

Influence of Nitrogen Deficiency on Ureide Mediated Heavy Metal Stress Resistance in *Arabidopsis thaliana*

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Abstract

The relationship between nitrogen availability and presence of heavy metal stress appear to affect development and growth of *Arabidopsis thaliana* plants. In this work, *Arabidopsis thaliana* seedlings were exposed to media containing varying concentrations of nitrogen in form of NH_4NO_3 or KNO_3 and cadmium in the form of CdCl_2 . When exposed to increasing cadmium stress, *aln* mutant lines, lacking a functional allantoinase enzyme, displayed enhanced growth and development when compared to wild type, most likely due to their high allantoin content. Increasing nitrogen concentrations appeared to decrease the amount of senescing tissue caused by exposure to cadmium stress. Growth appeared to be slightly increased in both wild type and *aln* mutant lines grown in media containing KNO_3 as the nitrogen source, thereby supporting the importance of nitrogen source on plant growth.

Keywords: nitrogen deficiency, *arabidopsis thaliana*, heavy metals, cadmium stress

Introduction

The growth and development of a plant primarily depends on the availability of a few key macronutrients such as carbon, oxygen and hydrogen. In general, plants are able to obtain these macronutrients from the atmosphere and surrounding water sources. However, an essential and potentially restrictive macronutrient for a plant is nitrogen (Vidal and Gutierrez, 2008). Nitrogen holds great significance in plants, as it is a major element of chlorophyll, the molecule responsible for producing energy for the plant via absorption of sunlight (Sanchez-Zabala et al., 2015). In addition, nitrogen is also a major building block for nucleic acids and amino acids, and

plays key roles in controlling gene expression (Vidal and Gutierrez, 2008).

The model plant *Arabidopsis thaliana* (*A. thaliana*) uses the purine oxidation pathway, otherwise referred to as the ureide metabolic pathway, to degrade purine or pyrimidine bases into functional nitrogen sources such as ammonium (Werner and Witte, 2011; Yang and Han, 2004). This pathway consists of a series of enzymatic reactions involving 10 different intermediates (Werner et al., 2013). Many of the intermediates associated with this pathway, specifically allantoin, appear to be nitrogen-rich and therefore serve an important role in nitrogen assimilation, transport and storage throughout the shoots and leaves of the plant (Yang and Han, 2004). In particular, the *AtALN* gene

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encoding for the allantoinase enzyme is responsible for synthesizing allantoin from allantoin. When mutated, the *AtALN* gene results in defective allantoinase, thereby triggering elevated allantoin levels that may be associated with increased tolerance to abiotic stresses (Watanabe et al., 2014). Watanabe et al. (2014) have proposed that increased allantoin levels may activate abscisic acid (ABA) metabolism, which in turn up-regulates various drought and osmotic stress response genes.

Although abiotic stress responses in *A. thaliana* loss of function mutants such as those of xanthine dehydrogenase (*XDH*), allantoinase (*ALN*) and allantoin aminohydrolase (*AAH*) have been studied, little work has been done on the effect of cadmium as an abiotic stress and the interaction that cadmium may have with critical plant nutrients such as nitrogen. Cadmium is a toxic heavy metal that is most commonly found in environments subject to anthropogenic pressure (Sanita di Toppi and Gabbriellini, 1999). It is rapidly taken up by plant roots and accumulated in plant tissue altering the rate of photosynthesis, uptake of nutrients and water use efficiency (Gill and Tuteja, 2011; Perez-Chaca et al., 2014). Exposure to cadmium also results in increased accumulation of hydrogen peroxide and reactive oxygen species (ROS) that serve as regulators of programmed cell death (Maksymiec and Krupa, 2006; Gechev et al., 2005). Concentrations of ROS are often dose and time dependent, therefore higher levels of cadmium result in increased death of plant tissue (Perez-Chaca et al., 2014).

The purpose of this study was to analyze the interaction between varying concentrations of environmental nitrogen and cadmium, and determine how this interaction may affect the growth of wild type (WT) and mutant *aln* *A. thaliana* seedlings. Nourimand and Todd (2016) have observed enhanced growth of *aln-3* mutant lines in both agar and soil environments subjected to cadmium. In addition, the increased tolerance of *aln* mutants to other abiotic stresses such as high osmoticum and enhanced drought has been attributed to their high levels of allantoin (Watanabe et al., 2014). It has also been noted that increased nitrogen levels decrease the amount of cadmium uptake by plant roots (Konotop et al., 2012). Based on the available literature, *aln* mutants were hypothesized to display better growth and enhanced tolerance to cadmium stress when compared to wild type plants, due to the characteristic allantoin accumulation observed in *aln* mutant lines.

