Analyzing the Ability of *Astragalus tennesseensis* to Accumulate Selenium

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Dedication

This thesis is dedicated to my mother, who wanted me to be a hematologist and hates the career path I chose, but still loves and supports me no matter what I'm studying.

Acknowledgments

Thank you to my thesis director, Dr. Frank Bailey, who agreed to direct a project on a topic he hasn't researched since his Ph.D. dissertation. I couldn't have had a better experience for my Honors Undergraduate Thesis. Thank you for being patient with my relentless questions and helping me overcome all of the difficulties I encountered.

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Abstract

Selenium (Se) is a naturally occurring essential micronutrient that can be accumulated by some plants. When accumulated above 1,000 µg Se per gram of plant dry weight (µg/g), the plant is labeled as a Se hyperaccumulator (Alford et al., 2012). *Astragalus bisulcatus* is one of the most well-studied Se hyperaccumulators. *Astragalus tennesseensis* is a related species, but there is currently no literature observing its ability to accumulate Se. This project investigated whether *A. tennesseensis* can accumulate Se. Both species were grown in a greenhouse to compare the amount of Se each would accumulate. Sodium selenate (Na_2SeO_4) was applied to test groups weekly at 1 µg Se per gram of soil for eight weeks. After treatment was completed, each plant was separated into above and below-ground parts to be dried and digested in nitric acid to analyze Se content. Samples were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) to determine the Se content. The average Se content in dosed *A. bisulcatus* shoots (219 µg/g), was not found to differ from *A. tennesseensis* shoots (205 µg/g), although the Se content was expected to be much higher in *A. bisulcatus* (above 1,000 µg/g) due to its status as a known hyperaccumulator. However, when compared to control groups (avg 1.72 µg/g in *A. bisulcatus* shoots, avg 1.46 µg/g in *A. tennesseensis* shoots), both species in test groups had much higher levels of Se. Therefore, it can be concluded that Se treatment did indeed increase levels of Se in both species, and that *A. tennesseensis* has the ability to accumulate Se from soil. Nonetheless, due to the unexpectedly low values in *A. bisulcatus*, it was not possible to determine if *A. tennesseensis* could be classified as a Se hyperaccumulator.

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Introduction

Selenium (Se) is a metalloid and essential micronutrient that is toxic in high amounts (Conrad and Moxon, 1980). This element is naturally occurring in many soils and can be accumulated by some plants. When accumulated in high amounts, generally considered to be above 1,000 µg Se per g DW (µg/g), the plant is described as a hyperaccumulator and is toxic if eaten by humans or animals. A study by Ohlendorf et al. showed that a diet high in Se given to aquatic birds resulted in severe and fatal birth defects, often involving developmental deformities of various organs and external body parts (1986). Se poisoning can also be directly fatal to the animal that consumes it; a large group of sheep that ate Se hyperaccumulator plants were reported to be wandering in circles, stumbling over obstacles, losing appetite, and ultimately collapsing from paralysis (Galston, 1981). However, animals tend to avoid plants that contain Se due to a musky odor from volatile dimethyl selenium compounds (Cornell College of Agriculture and Life Sciences, 2019).

Astragalus is one of the largest genera of plants, with an estimated 3000 species in the genus (Amiri et al., 2020; Li et al., 2014). This genus contains many Se-hyperaccumulating species; among these, one of the most well studied species is *Astragalus bisulcatus*, which is often used as an indicator plant to detect seleniferous soils (Beath et al., 1939a). *Astragalus tennesseensis* is native to Tennessee and surrounding states and is often found in Middle Tennessee's unique cedar glades. The environments this species typically grows in contain relatively low amounts of Se when compared to the environments of *A. bisulcatus* in the western United States in highly seleniferous soils (USGS, 2008). The average Se content in soils in the United States is 2

mg Se/kg soil, while the highest reported concentration in the country is below 100 mg/kg (Ihnat, 1989). A proposed rating for Se concentrations in soils is as follows: <0.3 mg/kg, very low; 0.3 to 0.5 mg/kg, low; 0.5 to 0.9 mg/kg, average; 0.9 to 1.5 mg/kg, high, and >1.5 mg/kg, very high (Wells, 1967).

Astragalus tennesseensis seeds have a tough outer seed coat which prevents germination. When planted in soil in buried pots with no pretreatment, Baskin and Quarterman (1969) found that only 3% of seeds germinated after one year. In this same study, Baskin and Quarterman tested several pretreatments to determine which factors inhibit germination, and which pretreatment methods yield the maximum germination rate. They found that seeds of this species have tough inner and outer seed coats, as well as a chemical inhibitor, all of which prevent germination. Germination rates were also influenced by light and temperature. They achieved their highest germination rate (100%) by removing both seed coats, leaching the chemical inhibitor, and allowing the seeds to germinate in moistened Whatman No. 1 filter paper in petri dishes at 25°C under a 12-hour photoperiod.

