cells or the nuclei of the cells could not be distinguished from each other, then the process would be repeated with new random values.

Statistical Analysis

An ANOVA test with a Tukey Kramer correction procedure was conducted. An alpha level of 0.05 was taken as significant. Calculations were performed manually using Google Sheets.

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