Materials and Methods

Plants and Growth Conditions

A. thaliana wild type (WT) and mutant *aln* lines were used in this work. The SALK T-DNA insertion line, *aln* was obtained from the Arabidopsis Biological Resource Center at Ohio State University. Surface sterilized seeds from both WT and transgenic plants were grown on 9 cm plates containing Murashige and Skoog (MS) basal salts, 0.5% sucrose, 1.2% agar and varying concentrations of both nitrogen and cadmium. Nitrogen was provided in the form of ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) in concentrations of 0 mM, 11.25 mM and 22.5 mM, while cadmium was provided in the form of cadmium chloride (CdCl_2) in concentrations of 0 μM , 50 μM and 100 μM . Following incubation at 4 °C for two days, plates were placed in a growth chamber with a light intensity of 70 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for fourteen days at 22 °C with a photoperiod of sixteen hours as described by Nourimand and Todd (2016).

Phenotypic Comparisons and Average Root Length Measurement

A. thaliana plants from each treatment were compared visually based on size, color, root length and root branching. Roots of *A. thaliana* plants were measured using Image J software version 1.46r. Average root length was measured and recorded in centimeters.

High Performance Liquid Chromatography

Following the protocol described by Nourimand and Todd (2016), approximately 60 mg of fresh tissue was sampled and homogenized with distilled water using a mortar and pestle. Following centrifugation at 2 °C at maximum speed (14,000 rpm) for 25 minutes, supernatant was removed and dispensed through a 3 mL syringe with a 13 mm filter (VWR International) into a 1.5 mL HPLC vial. Samples were run in an Agilent Technologies 1200 series HPLC with an Aminex HPX-87H Ion Exclusion column, using diluted sulfuric acid (H_2SO_4) solution consisting of 0.0025 N H_2SO_4 and distilled H_2O for 55 minutes. Concentrations of allantoin within the samples were compared to standard curves generated from pure allantoin (Sigma-Aldrich).

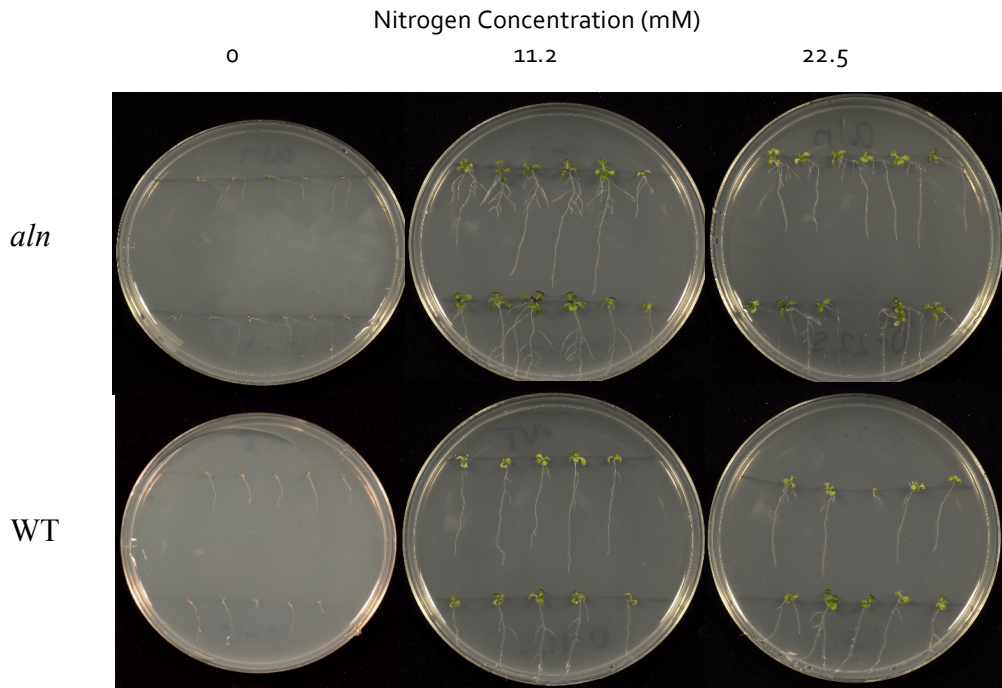


Figure 1: WT and *aln* *Arabidopsis* plants treated with varying concentrations of nitrogen (supplied as NH_4NO_3) in the presence of 0 μM cadmium. Representative images are shown.