Se-hyperaccumulation is a unique ability shared by some plants. The *Astragalus* genus has the highest number of Se-hyperaccumulating species (Sors et al., 2009). Because this genus is extremely large, not all species have been researched in the context of Se accumulation. This study aims to investigate whether *A. tennesseensis*, a G2 imperiled species unique to Middle Tennessee's cedar glades, shares this ability with relatives such as *A. bisulcatus*.

Methods

Germination and Growth

Astragalus bisulcatus seeds were obtained from Prairie Moon Nursery. *Astragalus tennesseensis* seeds were obtained from the Flat Rock Cedar Glades and Barrens Natural Area near Middle Tennessee State University's campus (Permit to collect seeds approved by TDEC, permit #2022-016). Seeds were pre-treated and germinated according to the methods of Baskin and Quarterman (1969) to promote the highest possible germination rate. These methods were tested on seeds from both species before beginning the main batch.

Seeds were first acid scarified by soaking in concentrated sulfuric acid (H_2SO_4) for 20 minutes. After being rinsed with deionized (DI) water, seeds were mechanically scarified. This was done using a dissection microscope and a scalpel to remove a section of the seed coat on the end opposite to the tip of the radicle. After each seed was acid and mechanically scarified, they were put between two pieces of Whatman No. 1 filter paper moistened with DI water and placed in Ziplock bags in a growth chamber at 25°C with a 12-hour photoperiod. For the first three days, filter papers were changed every twelve hours. All seeds were then transferred to Whatman No. 1 filter paper in petri dishes and re-moistened daily or as needed until maximum germination had been achieved.

After germinating, seeds were transplanted into soil. The following soil mixture was used following the methods of Baskin and Quarterman (1969): one-third peat moss, one-third sand, and one-third cedar glade soil. Cedar glade soil was passed through a ½ cm sieve to remove rocks, wood, and other unwanted objects. Once soil was mixed and

moistened, germinated seeds were gently placed on the surface and roots were covered with a thin layer of soil.

Plants were allowed to acclimate to transplanting in the greenhouse growth chamber. Pots were loosely covered with saran wrap to maintain high humidity and reduce transfer shock. Saran wrap was removed after one week. Plants were watered daily or as needed by pouring one liter DI water into each tray and allowing 15-30 minutes for pots to soak up water. Plants were fertilized weekly, starting at the third week after transferring to pots, by pouring diluted MiracleGro® (¼ teaspoon per 1 gallon water) into each tray and allowing the soil to soak.

This process began with ninety seeds of each species. After germination, transfer, and acclimation, twelve *A. bisulcatus* and eleven *A. tennesseensis* plants remained. These were split into two groups of each species: six plants in *A. bisulcatus* control (BC), six in *A. bisulcatus* dosed (BD), five in *A. tennesseensis* control (TC), and six in *A. tennesseensis* dosed (TD).

Selenium Treatment

Sodium selenate (Na_2SeO_4) was used to make a stock solution at a nominal concentration of 15 mg Se per liter of DI water. This stock was used to dose soil at a target concentration of 1 mg Se per kg soil during each treatment. Treatment began five weeks after plants were transferred to pots; 10ml of the sodium selenate stock solution was added to each test pot with a pipette once per week for eight weeks. This resulted in a final nominal concentration of 8 mg Se/kg soil. Each time test groups were treated, control groups were given 10 ml of DI water using the same methods.

Plant Harvest and Digestion

All plants were harvested on February 10th, 2023, after they had been in pots for twelve weeks, fertilized for nine weeks, and treated with Se or water for eight weeks. When harvested, each plant was removed from its pot and the soil was carefully removed from the root mass. Care was taken to minimize loss of roots in the soil. Roots were then rinsed with DI water to remove soil, and forceps were used to remove remaining chunks of soil. The soil from each of the four groups was combined into four composites, and three samples were taken from each composite to analyze the remaining concentration of Se in each group's soil. Each plant was then separated into above and below-ground parts, which were then weighed and placed in a drying oven at 40°C for seven days. The soil samples were also placed in the drying oven. After drying, samples were weighed again, then placed in glass digestion tubes with 3 ml concentrated nitric acid (HNO_3).