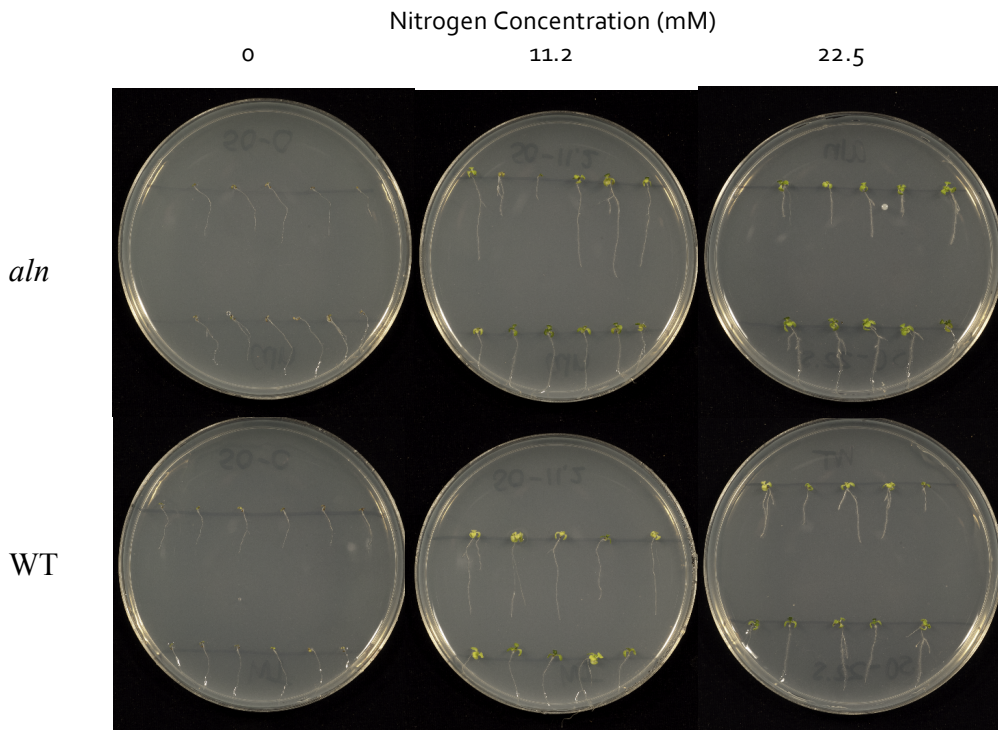


Figure 2: WT and *aln* *Arabidopsis* plants treated with varying concentrations of nitrogen (supplied as NH_4NO_3) in the presence of 50 μM cadmium (supplied as CdCl_2). Representative images are shown

Statistical Analysis

Data shown in figures are the means of three independent replicates, with exception to the seedlings grown in KNO_3 , which comprised only two independent replicates. Both means and \pm SEM values were calculated using Microsoft Excel.

Results

Arabidopsis WT and *aln* plants were plated on media containing varying concentrations of nitrogen at 0 mM, 11.2 mM or 22.5 mM and 0 μM , 50 μM or 100 μM of cadmium. Following a 14-day growth period, plants were compared visually based on size, tissue color and root length. When grown in the absence of cadmium, both WT and *aln* plants appeared to have better growth. However, when grown in the absence of nitrogen, both WT and *aln* mutants showed minimal growth. Furthermore, when treated with 11.2 mM and 22.5 mM nitrogen, *aln* mutants

were observed to have slightly better growth with increased root branching and lack of yellowing tissue, a hallmark of senescence (Figure 1).

Moreover, when grown in the presence of 50 μM cadmium, WT and *aln* mutants appeared to have very similar growth and color. In the absence of nitrogen, minimal growth was observed for both WT and *aln*. However, when treated with 11.2 mM and 22.5 mM nitrogen, *aln* mutants displayed increased root branching in comparison to WT (Figure 2).

Furthermore, when grown in the presence of 100 μM cadmium, drastic differences were observed in growth and health of WT and *aln* plants. In the absence of nitrogen, both WT and *aln* seeds were incapable of germinating, thereby producing no growth (Figure 3). However, when treated with 11.2 mM and 22.5 mM nitrogen, *aln* mutants displayed significantly better growth and very little tissue yellowing in comparison to WT (Figure 3).

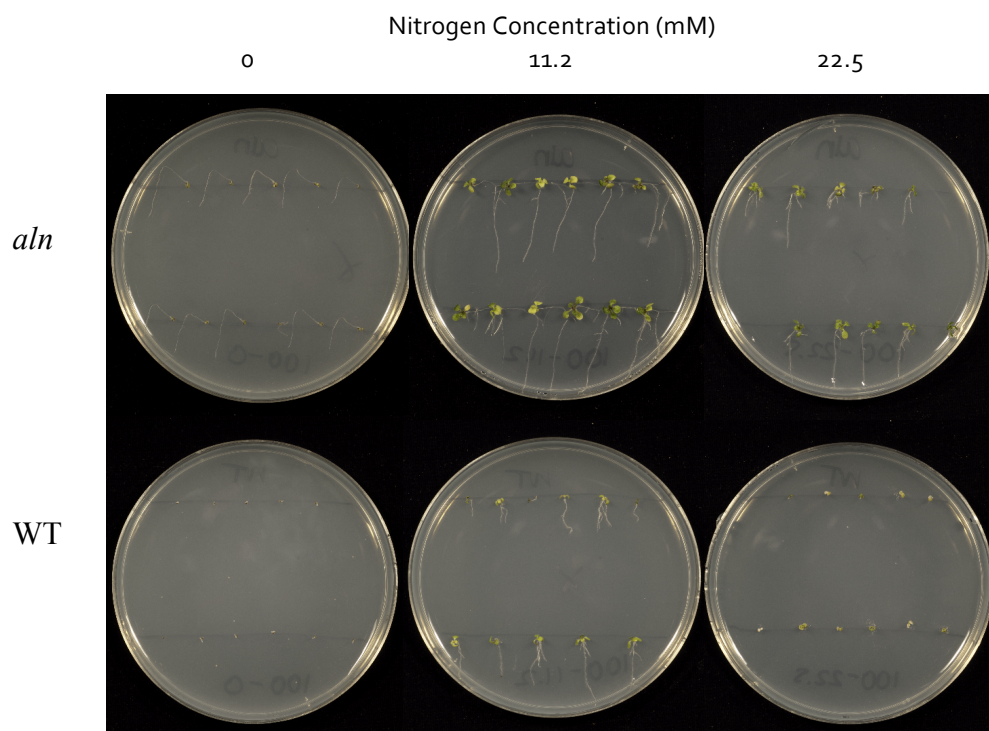


Figure 3: WT and *aln* *Arabidopsis* plants treated with varying concentrations of nitrogen (supplied as NH_4NO_3) in the presence of 50 μM cadmium (supplied as CdCl_2). Representative images are shown

Lastly, when comparing growth of WT and *aln* plants grown at 50 μM cadmium and 11.2 mM and 22.5 mM nitrogen in the form of KNO_3 , very similar growth was observed with distinct differences only found in root length. Both WT and *aln* appeared to be yellow in color.

Average root length was quantified using Image J software, allowing for numerical comparisons between WT and *aln Arabidopsis* plants treated with varying concentrations of nitrogen and cadmium. In general, root lengths between the two genotypes appeared to be more distinct with increasing cadmium concentration.

When grown in media lacking cadmium, WT and *aln* plants were observed to have very similar average root lengths. When grown in the absence of nitrogen as well, both WT and *aln* showed similar average root lengths of approximately 0.5 cm (Figure 4A). However, when treated with 11.2 mM nitrogen, WT plants appeared to have a 20% higher average root length in comparison to *aln* (Figure 4A). Conversely, when treated with 22.5 mM of nitrogen, *aln* plants appeared to have a 22% higher average root length in comparison to WT (Figure 4A).

In most cases, when grown in media containing 50 μM cadmium, *aln* mutants appeared to have longer average root length than WT. When grown in the absence of nitrogen, *aln* plants appeared to have 56% higher average root length than WT, yet WT plants appeared to have 20% higher average root length than *aln* when treated with 11.2 mM nitrogen (Figure 4B). Furthermore, when treated with 22.5 mM nitrogen, *aln* mutants displayed a 62% increase in root length in comparison to WT (Figure 4B).

When grown in media containing 100 μM cadmium, drastic differences in average root length were noticed between the two genotypes. When grown in media lacking nitrogen, WT plants showed no germination and therefore no root growth, while *aln* developed roots of approximately 1 cm in length (Figure 4C). In addition, when treated with 11.2 mM nitrogen, *aln* plants appear to have a 173% increase in root length when compared to WT (Figure 4C). Furthermore, *aln* mutants appeared to have an approximate 676% increase in root length in comparison to WT when treated with 22.5 mM nitrogen (Figure 4C).