Samples were left to sit in HNO_3 for ten days with daily stirring. They were then put on heating blocks to expedite digestion. Samples were first heated to 50°C, then gradually raised to 110°C to prevent them from boiling over. Large glass marbles were placed on top of each tube to reflux HNO_3 fumes while allowing pressure to escape. When the samples were thoroughly digested, the marbles were removed to allow for evaporation down to 2 ml. The samples were then left to cool, then one to two ml of concentrated HNO_3 was once again added. The process was then repeated until the liquid in each tube was clear, then they were allowed to evaporate below 2 ml.

After cooling, 3 ml of 30% hydrogen peroxide (H_2O_2) was added to each tube to digest remaining organic matter. Samples were left to sit for two days, then placed on

heat blocks and evaporated below 2 ml. DI water was added to each tube to achieve a final volume of 10 ml for each sample, finalizing sample preparation for analysis.

Inductively Coupled Plasma Optical Emission Spectroscopy

Selenium standard solutions were made to create a standard curve. Solutions were made at 0.1, 1, 5, and 10 µg/ml. These standards were used to calibrate the ICP-OES before analyzing samples. Afterwards, all shoot, root, and soil samples were analyzed. The Na_2SeO_4 stock solution was also analyzed to confirm the actual Se concentration used in dosing the plants. Selenium in plant tissues is reported in µg Se/g dry weight of plant material.

Statistical analysis

Differences in Se concentrations among groups were tested by ANOVA using SPSS statistical software.

Results

The actual concentration of Se in the stock solution was 18.27 mg/l (nominal 15 mg/l). The average Se concentrations in each group by species, dose, and material, are shown in Table 1. Average Se concentrations for shoots and roots were log10 transformed in order to meet assumptions of homogeneity of variances and normality.

There were no differences in Se concentrations in shoots by species (F (1, 23) = 0.412, p = 0.529), but the Se concentrations in dosed tissues were found to be higher than control tissues (F (1, 23) = 1377.547, $p < 0.001$). (Figure 1). There was also no significant difference in Se concentrations in roots by species (F (1, 23) = 0.519, p = 0.480), but dosed roots contained higher Se concentrations than control (F (1, 23 = 558.6, $p < 0.001$) (Figure 2). There were significant differences between remaining Se concentration in soil by species (F (1, 12) = 18.32, p = 0.003), with *A. tennesseensis* dosed soil having a higher Se concentration than the *A. bisulcatus* dosed soil (F (1, 6) = 19.2, p = 0.012). Dosed soil also had higher soil Se concentrations than control soil (F (1, 12) = 392.5, $p < 0.001$) (Figure 3).

Table 1: Average concentrations of selenium found in each group by species, treatment, and material.

Species	Treatment	Material	µg Se/g DW
A. tennesseensis	Dosed	Shoots	205.03
A. tennesseensis	Dosed	Roots	109.72
A. tennesseensis	Dosed	Soil	1.22
A. tennesseensis	Control	Shoots	1.46
A. tennesseensis	Control	Roots	1.44
A. tennesseensis	Control	Soil	0.18
A. bisulcatus	Dosed	Shoots	219.29
A. bisulcatus	Dosed	Roots	96.67
A. bisulcatus	Dosed	Soil	0.85
A. bisulcatus	Control	Shoots	1.72
A. bisulcatus	Control	Roots	2.19
A. bisulcatus	Control	Soil	0.18

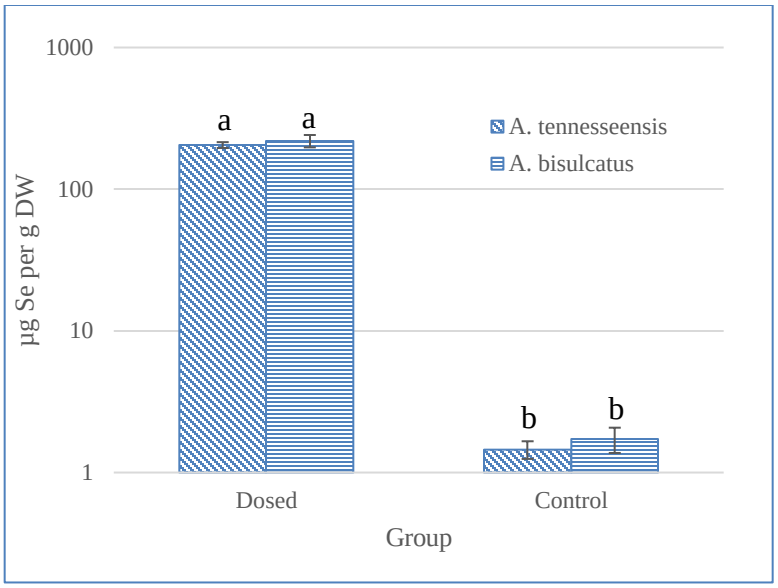

Figure 1: Logarithmic scale of the average concentrations of selenium in µg per gram of dry weight in shoots of each species, dosed vs control. There was no difference between selenium concentration in shoots by species (F (1, 23) = 0.412, p = 0.529), but there were significant differences between dosed and control selenium shoot concentrations (F (1, 23) = 1377.547, p < 0.001). Different lowercase letters above bars denote significant differences.