In order to analyze the effect of nitrogen source on growth of WT and *aln* mutants under cadmium stress, average root length was also quantified for *Arabidopsis* plants grown in media containing KNO_3 as the nitrogen source. Overall, average root length appeared to be longer for both WT and *aln* plants exposed to 50 μM cadmium when treated with KNO_3 instead of NH_4NO_3 . When treated with 11.2 mM nitrogen in the form of KNO_3 ,

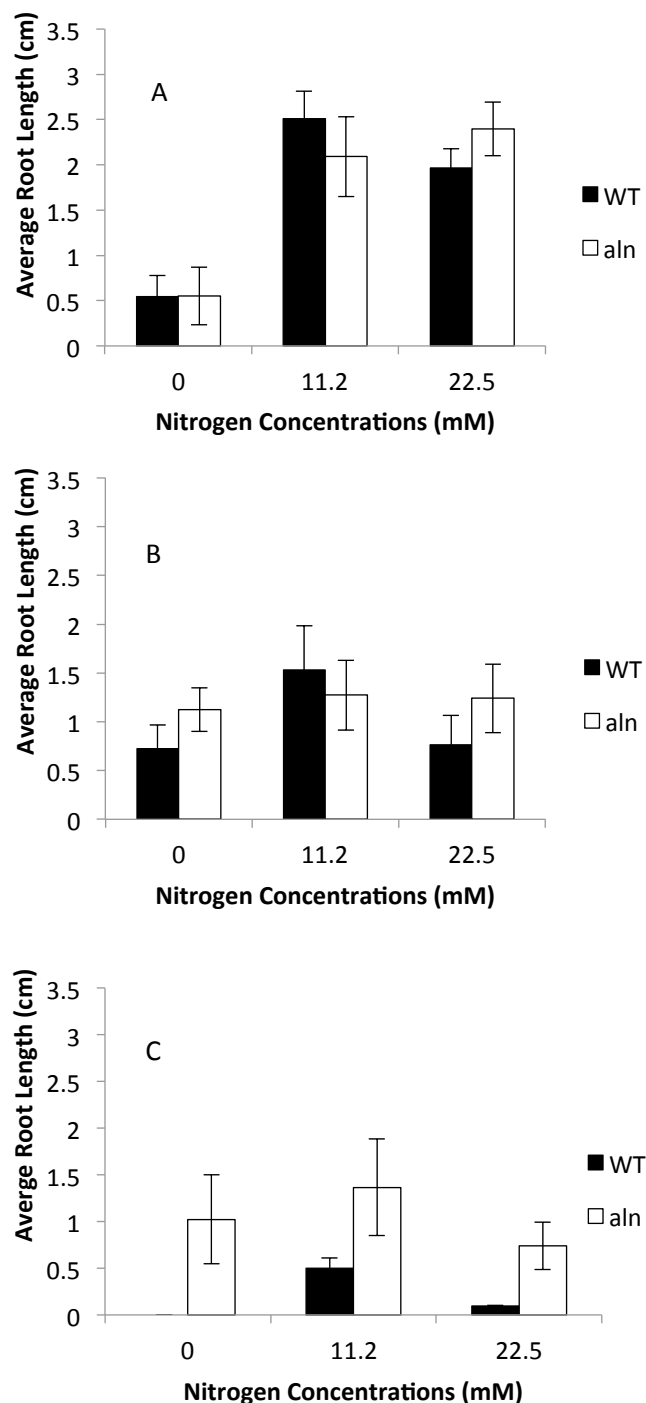
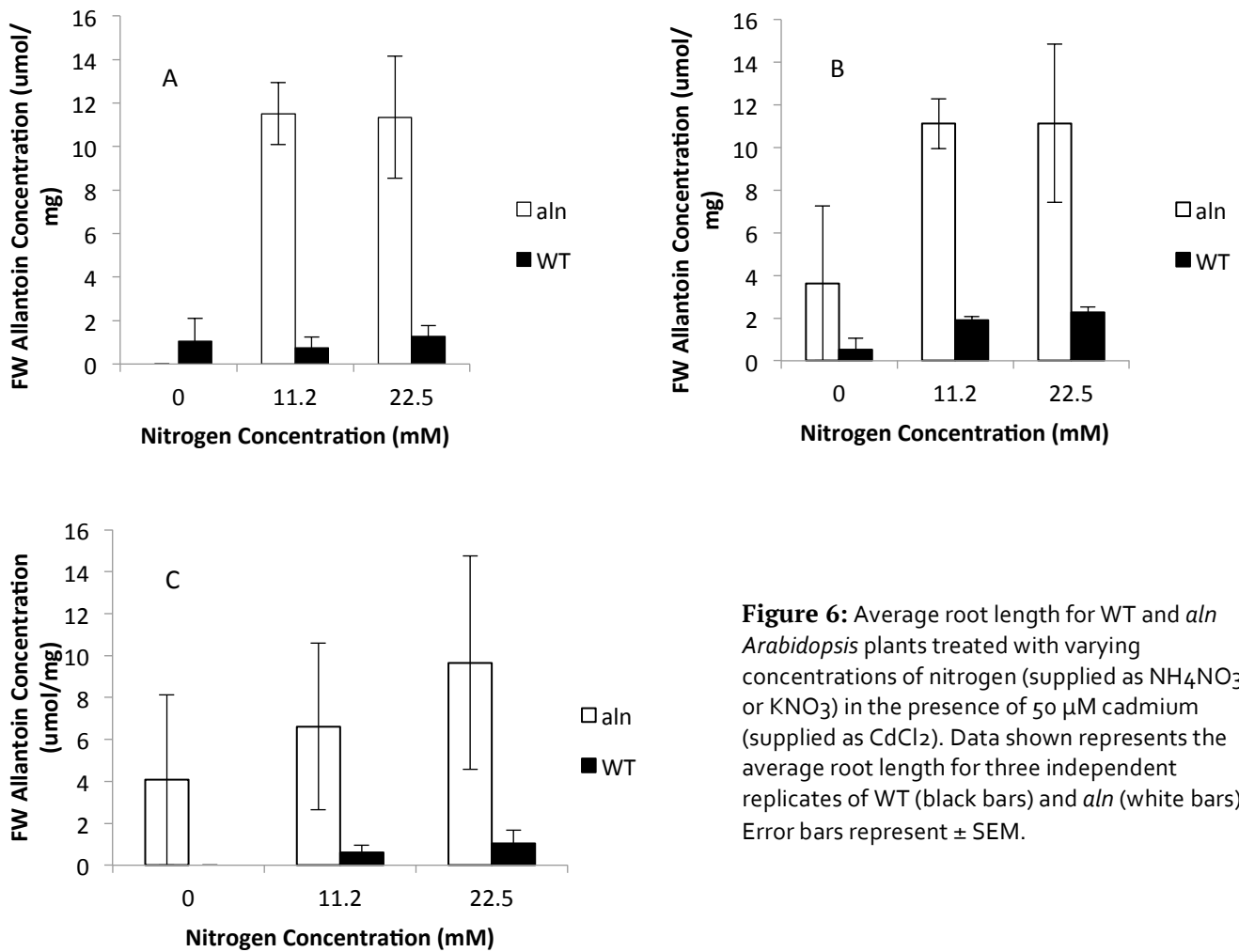
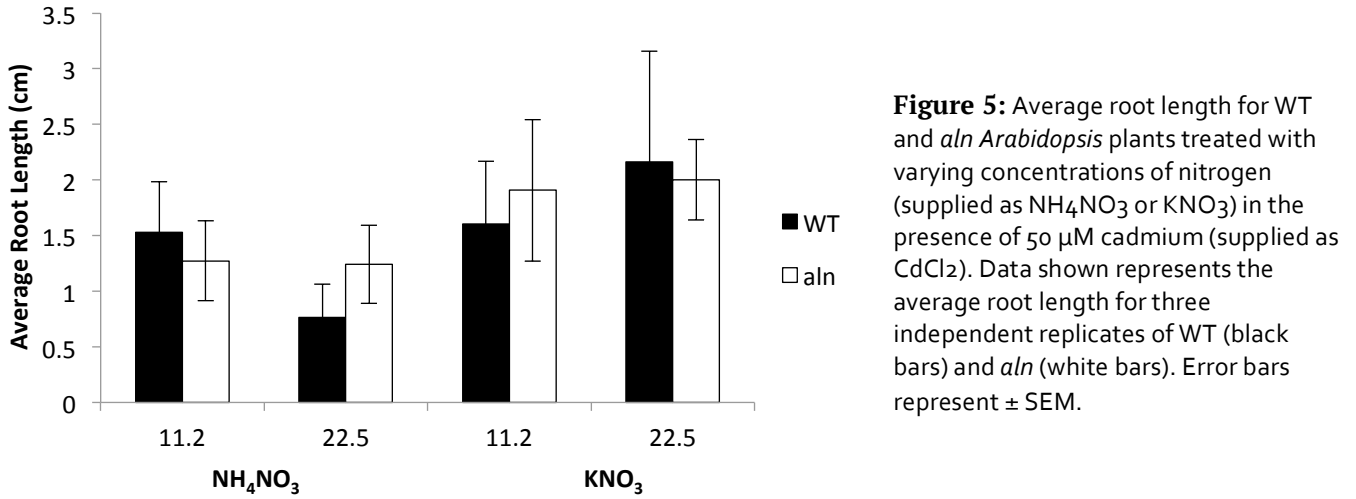


Figure 4: Average root length for WT and *aln Arabidopsis* plants treated with varying concentrations of nitrogen (supplied as NH_4NO_3) in the presence of A. 0, B. 50, C. 100 μM cadmium (supplied as CdCl_2). Data shown represents the average root length for three independent replicates of WT (black bars) and *aln* (white bars). Absence of bar indicates no allantoin detected. Error bars represent \pm SEM.



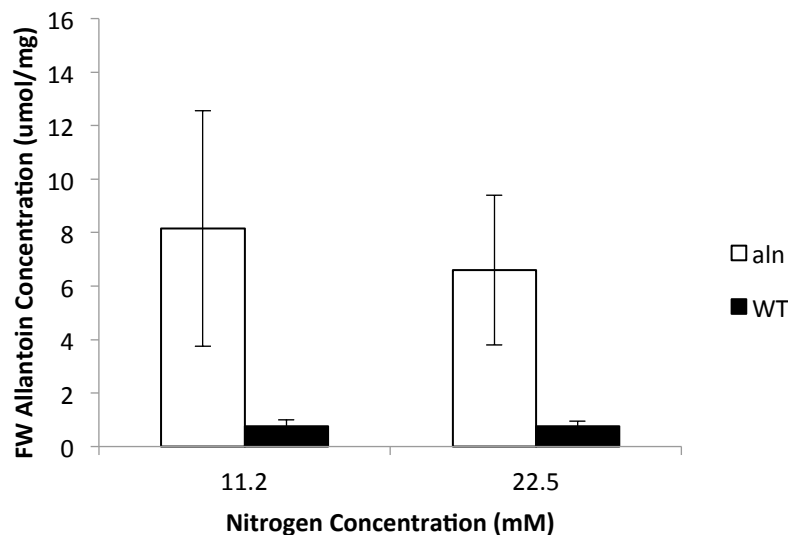


Figure 7: Average concentration of allantoin in fresh weight (FW) samples of WT and *aln* Arabidopsis plants treated with varying concentrations of nitrogen (supplied as KNO_3) in the presence of $50 \mu\text{M}$ cadmium (supplied as CdCl_2). Data shown represents the average allantoin concentration for two independent replicates for WT (black bars) and *aln* (white bars). Error bars represent \pm SEM.

aln mutants showed a 19% increase in average root length compared to WT (Figure 5). However, when treated with 22.5 mM nitrogen in the form of KNO_3 , WT plants displayed an 8% increase in average root length in comparison to *aln* mutants (Figure 5). Similarly, WT plants appeared to have 20% higher average root length than *aln* when treated with 11.2 mM of nitrogen in the form of NH_4NO_3 (Figure 5). However, *aln* mutants appeared to have a 62% longer average root length in comparison to WT (Figure 5).

Next, allantoin concentration was quantified for WT and *aln* samples treated with varying concentrations of CdCl_2 , NH_4NO_3 and KNO_3 , using HPLC. As predicted, allantoin concentrations appeared to be significantly higher in *aln* mutant lines. When grown in media lacking cadmium and nitrogen, *aln* mutants had zero allantoin content, while WT had a minimal average allantoin concentration of approximately $1 \mu\text{mol/mg}$ (Figure 6A). Furthermore, when treated with 11.2 mM and 22.5 mM nitrogen, *aln* mutants had allantoin content 15 fold and 8.9 fold higher than WT respectively (Figure 6A).

Similar allantoin concentrations were also observed in WT and *aln* plants subjected to $50 \mu\text{M}$ cadmium. When grown in media lacking nitrogen, *aln* mutants appeared to have a 7-fold increase in allantoin content in comparison to WT (Figure 6B). In addition, plants grown in media containing 11.2 mM and 22.5 mM of nitrogen, *aln* mutants appeared to have respective 6-fold and 5-fold increases in allantoin concentrations in comparison to WT (Figure 6B).

Lastly, allantoin concentrations for plants exposed to $100 \mu\text{M}$ cadmium appeared to be slightly

decreased in *aln* mutants but still significantly higher than WT plants. When grown in media lacking nitrogen, zero allantoin was observed in WT, while *aln* mutants were observed to have an allantoin concentration of approximately $4 \mu\text{mol/mg}$ (Figure 6C). In addition, when treated with 11.2 mM and 22.5 mM of nitrogen, *aln* mutants were observed to have allantoin concentrations 10.7 and 9.2-fold higher than WT respectively (Figure 6C).