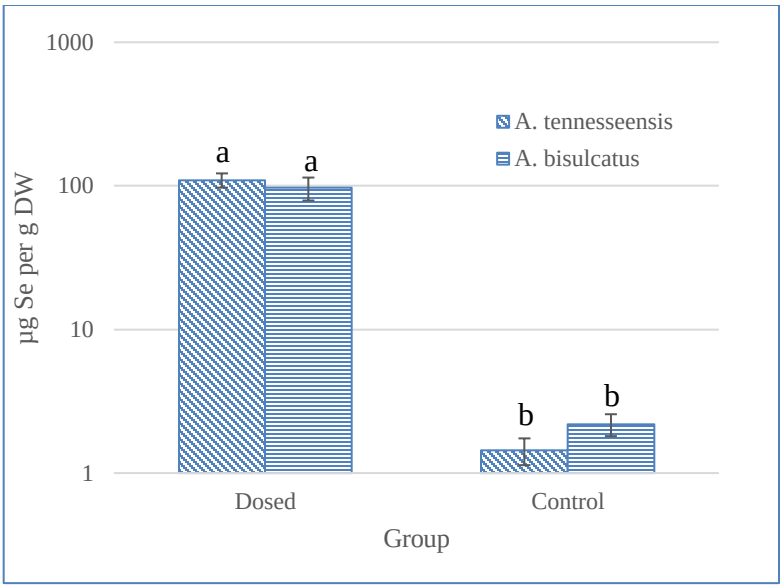

Figure 2: Logarithmic scale of the average concentrations of selenium in µg per gram of dry weight in roots of each species, dosed vs control. There was no difference between selenium concentration in roots by species (F (1, 23) = 0.519, p = 0.480), but there were significant differences between dosed and control selenium root concentrations (F (1, 23) = 558.6, $p < 0.001$). Different lowercase letters above bars denote significant differences.

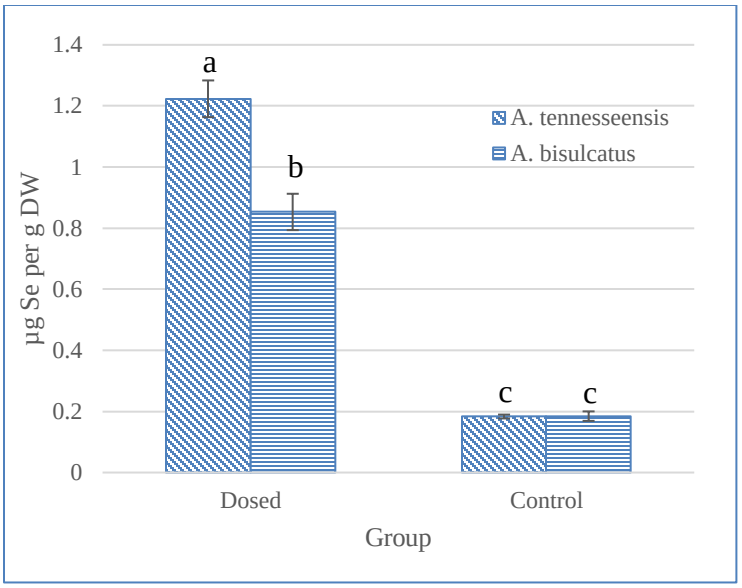

Figure 3: Average concentrations of selenium left behind in the soil by group, in µg Se per g dry weight. There were significant differences between selenium concentration in soil by species (F (1, 12) = 18.32, p = 0.003). Further analysis found that *A. tennesseensis* dosed soil had a higher Se concentration than *A. bisulcatus* dosed soil (F (1, 6) = 19.2, p = 0.012). There were also significant differences between dosed and control selenium soil concentrations (F (1, 12) = 392.5, p < 0.001). Different lowercase letters above bars denote significant differences.

Discussion

The expected concentration of Se in *A. bisulcatus* shoots is 1,000 µg/g to 10,000 µg/g due to its status as a known Se hyperaccumulator (Pilon-Smits, 2019; Sors et al., 2009; Pickering et al., 2003; Sors et al., 2005a). One study claims that in the wild, this species can accumulate up to 15,000 µg/g (Valdez Barillas et al., 2012). However, the average concentration achieved in *A. bisulcatus* shoots in this study was only 219.29 µg/g. There are several possible explanations for the unexpectedly low amount of Se in *A. bisulcatus*. The seeds were purchased online from Prairie Moon Nursery. Ideally, for a study such as this one, seeds would be obtained from the field from plants that are confidently identified as *A. bisulcatus* growing in soil that is known to be seleniferous. However, travel for obtaining seeds from the wild was not within the budget of this project. Because seeds were purchased online, it's possible that the parent plants were not exposed to Se, were a non-accumulating variation of *A. bisulcatus*, or were an entirely different species.

We also speculated that the form of Se that was given to these plants, selenate, may not have been particularly available to them. However, several studies successfully treated this species with selenate, researched the biochemical processes of accumulating selenate, and/or claim that selenate is one of the predominant forms of Se available to plants (Pickering et al., 2003; Sors et al., 2005a; Sors et al., 2009; Pilon-Smits, 2019; Banuelos and Meek, 1990; Sors et al., 2005b).

Another possibility is that the plants did accumulate higher amounts of Se, but it was volatilized when the plants were in the growth chamber and the drying oven, both of which had quite heavy airflow. I noticed a strong Se odor in both the growth chamber and

the drying oven, indicating volatilization. Studies claim that Se hyperaccumulators do indeed lose Se due to volatilization of compounds such as dimethylselenide (Sors et al., 2005b; Pickering et al., 2003). The expression of certain genes, and in turn the use of certain enzymes, can cause hyperaccumulators to convert more accumulated Se to these volatile forms; one study found that increased expression cystathionine-γ-synthase led to higher volatilization and 20-40% lower Se shoot concentrations (Sors et al., 2005b). However, gene expression was not purposefully altered in this study, so it is unknown whether the overexpression of certain genes decreased *A. bisulcatus* shoot Se concentrations.

One more factor that could have affected Se accumulation is the soil microbiome. Legumes are known to have associations with nitrogen-fixing bacteria (Pilon-Smits, 2019). One study found that when *A. bisulcatus* plants are grown with nitrogen fixing bacteria, Se accumulation was increased, particularly in the form of gamma-glutamyl-methyl-SeCys (Alford et al., 2012). They hypothesized that the bacteria provided the nitrogen needed to synthesize this compound. Mycorrhizal fungi can also affect selenium accumulation. In one study, garlic was grown in untreated soil, soil with mycorrhizae, soil with selenate, and soil with both mycorrhizae and selenate; these plants accumulated 1.5, 15, 853, and 969 µg/g, respectively (Larsen et al., 2006). Interestingly, another study conducted on maize found that mycorrhizal inoculation increased accumulation of selenate, but reduced accumulation of organic Se (Yu et al., 2011). It is apparent that soil microbes can influence Se concentrations in plants by enhancing or inhibiting accumulation. Because we did not evaluate microbes in this study, it is unknown which microbes were present and how they influenced Se accumulation, if at all.

Regardless of the reason that *A. bisulcatus* did not accumulate the expected amount of Se, *A. tennesseensis* accumulated more than expected. Most non-accumulating plants rarely exceed 100 µg/g in shoots (Sors et al., 2005b). We found an average Se concentration of 205.03 µg/g in *A. tennesseensis* shoots, which is more than most non-accumulators, but not significantly lower than the concentration achieved in *A. bisulcatus* shoots in this study. Although these results show that *A. tennesseensis* can accumulate more than most non-accumulators, due to the unexpectedly low concentration in *A. bisulcatus*, no claims can be made on *A. tennesseensis*'s status as a Se accumulator.

Several measures can be taken to increase the efficacy of a similar study in the future. The sample sizes were quite low due to a high percentage of plants dying after germination and transplant; we had only five *A. tennesseensis* plants in the control group and six plants of each species in the remaining groups. A higher sample size would allow for more robust results, as well as more test groups with varying concentrations of Se dosage. It would be ideal to obtain *A. bisulcatus* seeds from the field and test the soil for Se content. Lastly, although selenate is one of the most available forms of Se to plants, the method of exposure could be changed. For example, soil could be treated with the entire desired dose before planting rather than treating weekly. To maximize the efficiency of a similar study in the future, all seeds should be obtained from the field, a larger sample size and more test groups should be used, the digestion should be performed more carefully, different methods of exposure could be tested, and soil microbes should be considered.

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