Allantoin content was also quantified for WT and *aln* plants grown in media containing $50 \mu\text{M}$ of cadmium and 11.2 mM and 22.5 mM of nitrogen in the form of KNO_3 . In plants treated with 11.2 mM of nitrogen, *aln* plants were observed to have allantoin concentrations 10.8-fold higher than WT plants (Figure 7). Furthermore, when treated with 22.5 mM of nitrogen, *aln* plants had allantoin concentrations 8.7-fold higher than WT (Figure 7)

Discussion

In this study, phenotypic comparisons and HPLC analysis provided evidence to suggest that *aln* mutant *Arabidopsis* lines display superior growth over WT when exposed to abiotic stress in the form of cadmium. Since allantoin has been known to increase abiotic stress tolerance in plants by activating ABA stress response genes such as RD29A, RD29B and RD26, this may explain why the *aln* mutants displayed increased growth in comparison to WT (Watanabe et al., 2014). When grown in media lacking cadmium, *aln* mutant lines and WT plants were observed to have differences in color, but relatively similar average root lengths. Furthermore, with the addition of nitrogen

to the media, both WT and *aln* mutants appeared to have better growth, which was expected in the absence of stress. Although WT plants appeared to have a slightly higher average root length than *aln* mutants when treated with 0 μM cadmium and 11.2 mM nitrogen, *aln* mutants displayed better growth with increased root branching and less tissue yellowing in comparison to WT when treated with 22.5 mM nitrogen. Furthermore, since allantoin concentrations were found to be significantly higher in *aln* mutants, it may be that high allantoin concentrations allow for better growth even in the absence of stress (Watanabe et al., 2014).

When grown in media containing 50 μM cadmium, WT and *aln* plants displayed very similar growth. In the presence of 11.2 mM and 22.5 mM nitrogen, *aln* mutants were observed to have similar color but slightly more root branching in comparison to WT. Although *aln* mutants displayed longer average root length when exposed to 22.5 mM nitrogen, WT plants displayed slightly longer average root length when exposed to 11.2 mM nitrogen. This is not surprising, as similar increases in root length have also been observed in WT *Glycine Max* plants exposed to cadmium and medium doses of nitrogen (Konotop et al., 2012). This slight increase in root length observed in WT may be due to low replicate number. However, it may be possible that at 50 μM cadmium and 11.2 mM nitrogen, WT plants act as they would in a soil environment and are able to develop longer roots to aid in pursuing nutrients that would allow for survival.

In the presence of 100 μM cadmium, WT and *aln* plants displayed significant differences in growth. In the absence of nitrogen, both WT and *aln* seedlings were incapable of germinating. This result was expected due to the presence of stress and lack of nutrients. *aln* mutants also displayed considerably longer average root length and better growth at 11.2 mM and 22.5 mM nitrogen in comparison to WT. This increase in root length and enhanced growth correlates with the high levels of allantoin observed in *aln* mutants. Furthermore, WT plants were observed to have very minimal allantoin content, which may explain why they displayed little growth under high abiotic stress conditions. As observed in previous studies, allantoin accumulation may lead to activation of ABA stress response genes (Watanabe et al., 2014). Increased expression of ABA stress response genes may further explain why *aln* mutants display better growth than WT, specifically when exposed to considerable abiotic stress.

The results suggest that there is an interaction between nitrogen and abiotic stresses such as cadmium and that this interaction aids in determining the amount

of plant growth for both WT and *aln*. However, this interaction may be more complex than originally thought. Analysis of WT and *aln* plants exposed to 50 μM cadmium and 11.2 mM and 22.5 mM nitrogen in the form of KNO_3 confirms that the nitrogen source may also play a key role in determining plant growth in the presence of abiotic stress. When comparing WT plant growth, we see increased root length in those treated with KNO_3 as the nitrogen source. Given the limited number of replicates used in this study, future studies may focus on reproducing these findings using a larger sample size to determine whether the associations between nitrogen presence and/or cadmium stress, and plant growth are significant.

Conclusion

The purpose of this study was to analyze the interaction between cadmium and nitrogen and determine how this interaction may affect plant growth. It was hypothesized that *aln* mutant *Arabidopsis* plants would display better growth in comparison to WT when exposed to cadmium, due to their characteristic accumulation of allantoin. In addition, it was also predicted that both WT and *aln* plants grown in nitrogen containing media would show enhanced growth in comparison to plants grown in the absence of nitrogen. In general, results from this study have supported the original hypothesis. Under cadmium stress, *aln* mutant lines appeared to display better growth with longer and more branched roots and little senescing tissue, particularly in media containing nitrogen. Increased growth and resistance to cadmium stress was most likely attributed to the high allantoin concentrations observed in *aln* mutants. Furthermore, WT plants appeared to have better growth under cadmium stress when grown in the presence of nitrogen. Lastly, it appears that the interaction between cadmium and nitrogen is also dependent on the nitrogen source. Although only slight differences were noticed between the phenotypes of plants treated with NH_4NO_3 and KNO_3 , it may be beneficial to analyze the interaction between cadmium and other nitrogen sources to further understand the effects that this interaction may have on plant growth.

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